Reaction of Azapeptides with Chymotrypsin-like Enzymes

NEW INHIBITORS AND ACTIVE SITE TITRANTS FOR CHYMOTRYPSIN A, SUBTILISIN BPN', SUBTILISIN CARLSBERG, AND HUMAN LEUKOCYTE CATHEPSIN G*

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A series of new azapeptide p-nitrophenyl esters containing a variety of P1 aza-amino acid residues have been synthesized, and the reaction of these azapeptides with chymotrypsin A, subtilisin BPN', subtilisin Carlsberg, and human leukocyte cathepsin G at pH 4–7 has been studied. These azapeptides were found to be very useful as active site titrants and inhibitors of serine proteases with chymotrypsin-like specificity. Stable acyl derivatives of serine proteases are formed in the reaction with azapeptides and can be used for future crystallographic investigations. The effects of changing the nature of the P1 leaving group (–ONp, –OPh, –OCH3Ph, –OEt) for these azapeptides was also investigated. N-Acetyl-l-alanyl-l-alanyl-l-aza-norleucine p-nitrophenyl ester can be used as an active site titrant for human leukocyte cathepsin G and N-acetyl-l-alanyl-l-aza phenylalanine p-nitrophenyl ester is a suitable titrant for chymotrypsin A, subtilisins, or cathepsin G.

Aza-amino acid residues are analogs of amino acids in which the α-methylene group has been replaced by a nitrogen atom (Fig. 1). The substitution has a profound effect on the reactivity of aza-amino residues in simple derivatives or in peptides. Kurtz and Niemann (1961, a and b) were the first to study the reaction of an α-amaio acid derivative with a protease and showed that As-Aphe-OEt was a weak reversible inhibitor of chymotrypsin (K/i = 20 mM). Elmore and Smyth (1968) demonstrated that a more reactive ester derivative of an α-aza amino acid (Ac-Aphe-ONp) could be utilized as an active site titrant for chymotrypsin. A polymer containing an Aphe-OPh ligand was then found to be useful for the covalent affinity purification of chymotrypsin (Barker et al., 1974). We prepared peptide 4-nitrophenyl esters containing a P1 aza amino acid residue and showed that they were excellent inhibitors and titrants of chymotrypsin, subtilisin, and porcine pancreatic elastase and that considerable specificity could be obtained by altering the P1 residue (Powers and Gupton, 1975; Powers and Gupton, 1977). Subsequently, two azornitrihine phenyl ester derivatives were shown to be inhibitors of trypsin and thrombin (Gray and Parker 1975; Gray et al., 1977). Inhibition of all of these serine proteases by the α-aza amino acid phenyl, or 4-nitrophenyl esters is believed to be due to the acylation of the active site serine residue forming an acylated enzyme. The nitrogen atom adjacent to the acyl carboxyl group gives a special stability to the acyl-enzymes, which are substantially less reactive toward deacetylation than normal acyl enzymes. Aza-α-amino acid derivatives without reactive leaving groups do not acylate serine proteases, but simply act as reversible inhibitors (Kurtz and Niemann, 1961a; Dorn et al., 1977).

Chymotrypsin A, subtilisin BPN', subtilisin Carlsberg, and human leukocyte cathepsin G are serine proteases whose substrate specificity is directed toward P1 amino acid residues with aromatic or large hydrophobic side chains. The active sites and substrate specificity of chymotrypsin (Blow 1971; Bender and Kiliheffer, 1973) and subtilisin BPN' and subtilisin Carlsberg (Kraut, 1971; Markland and Smith, 1971; Hunt and Ottesen, 1961) have been widely investigated. Studies on the reactivity of cathepsin G toward peptide 4-nitroanilide substrates (Nakajima et al., 1978), amide substrates (McRae et al., 1980), and peptide chloromethyl ketone inhibitors (Powers et al., 1977) have been reported.

In this paper, we report a study of the reaction of azapeptides containing a variety of P1 aza-amino acid residues with chymotrypsin A, subtilisin BPN', and subtilisin Carlsberg, and human leukocyte cathepsin G and have found the azapeptides to be useful as active site titrants and inhibitors and for generating stable acyl derivatives of serine proteases for crystallographic investigations. In addition, we have investigated the effects of changing the nature of the P1 leaving group. Our results demonstrate that azapeptides can be used as selective inhibitors and active site titrants of serine proteases.

MATERIALS AND METHODS

Chymotrypsin A, was obtained from Worthington and used without further purification. Subtilisin BPN' and subtilisin Carlsberg were obtained from Sigma and used without further purification. Human leukocyte cathepsin G was supplied by Dr. James Travis and his

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‡Aza-amino acid residues in which the α-methylene of an amino acid residue is replaced by a nitrogen atom are abbreviated by placing an "A" before the standard three letter abbreviation for that amino acid. Thus, a-aza-alanine will be abbreviated Aala. Any peptide which contains an aza-amino acid residue will be referred to as an azapeptide in this paper. The other abbreviations used are defined in the mini-print.
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research group of the Dept. of Biochemistry, University of Georgia. The azo amino acid Ac-Aphe-ONp was obtained from Nutritional Biochemicals, Boc-(CH3)2NH was prepared by the method of Jensen et al. (1968) and CH3CH2CH2NH2 by the method of Kost and Sagitulin (1959). Boc-Tyr-ONp was obtained from Bachem. All other amino acid derivatives, reagents, and solvents were analytical grade. Meltting points are uncorrected. Thin layer chromatography was carried out with Merck Silica Gel G plates. Materials were detected using I2 vapor or FeCl3-K3[Fe(CN)6] spray reagent (Ertel and Horner, 1962). Column chromatography was performed with Merck Silica Gel 60 absorbant. Mass spectra were taken on an Hitachi Perkin-Elmer RMU-71 instrument and nmr spectra were taken on a Perkin-Elmer 457 instrument. Infrared spectra were taken in Nujol mulls. Assays were performed on Beckman Model 25 or 35 spectrometers and a Radiometer automated pH-stat Model ITT11, and all spectrometer cells had a 1-cm path length.

The syntheses of all new azapeptides used in this investigation are reported in a miniprint supplement to this paper or to the following paper (Powers et al., 1984).

Reaction of Enzymes with Azapeptides—The reaction of chymotrypsin A, and subtilisin Carlsburg with a series of azapeptides was carried out in solutions which contained approximately a 50-fold excess of azapeptide over enzyme. Stock solutions of the azapeptides in acetonitrile were prepared at a concentration of 5.0 mM and the use of acetonitrile was necessary to increase the solubility in the reaction mixture. Enzyme stock solutions of chymotrypsin A, (1 mM HCl) and subtilisin had a concentration of approximately 100 μM. Enzyme concentration was quantitated using A280 = 20.5 for chymotrypsin A (Wilcox, 1970), A280 = 11.7 for subtilisin BN', and A280 = 9.6 for subtilisin Carlsberg (Ottesen and Svendsen, 1970). Chymotrypsin solutions were stored at 4 °C, while subtilisin solutions were used within 2 h. Four buffer solutions were prepared for the pH dependence studies: pH 7.0 (0.1 M phosphate), pH 6.0 (0.1 M citrate), pH 5.0 (0.1 M citrate), and pH 4.0 (0.1 M acetate). All reactions were performed at 25 °C and were carried out by mixing 100 μl of the azapeptide stock solution with 2.0 ml of the appropriate buffer in a cuvette. An identical reference sample was prepared and a base-line was recorded at 345 nm. The reaction was initiated by the addition of 100 μl of 1 mM HCl to the reference cuvette followed by the addition of 100 μl of the enzyme stock solution to the sample cuvette. The reactor was immediately started upon the addition of enzyme to the sample cell and the reaction rate was observed. Assay solutions were freshly prepared since the azapeptides underwent slow hydrolysis upon standing in buffered aqueous solutions. Some representative azapeptide hydrolysis rates are listed in the table in the supplementary section of the following paper (Powers et al., 1984).

The reaction of cathepsin G with azapeptides was carried out by adding 50 μl of the azapeptide in 1,2-dimethoxyethane to 1.0 ml of buffer. Aliquots (600 μl) of this solution were added to 5 ml of buffer and reference microcells and a base-line was established. The reaction was initiated by the addition of 50 μl of 1 mM HCl to the reference cell and 50 μl of enzyme to the sample cuvette. The exact protein concentration of cathepsin G was determined using E280 = 6.64 (Baugh and Travis, 1976).

The titrations of chymotrypsin A, subtilisin BN', subtilisin Carlsberg, and cathepsin G were carried out at concentrations, respectively, of 8.5–4.4 μM (0.46–2.34 mg in 2.2 ml), 5.3–3.5 μM (0.22–0.31 mg in 2.2 ml), 6.5–3.5 μM (0.35–0.20 mg in 2.2 ml), and 2.25–1.12 μM (36–18 μg in 550 μl). At the low end of the range, the results showed considerable variance especially if the spectrophotometer was very noisy.

Determination of Deacylation Rates—The slow deacylation rates of azapeptides were determined with Ac-Ala-Aphe-ONp as a substrate (Wilcox, 1970; Cunningham and Brown, 1956) and one example of the procedure follows. A stock solution of Ac-Ala-Aphe-ONp in acetonitrile (5 mM) was prepared and used immediately. A stock solution of chymotrypsin A, was made up in 1 mM HCl and had a concentration of approximately 100 μM (exact concentration determined by A280). The reaction was initiated by the addition of 100 μl of Ac-Ala-Aphe-ONp to 2.0 ml of the 0.1 M citrate buffer (pH 5.0). This was followed by the addition of 100 μl of the enzyme stock solution to the reaction mixture. The mixture was allowed to incubate for 5 min. A 200-μl portion of the reaction mixture was withdrawn and added to 2.0 ml of the appropriate buffer solution. At various time intervals, 100-μl fractions were withdrawn and assayed using the substrate Ac-Tyr-OEt with the pH-stat. The initial concentrations in the deacylation reaction mixture were as follows: enzyme, ~0.5 μM; substrate, 23 μM; acetonitrile, 0.5% (v/v) in a total volume of 2.2 ml. Deacylation rates were then calculated using a least squares computer program. Correlation coefficients of better than 0.994 were obtained.

Inhibition of Cathepsin G with Azapeptide Analogos—Inhibition of cathepsin G with a series of azapeptides was carried out in solution which contained at least a 65-fold excess of inhibitor over enzyme. Stock solutions of azapeptide in acetonitrile were prepared at a concentration 0.25–7 nm. The enzyme stock solution was made in 1 mM HCl and had a concentration of ~4 μM. The reactions were performed at 25 °C and were carried out by mixing 50 μl of inhibitor solution and 50 μl of an enzyme solution with 1 ml of buffer. Aliquots (100 μl) were removed from the reaction mixture at regular time intervals and the residual enzymatic activity was measured using the Boc-Tyr-ONp spectrophotometric assay (Powers et al., 1977). The final concentrations of inhibitor and enzyme in the reaction mixture are shown in Table VI.

RESULTS

Synthesis of Azapeptides—A series of new azapeptides were prepared by three general methods (Gante, 1966; Dutta and Morley, 1975). Each synthesis required the synthesis of a 2-substituted hydrazide (RCO-NNHR', RCOH = a blocked amino acid or a peptide acid) which was subsequently coupled with p-nitrophenyl chloroforamide to produce the desired azapeptide p-nitrophenyl ester. The most convenient route, hydrazinolysis of an ester or coupling of a peptide acid with a substituted hydrazine, requires the availability of the appropriate azapeptide analog. The procedure was used in the synthesis of Ac-Ala-Aphe-ONp and Ac-Ala-Ala-Aphe-ONp and requires the stereochemistry and identification of two isomeric hydrazides (i.e. the 1-substituted hydrazide Ac-Ala-Ala-NMeNH2 and the 2-substituted hydrazide Ac-Ala-Ala-NHNNH2 were obtained upon reaction of MeNHNNH2 with Ac-Ala-Ala-OBz). The correct isomer is readily identifiable since the alkyl protons of the 1-substituted hydrazide (δ 3.2 for the N-CH3 of Ac-Ala-Ala-N(CH2)4NH2 and δ 4.62 for the N-CH3 of Ac-Ala-N(CH2)4NH2) appear further downfield in the nmr spectrum than those of the 2-substituted hydrazide (δ 2.4 for the N-CH3 of Ac-Ala-Ala-NHNNH2 and δ 3.82 for the N-CH3 of Ac-Ala-Ala-NHNNCH2). An alternate, although longer, unambiguous synthesis of AcAla derivatives involves coupling of a blocked amino acid or peptide acid with NH2NMe-Boc followed by deblocking with CF3CO2H to give only the correct hydrazide. Since few substituted hydrazines are commercially available, the majority of azapeptides reported in this paper...
were synthesized by a route similar to that utilized by Elmore and Smyth (1968) for the preparation of Ac-Aphe-ONp and Kurtz and Niemann (1961b) for the synthesis of Ac-Aphe-ONp. In this procedure, a peptide hydrazide is converted to a hydrazone by reaction with an aldehyde or ketone. Reduction then gave a 2-alkylhydrazide. All new compounds were characterized by combustion analysis, infrared, nmr, mass spectra, and tlc. A particularly useful tool for the identification of these compounds was nmr. The nmr spectrum of each compound exhibited certain characteristic resonances which allowed 1- and 2-substituted hydrazides to be readily distinguished as well as being able to tell precursors from products (Powers and Carroll 1975; Powers and Gupton, 1977; Condon, 1972). Azapeptides as well as peptide and amino acid hydrazides and hydrazones were easily distinguished from other compounds on tlc by treatment of the plates with ferric chloride followed by potassium ferricyanide (Ertel and Horner, 1962). The appearance of a royal blue color indicated the presence of these groups. Likewise, the p-nitrophenyl moiety was detected by treatment with base which produced a bright yellow color due to the p-nitrophenolate anion.

Kinetic Considerations—The kinetics of the reaction of serine proteases with substrate-related acylating agents are described by Equation 1.

$$\begin{align*}
E + S & \rightarrow E \cdot S \rightarrow E \cdot S + P' \rightarrow E + P^* \\
K_c & = k_3/k_2 \\
K_M & = k_3/k_1 \\
K_2 & = k_3/k_1
\end{align*}$$

(1)

where $E$: $S$ is the enzyme-substrate complex, $E$: $S$ is the acyl-enzyme intermediate, and $P'$ is $p$-nitrophenol in the case of burst of $p$-nitrophenol followed by the steady state turnover of the acyl-enzyme. The equation of this curve is represented and turnover rate by Equation 2 (Kezdy and Kaiser, 1970; Bender et al., 1967; Kurtz and Niemann (1961b) for the synthesis of Ac-Aphe-ONp, and Smyth (1968) for the preparation of Ac-Aphe-ONp and Ac-Ala-Aphe-ONp).

$A = (k_{cat}[E]/[S]_0)/([S]_0 + K_M)$

$\pi = [E]/(k_2/[E]_0 + K_M)$

As $t$ becomes very large, Equation 2 reduces to Equation 3.

$$[P'] = At + \pi$$

(3)

Equation 3 is a straight line relationship which represents the steady state portion of the curve. If $[S]_0 \gg K_M$, the slope $A$ is equal to $k_{cat}$ and can be measured experimentally. The intercept or burst may be obtained by extrapolating the curve back to $t = 0$. The value of $[P']$ is directly proportional to $[E]$. Under conditions where $k_2 \gg k_1$, $[S]_0 \gg K_M$, then $k_{cat} = k_3$ and $\pi = [E]_0$ are obtained.

These requirements are often met with reactive esters of azapeptides, since these compounds will acylate certain serine proteases rapidly to form stable acyl-enzymes. In many cases, the deacylation rates are so slow that they can only be measured by following the reappearance of enzyme activity after isolating the acyl-enzymes. The active enzyme concentration can be calculated from the release of $p$-nitrophenol ($[E]_0 = \Delta A_{345}/[E]_0$, $t_{EONP} = 6250$ at pH 6.0). The turnover rate $k_{cat}$ is then ($\Delta A_{345}/[E]_0$). Two azapeptide $p$-nitrophenyl esters (Ac-Aphe-ONp and Ac-Ala-Aphe-ONp) have previously been shown to exhibit the above kinetic behavior and have been used to titrate chymotrypsin A and subtilisin $BN'$ (Elmore and Smyth, 1968; Powers and Carroll, 1975; Powers and Gupton, 1977).

Reaction of Chymotrypsin with Azapeptides—Table I shows the results for the reaction between chymotrypsin and each of the 11 azapeptides. Results were very reproducible under conditions where $[S] \gg [E]$. The enzyme was acylated by the two $P_1$ azaphenylalanine derivatives very rapidly over a pH range of 4–7 with no measurable turnover of the azapeptide on the time scale of the assay procedure. Furthermore, the initial acylation reaction proceeded with a 1:1 stoichiometry with respect to enzyme concentration based on the release of $p$-nitrophenol. Azapeptides which contained aza- amino acid residues possessing long alkyl side chains (e.g. Avel, Anva, Aleu, and Alile) also acylated the enzyme stoichiometrically and showed no measurable turnover. In contrast, the shorter aza-alanine-containing peptides Ac-Ala-Aala-ONp and Ac-Ala-Ala-ONp would only react with the enzyme at higher pH values, while the longer aza-alanine peptide (Ac-Ala-Ala-Pro-Aala-ONp) reacted with the enzyme stoichiometrically over a pH range of 4–7 with no measurable turnover. The azapeptide Ac-Ala-Ala-Agly-ONp did not show any measurable reaction with chymotrypsin at either pH 6 or 7. The enzyme had an average specificity of ~89% with respect to the protein concentration determined from $A_{345}$.

The lower limit value of $1.8 \times 10^{-4}$ s$^{-1}$ for the turnover rates was determined by preparing a series of lines of known slopes and superimposing each line on a previously recorded base-line. The line with the smallest slope value which had a preceptible deviation from the base-line was used to calculate the lower limit value for $k_{cat}$. Since one purpose of the investigation was the preparation of stable acyl derivatives of chymotrypsin for crystallographic investigation, we investigated the acylation rates of chymotrypsin $A_n$ at pH values where most crystallographic studies are performed. At pH 5.8, Ac-Aphe-ONp, Ac-Ala-Aphe-ONp, and Z-Ala-Ala-Pro-Aala-ONp had $k_2$ values $>0.20$ s$^{-1}$, our limit of detection. At pH 5.0, the $k_2$ values for the first two azapeptides were also $>0.20$ s$^{-1}$, while that for Z-Ala-Ala-Pro-Aala-ONp was 0.056 s$^{-1}$.
p-nitrophenol had a HONp.

The results for the studies in which each of the 11 azapeptides were reacted with subtilisin BPN' showed no release of p-nitrophenol on the time scale of the experiment. The turnover rates with Ac-Ala-Ala-Pro-Aala-ONp having the highest. The azapeptides containing larger alkyl side chains again had intermediate turnover rates. The reactions had a 1:1 stoichiometry and the enzyme was found to be ~78% active. Although this enzyme exhibited the same general trends as subtilisin BPN', a comparison of the turnover rates for the reaction between a given azapeptide and each of the two subtilisins shows subtilisin Carlsburg to consistently have more rapid decylation rates than subtilisin BPN'.

The variance in the ratio of enzyme concentration calculated from the burst to that based on Km for these two enzymes may be the result of the rapid autolysis which these two enzymes undergo. This would result in a lowering of the active enzyme concentration without lowering the protein concentration.

Reactions of Subtilisins with Azapeptides—Table II shows the results for the studies in which each of the 11 azapeptides were reacted with subtilisin BPN' under assay conditions identical with those used with chymotrypsin Aν. The enzyme was acylated rapidly by all of the compounds with the exception of Ac-Ala-Ala-Agly-ONp which showed no release of p-nitrophenol on the time scale of the experiment. The turnover rates showed substantial variance for the series of compounds. They were very slow for the reactions involving the two azaphenylalanine peptides. In contrast, the reactions involving the three aza-alanine peptides had high turnover rates with Ac-Ala-Ala-Pro-Aala-ONp having the highest. The azapeptides possessing longer side chains on the P1 aza-amino acid showed intermediate turnover rates. The initial burst of p-nitrophenol had a 1:1 stoichiometry with respect to enzyme concentration and commercial subtilisin BPN' was found to be ~82% active. The acylation rates (kν) for Ac-Aphe-ONp, Ac-Ala-Aphe-ONp, Ac-Ala-Aphe-ONp, and Z-Ala-Ala-Pro-Ala-ONp were >0.20 s⁻¹ at pH 5.8 and 5.0, although the rates at pH 5.0 for Ac-Aphe-ONp and Ac-Ala-Ala-ONp appeared to be slightly slower than the others.

Table III shows the results for the experiments in which the azapeptides were reacted with subtilisin Carlsburg. The two azaphenylalanine peptides were again found to have the lowest turnover rates while the aza-alanine peptides were turned over very rapidly. The azapeptides containing larger alkyl side chains again had intermediate turnover rates. The Azapeptide PH IE f(En,En) Ac-Ala-Ala-Pro-Ala ONp 5.0 4.9 3.5 4.5

**Table I**

| Azapeptide       | pH | [En]_{110} | [En]_{100} % purity | k_{100} x 10^6 |
|------------------|----|------------|---------------------|----------------|
| Ac-Aphe-ONp      | 4.0| 4.4        | 3.7 84               | <1.8           |
|                  | 5.0| 4.4        | 3.7 84               | <1.8           |
|                  | 6.0| 4.4        | 3.5 80               | <1.8           |
| Ac-Ala-Aala-ONp  | 4.0| 4.4        | N.R.                |                |
|                  | 5.0| 4.4        | N.R.                |                |
|                  | 6.0| 4.4        | N.R.                |                |
| Ac-Ala-Aleu-ONp  | 4.0| 4.6        | 3.4 74               | <1.8           |
| Ac-Ala-Aphe-ONp  | 4.0| 5.4        | 4.5 83               | <1.8           |
| Ac-Ala-Ava-ONp   | 4.0| 5.4        | 4.3 80               | <1.8           |
| Ac-Ala-Aleu-ONp  | 7.0| 5.4        | 4.3 91               | <1.8           |
| Ac-Ala-Aala-ONp  | 4.0| 4.4        | 3.8 96               | <1.8           |
|                  | 5.0| 4.4        | 3.8 96               | <1.8           |
|                  | 6.0| 4.4        | 3.8 96               | <1.8           |
| Ac-Ala-Anle-ONp* | 5.0| 4.4        | 3.8 96               | <1.8           |
| Ac-Ala-Anle-ONp* | 6.0| 4.4        | 3.8 96               | <1.8           |
| Ac-Ala-Anle-ONp* | 7.0| 4.4        | 3.8 96               | <1.8           |
| Ac-Ala-Anle-ONp* | 8.0| 4.4        | 3.8 96               | <1.8           |
| Ac-Ala-Anle-ONp* | 9.0| 4.4        | 3.8 96               | <1.8           |
| Ac-Ala-Anle-ONp* | 10.0| 4.4      | 3.8 96               | <1.8           |
| Ac-Ala-Pro-Ala-ONp| 4.0| 4.5        | 4.5 83               | <1.8           |
|                  | 5.0| 4.5        | 4.3 80               | <1.8           |
|                  | 6.0| 4.5        | 4.3 80               | <1.8           |
|                  | 7.0| 4.5        | 4.3 80               | <1.8           |

*Enzyme concentration determined from the burst (ΔA_{110}) of HONp.

**Table II**

| Azapeptide       | pH | [En]_{110} | [En]_{100} % purity | k_{100} x 10^6 |
|------------------|----|------------|---------------------|----------------|
| Ac-Ala-Aala-ONp  | 4.0| 4.4        | 3.7 84               | <1.8           |
|                  | 5.0| 4.4        | 3.7 84               | <1.8           |
|                  | 6.0| 4.4        | 3.4 77               | <1.8           |
| Ac-Ala-Aala-ONp  | 4.0| 4.4        | 3.4 77               | <1.8           |
|                  | 5.0| 4.4        | 3.4 77               | <1.8           |
|                  | 6.0| 4.4        | 3.4 77               | <1.8           |
| Ac-Ala-Ala-ONp   | 4.0| 4.2        | 3.1 88               | <1.8           |
| Ac-Ala-Ala-ONp   | 4.0| 4.1        | 3.2 78               | <1.8           |
| Ac-Ala-Ala-ONp   | 5.0| 4.1        | 3.5 85               | <1.8           |
| Ac-Ala-Ala-ONp   | 6.0| 4.1        | 3.7 88               | <1.8           |
| Ac-Ala-Ala-ONp   | 7.0| 4.1        | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 8.0| 4.1        | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 9.0| 4.1        | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 10.0| 4.1      | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 11.0| 4.1      | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 12.0| 4.1      | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 13.0| 4.1      | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 14.0| 4.1      | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 15.0| 4.1      | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 16.0| 4.1      | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 17.0| 4.1      | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 18.0| 4.1      | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 19.0| 4.1      | 3.8 92               | <1.8           |
| Ac-Ala-Ala-ONp   | 20.0| 4.1      | 3.8 92               | <1.8           |

*Enzyme concentration determined from the burst (ΔA_{110}) of HONp.

Reaction of Chymotrypsin G with Azapeptides—Table IV shows the results of the reactions between chymotrypsin G (several different preparations) and each of seven azapeptides. Azapeptides which contained Aphe, Aleu, and Aval acylated the enzyme very rapidly and with 1:1 stoichiometry with respect to the enzyme concentration. With Ac-Ala-Aphe-ONp, the reactions had very small turnover rates and K_{m} remained constant at inhibitor concentrations of 0.06-0.2 mM. The peptides with long aliphatic aza-amino acid (e.g. Aval, Anva, Aleu, Anle) acylate the enzyme with no measurable turnover rates. Ac-Ala-Ala-Ala-ONp does not react with chymotrypsin G at pH 6-7. Three different batches of chymotrypsin G were found to be ~62%, 88%, and 45% active with respect to protein concentration determined as A_{110}.
Previous studies in this laboratory have shown that Ac-Ala-Ape-ONp does not react with trypsin at pH 5.8 (Powers and Carroll, 1975), while Ac-Ala-ONp has been reported to acylate both chymotrypsin A, and trypsin at pH 7.04, but with an acylation rate much slower for trypsin (Elmore and Smyth, 1968).

**Determination of Deacylation Rates**—The rates of deacylation for the reaction of chymotrypsin with Ac-Ala-Ape-ONp were measured by employing the substrate Ac-Ala-Ape-ONp to monitor the increase in enzymatic activity as excess azaepitope was removed by hydrolysis and dilution. Although the reaction mixture was diluted by a factor of 10, the relative ratios of azaepitope to enzyme were essentially identical with those used in the previous experiments and comparison with the previous results can be made. Table V shows the results. All the deacylation rates were slower than the lower limit (1.8 \times 10^{-4} s^{-1}) observed in the spectrophotometric assay.

**Inhibition of Cathepsin G with Azaepitope Analogs**—Ac-Ala-Ala-Anle-ONp acylated cathepsin G stoichiometrically forming an acyl-enzyme with a very small turnover rate and behaves like an irreversible inhibitor. Therefore, we decided to investigate the inhibition of cathepsin G with azaepitopes possessing three different leaving groups (—OPh, —OCH2CF3, and —OC2H5) in place of p-nitrophenol. Table VI shows the results. Ac-Ala-Ala-Anle-OPh and Ac-Ala-Ala-Anle-OCH2CF3 irreversibly inhibited cathepsin G, while Ac-Ala-Ala-Anle-OC2H5 was a poor inhibitor. The azaepitopes with a P3 Anle residue were much poorer inhibitors and the ethyl ester did not react at all with the enzyme over the course of 20 min. Preliminary studies with chymotrypsin also indicate that both the azaepitope phenyl ester and trifluoromethyl ester...
ester good inhibitors (data not shown).

The kinetics of inhibition was investigated over a range of inhibitor concentrations in order to treat the data according to Kitz and Wilson (1970). None of the inhibitors showed any variation in $k_{\text{obs}}$ over the concentration range investigated. This indicates that $K_i$ was much greater than the inhibitor concentrations utilized and that $k_{\text{cat}}/K_i$ under the experimental conditions utilized.

**DISCUSSION**

The goals of our research with azapeptides were 3-fold. First, we wished to prepare suitable stable acyl-enzyme derivatives of serine proteases for possible future crystallographic investigations. We were interested in developing new series of active site titrants for various serine proteases. It is evident that azapeptides are suitable for all three uses.

Several azapeptides containing a PI aza-amino acid residue have previously been shown to react stoichiometrically with serine proteases (Kurtz and Niemann, 1961, a and b; Elmore and Smyth, 1968; Powers and Carroll, 1975; Powers and Gupton, 1977). Although there is no direct evidence, it is almost certain that the azapeptides are acylating the active site serine of serine proteases to form a stable acyl-enzyme derivative. The acyl-enzyme mechanism for the serine protease-catalyzed hydrolysis of amides and esters containing a PI aza-amino acid residue investigated in those of the shorter Aphe derivatives. This is consistent with the deacylation rate being 15-fold faster at pH 7 compared to pH 5. The stability of the acyl derivatives formed from azapeptides and their close resemblance to true acyl derivatives makes azapeptide derivatives quite suitable for crystallographic investigations of acyl-enzyme intermediates of serine proteases.

The stability of acyl derivatives formed upon reaction of azapeptides with P1, aza-amino acid residues with serine proteases can be attributed to two factors. The first is electronic. The acyl derivative formed upon acylation of the active site serine residue with an azapeptide is a carbazic acid ester or carbanzyl carbon (RCO-NHNH-CO-O-serine) compared to the ester which is formed from normal amide or ester substrates (RCO-NHCH(R)-CO-O-serine). In the carbazyl, the carbanzyl carbon is much less electropositive than the ester due to the resonance effect of the adjacent nitrogen atom. Thus, it would be much less susceptible to nucleophilic attack by water which is required for deacylation to take place. Carbamates, which also share this same structural feature with carbazates, are well known to form stable acyl derivatives with serine proteases and indeed the crystal structure of carbamyl chymotrypsin (NH2-CO-O-serine) has been determined by x-ray crystallography (Robillard et al., 1972).

The second reason for the stability of the acyl derivatives formed from azapeptides is steric. When one examines the structures of the two acyl-envelope derivatives of chymotrypsin whose structures have been examined crystallographically, it is evident that there are significant differences with the hypothetical structure of a true acyl-enzyme intermediate. In both carbamyl chymotrypsin and in indoleacryloyl chymotrypsin (Henderson, 1970), the plane of the acyl-envelope group is twisted away from that which would allow optimum attack by the water molecule which must be deprotonated by histidine-57 (Robillard et al., 1972). In the case of indoleacryloyl chymotrypsin, binding of the rigid indole-acryloyl moiety in the S1 pocket of the enzyme forces the carbazyl out of proper alignment. In contrast, the carbamyl derivative has nothing forcing it to occupy any particular conformation and it simply adopts a conformation which allows it to participate in a favorable hydrogen bonding network.

The change in the P1 residue of a peptidyl acyl-enzyme from an amino acid residue to an aza-amino acid residue would have considerable influence on the geometry of the carbonyl group of the acyl-enzyme. The α-carbon of an amino acid residue is tetrahedral, while the α-nitrogen atom of an aza-amino acid residue is probably trigonal and the N-CO-0 atom. Thus, it would be much less susceptible to nucleophilic attack by water. The change in the PI residue of a peptidyl acyl-enzyme has a half-life of over 5 days. This is 5–6 orders of magnitude more stable than the corresponding acyl derivative formed from substrates. For example, Ac-Aphe-chymotrypsin has a deacylation rate of 1.2 $\times$ 10^{-4} s^{-1} at pH 7.0 and 37 °C (Barker et al., 1974) compared to 72 s^{-1} for Ac-Phe-chymotrypsin at pH 7.0 and 25 °C (Zerner et al., 1964). The deacylation rate for Ac-Ala-Aphe-chymotrypsin at pH 7.0 and 25 °C which we found to be 0.9 $\times$ 10^{-4} s^{-1} is quite comparable to the value of 1.2 $\times$ 10^{-4} observed with Ac-Aphe-chymotrypsin. The deacylation rate is pH-dependent with the deacylation rate being 15-fold faster at pH 7 compared to pH 5. The stability of the acyl derivatives formed from azapeptides and their close resemblance to true acyl derivatives makes azapeptide derivatives quite suitable for crystallographic investigations of acyl-enzyme intermediates of serine proteases.
appears that the azapeptides with the larger side chains interact with the S$_1$ pocket to twist the acyl carbonyl group into a conformation or conformations which are unsuitable for deacylation. As the side chain decreases in size, it can slide around more freely in the S$_1$ pocket and it becomes more likely that a proper deacylation conformation can be formed. Thus, deacylation rates increase as the size of the side chain decreases. The behavior of cathepsin G resembles that of chymotrypsin since all the acyl derivatives except one were stable.

**Active Site Titration—Azapeptide p-nitrophenyl ester**

should be quite useful as active site titrants for serine proteases. Elmore and Smyth (1968) first showed that Ac-Aphe-ONp was a useful active site titrant for chymotrypsin and in this and the following paper (Powers et al., 1984) we have considerably expanded the number of reagents which are available for use with serine proteases of varying specificity. There are several conditions which must be met for an azapeptide p-nitrophenyl ester to be a suitable active site titrant. These are: (a) [S] > [E], (b) $k_d > k_a$, and (c) $S > K_M$. The first is easily met by choice of reaction conditions. But the last two must be checked with each new enzyme or set of titration conditions. The second condition, $k_d > k_a$, is easily met with most of the enzymes and azapeptides which we studied. In most cases, the acylation rates $k_a$ were greater than 0.2 s$^{-1}$ and the deacylation rates $k_d$ were slower than $1.8 \times 10^{-4}$ s$^{-1}$. However, with subtilisin at pH 7.0, some of the acyl derivatives deacylated very rapidly and it is likely that this condition is not met. In these instances, it would be necessary simply to carry out the titrations at lower pH values to avoid this problem. The final condition for an accurate titration requires determination of $K_M$ or study of the concentration dependence of the titration. With chymotrypsin and the subtilisins, we did not carry out such studies. Instead, we simply checked our titration results using the more widely studied titrant 2-hydroxy-5-nitro-o-toluenesulfonic acid sulfone (Kezdy and Kaiser, 1970) to demonstrate that the titration conditions were giving valid results. However, in the case of cathepsin G, an enzyme for which no other titration procedure has been reported, we carried out concentration dependence studies at pH 6.0 with some of the azapeptides. As can be seen from the data in Table IV, titration at low concentrations of some of the azapeptides resulted in enzyme concentrations which were too low. However, as the concentration of azapeptide was increased, the $k_{cat}$ (e.g. Ac-Ala-Aphe-ONp) or the burst reached a plateau indicating that the [S] > $K_M$ condition was being met.

The most useful titrant for any particular titration would be dependent on the needs of the individual investigation. For titrations of cathepsin G in our laboratory, we frequently use Ac-Ala-Ala-Anle-ONp at concentrations of $\sim$0.20 mM at pH 6.0 or 7.0. We average three or more replicates to obtain the final enzyme concentration. Many of the other azapeptide derivatives are equally suitable, but this titrant is easily synthesized and is also suitable for titration of human leukocyte elastase (Powers et al., 1984). If specificity was an important consideration, then it would be better to use Ac-Aphe-ONp or Ac-Ala-Aphe-ONp. Ac-Aphe-ONp will react with both chymotrypsin and trypsin at pH 7.04 (Elmore and Smyth, 1968), while we have found that Ac-Ala-Aphe-ONp reacts with chymotrypsin A$_1$ and A$_2$, subtilisins BPN' and Carlsberg, and cathepsin G but does not react with trypsin, porcine pancreatic elastase, and human leukocyte elastase (Powers and Carroll, 1975; Powers et al., 1984). These reagents would be useful if were necessary to determine the concentration of one enzyme in the presence of small amounts of other contaminating serine proteases. Similarly, Ac-Ala-Ala-ONp could be used to titrate subtilisins in the presence of chymotrypsin or cathepsin G, although it is not likely that this would be necessary in many instances.

**Inhibitors**—Finally, we examined azapeptides as potential inhibitors of serine proteases. Azapeptides which acylate serine proteases rapidly to form stable acyl-enzymes are in essence good inhibitors. With chymotrypsin and cathepsin G, these criteria are met with almost all the azapeptide p-nitrophenyl esters. In contrast, none of the azapeptides can be considered to be suitable inhibitors for the two subtilisins. The most stable acyl derivatives formed from subtilisin had half-lives of less than 6 min at pH 7.0.

The nature of the leaving group (P,') has a considerable influence on the rate at which a peptide will react with a serine protease. For example, in the series of 4-nitroanilide substrates Ac-Ala-Ala-Pro-AA-NA, Zimmerman and Ashe (1977) showed that cathepsin G would only hydrolyze the AA = Phe and Leu derivatives, while Ala, Val, and Ile were untouched. In contrast, with a set of more reactive thienobenzyl ester substrates Boc-Ala-Ala-AA-SBzl, we have observed that the AA = Phe, Nle, Leu, and Nva derivatives were hydrolyzed by cathepsin G. The azapeptide p-nitrophenyl esters are in the same high reactivity category and show very little specificity in their reaction with any particular serine protease. One way of increasing the specificity would be to change the nature of the leaving group in the azapeptide structure. The use of p-nitrophenol allows the reaction to be followed quite readily, but is not an optimum choice if specificity is the major goal.

The results in Table VI show that cathepsin G can be inhibited by azapeptides with a variety of leaving groups. In the series of compounds which we examined, the order of reactivity is --ONp > --OPh > --OCH$_2$CF$_3$ > --OEt. It is clear that the ethyl ester is just at the borderline in terms of reactivity. In a favorable case, Ac-Ala-Ala-Anle-OEt, acylation of the enzyme and irreversible inhibition occurs, although quite slowly. In a less favorable situation (Ac-Ala-Ala-Anva-OEt), no irreversible inhibition occurs. A similar situation has been observed with chymotrypsin. Ac-Aphe-ONp has been shown to acylate chymotrypsin stoichiometrically (Elmore and Smyth, 1968). The corresponding ethyl ester Ac-Aphe-OEt was initially believed to be a competitive inhibitor with $K_I = 20$ mM at pH 7.9 and 25°C (Kurtz and Niemann, 1964a and b). However, Brown et al. (1974) later showed that chymotrypsin A$_1$ slowly lost its activity when incubated with a large excess of Ac-Aphe-OEt at pH 7.0 and 37°C. If one wishes to use azapeptides as inhibitors, it is likely that trifluoroethyl esters, phenyl esters, or something similar would be the most suitable. These derivatives would be more reactive than ethyl esters, but would probably exhibit more specificity than the p-nitrophenyl esters.

In conclusion, we have reported a new series of azapeptides which are useful as active site titrants and inhibitors of serine proteases with chymotrypsin-like specificity. In the following paper we show that these compounds can also be utilized with elastase (Powers et al., 1984).

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Reaction of Azapeptides with Chymotrypsin-like Enzymes

Azapeptide-labeled-azapeptide reaction: 3-Dimensional Letter (Ac-Ala-Val-Ala-Ala) was prepared similarly to Ac-Ala-Val-Ala-Ala test reaction from Ac-Ala-Val-Ala-Ala and isotopically labeled leucine. The product was crystallized as chymotrypsin-like 3-Dimensional Letter (Ac-Ala-Val-Ala-Ala-Asp) and isolated in pure form. The mass spectrum of the product was consistent with the expected mass of 592.866 u. The IR spectrum of the product showed bands at 1690, 1540, and 1450 cm^-1. The mass spectrum of the product showed peaks at m/z 589 (M+1), 587 (M), 585 (M-2H), and 583 (M-4H). The product was analyzed by HPLC and showed a single peak at retention time of 17.2 min.

Azapeptide-labeled-azapeptide reaction: 4-Dimensional Letter (Ac-Ala-Val-Ala-Ala) was prepared similarly to Ac-Ala-Val-Ala-Ala test reaction from Ac-Ala-Val-Ala-Ala and isotopically labeled leucine. The product was crystallized as chymotrypsin-like 4-Dimensional Letter (Ac-Ala-Val-Ala-Ala-Asp) and isolated in pure form. The mass spectrum of the product was consistent with the expected mass of 696.866 u. The IR spectrum of the product showed bands at 1690, 1540, and 1450 cm^-1. The mass spectrum of the product showed peaks at m/z 693 (M+1), 691 (M), 689 (M-2H), and 687 (M-4H). The product was analyzed by HPLC and showed a single peak at retention time of 17.2 min.
Reaction of azapeptides with chymotrypsin-like enzymes. New inhibitors and active site titrants for chymotrypsin A alpha, subtilisin BPN', subtilisin Carlsberg, and human leukocyte cathepsin G.
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