THE REACTION OF TRANS-CINNAMOYL-PAPAIN WITH A SERIES OF POLYGLYCINAMIDES OF VARYING CHAIN LENGTH*

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INTRODUCTION

The active site of lysozyme may be described as a groove running through the entire length of the molecule. (Blake, et. al., 1965; Johnson and Phillips, 1965). This finding is consistent with the substrate on which lysozyme acts, a polysaccharide. If this enzyme acting on a polysaccharide has an active site capable of interacting with six monosaccharide units, then it may be presumed that an enzyme acting on a polypeptide may have an active site of comparable complexity and length, capable of interacting with multiple amino acids of the polypeptide. Such a complex site (perhaps such sites should be called compound sites) might occur in the pancreatic proteolytic enzyme such as chymotrypsin or trypsin or the proteolytic enzymes of plant origin such as papain or ficin.

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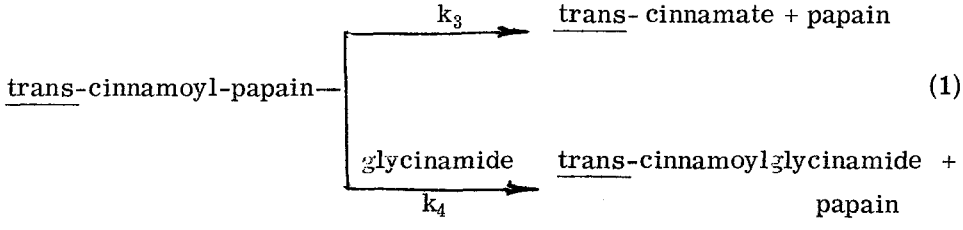
Recently it has been shown that of a series of polyalanines from L-alanyl-L-alanine to hexa-L-alanine, the last is the most specific substrate for papain and carboxypeptidase. (Schechter, Abramovitz and Berger, 1965; Schechter and Berger, 1966). Diastereomeric peptides show retarding effects of D residues even at great distances from the position of cleavage, indicating multiple attachment of the substrate to the active site. (Schechter, Abramovitz, and Berger, 1967; Schechter and Berger, 1967). These polypeptides contain negative charges, however, which may affect their overall reactivity. Further, the possibilities for non-productive binding of the polypeptides are manifold, an occurrence which may complicate the interpretation of the results. We have therefore investigated the reactivity of a series of neutral polyglycinamides in the deacylation of trans-cinnamoyl papain which follows equation 1. Glycinamide is known to be more than 100-fold poorer as a nucleophile than L-tryptophanamide toward trans-cinnamoyl papain. This difference in reactivity is ascribed to differences in binding of these two nucleophiles to the acyl-enzyme (Brubacher and Bender, 1966). Thus the nucleophilic reactivity in deacylation is a convenient and sensitive measure of binding capability to a portion of the active site. By using the polyglycinamides, we can probe the extent of a limited region of the active site, the region from the reactive sulfhydryl group to the carboxyl terminus of the site, thus reducing the possibilities of non-productive binding. Furthermore, by using the glycinamides, we can do so with neutral compounds where electrostatic interaction do not interfere.



(1)

EXPERIMENTAL

Diglycinamide hydrochloride (Grade I, Lot K-5302) was purchased from the Cyclo Chemical Corporation. After extracting with hot absolute ethanol to remove a small amount of water-insoluble material, the colorless residue melts at 191-193° C (Lit. (Pfeiffer and Saure, 1941), 185°). Analysis: Calcd. for $C_4H_{10}N_3O_2Cl$; N, 25.07%. Found;* N, 24.9%. A neutralization equivalent of 168 (M. W. 167.6) was determined by titration with standard base using a Radiometer Autotitrator Type TTTlc and Recorder Type SBR2c.

Triglycinamide acetate (Grade I, Lot K-2597) was obtained from Cyclo Chemical Corporation. Eight hundred mg of the yellow material was extracted with 30 ml of hot absolute ethanol and 250 mg of off-white material crystallized from the cooled ethanol solution. Recrystallization of this latter material from absolute ethanol gave a product which was still off-white. However, the cold mother liquor from the latter treatment, when stripped of solvent, yielded 35 mg of a white powder. After drying in vacuo over P_4O_{10}, this material begins to char at 160° but does not melt even up to 300°. It has a neutralization equivalent of 264 (M. W., 248.2). Analysis: Calcd. for $C_8H_{16}N_4O_5$; N, 22.57%. Found:‡ N, 22.83%.

Tetraglycinamide acetate (Grade I, Lot K-1671/R) was also obtained from Cyclo Chemical Corporation. Two recrystallizations from absolute ethanol yielded a white material which is so hygroscopic that neither a good melting point nor analysis can be obtained. The neutralization equivalent is 335 (M. W., 305.3).

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* Analyzed by Cyclo Chemical Corporation.

‡ Analysis by Micro-Tech Laboratories, Skokie, Illinois.

The procedures for preparing and following the deacylation of trans-cinnamoyl-papain have been described in a previous publication (Brubacher and Bender, 1966).

RESULTS

The deacylations of trans-cinnamoyl-papain in the presence of the polyglycinamides were first-order over more than 90% of reaction. The pseudo first-order reactions were assumed to follow equation 2, which stems directly from equation 1 (assuming no observable binding of the nucleophile). Plots of the observed first-order rate constants (k_{obs}) versus nucleophile concentration, shown in Fig. 1, indicate that equation 2 is being obeyed. The value

$$k_{obs} = k_3 + k_{4_{II}} \text{(Nucleophile)} \qquad (2)$$

of the second-order rate constant, $k_{4_{II}}$, was obtained from the slopes of the plots. These results are recorded in Table I.

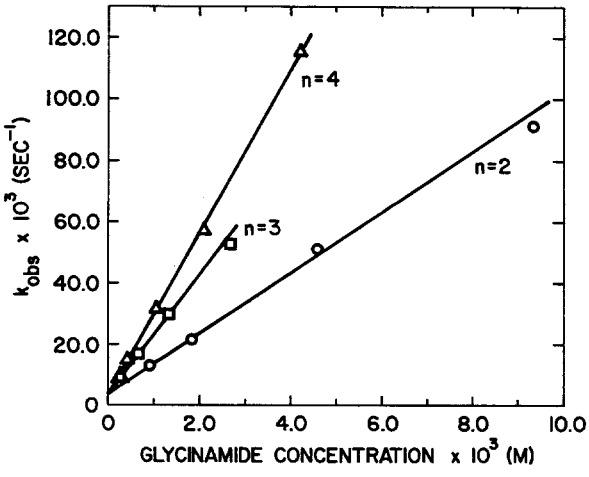

Fig. 1. The effect of nucleophile concentration on the observed first-order rate constant in the deacylation of trans-cinnamoyl-papain at pH 9.1, μ = 0.100, 25°. Nucleophiles are diglycinamide (⊙), triglycinamide (⊡), and tetraglycinamide (△).

TABLE I

Deacylation of trans-Cinnamoyl-Papain in the Presence of Polyglycinamides[a]

Nucleophile	pK_a	Conc. Range (free base) x 10^3 (M)	No. of Det'ns.	k_{4II} ($M^{-1}sec^{-1}$)	Relative k_{4II}
Glycinamide[c]	7.8	4.5 - 45.	4	0.533	1.0
Diglycinamide	7.7[b]	0.92 - 9.3	4	9.89	19
Triglycinamide	7.8[b]	0.27 - 2.7	4	19.5	37
Tetraglycinamide	7.8[b]	0.21 - 4.2	5	26.4	50
L-Tryptophanamide[c]	7.6	0.10 - 1.0	4	64.5	121

a. 0.0125 M borate buffer, μ = 0.100, pH 9.1, 25.0 $\pm$ 0.2°.

b. As determined from titration curves; uncertainty is $\pm$ 0.1 pH unit.

c. See Brubacher and Bender, 1966.

DISCUSSION

If the nucleophile binds to trans-cinnamoyl-papain before reacting with it, as postulated in an earlier paper, (Brubacher and Bender, 1966), $k_{4_{II}} = k_4/K_n$, assuming for simplicity that water is not an effective competitive inhibitor. Since the amines in Table I are of essentially identical basicity, they should have identical k_4 values (assuming identical orientation of the bound species); hence the differences in reactivity reflect differences in binding. No nucleophile saturation is observed with the glycinamides (see Fig. 1); hence all binding modes, whether productive or non-productive, have dissociation constants much greater than the highest nucleophile concentrations. In this circumstance, the concentration of any productive acyl-enzyme:nucleophile complex, is not affected by the existence of non-productive acyl-enzyme:nucleophile complexes. Thus deacylation kinetics observed under non-saturating conditions are not affected by non-productive modes of binding, if such occur. Therefore, in the present case, increasing $k_{4_{II}}$ values for the amines (in descending order) in Table I reflect decreasing values of the dissociation constants of the respective productive acyl-enzyme: nucleophile complexes.

Diglycinamide exhibits a dramatically greater reactivity than glycinamide; however, further lengthening of the nucleophile (by inserting more glycine residues) has only a small additional enhancement of the reactivity of the nucleophile. There appears to be a sort of "saturation" effect with respect to the number of glycine residues, n, in the nucleophile. A plot of $\log(1/k_{4_{II}})$ *vs* $1/n$, although slightly curved, gives an approximate intercept corresponding to a value of $k_{4_{II}}$ between 50 and 100 M^{-1} sec^{-1} for $n = \infty$. Whether this "maximal" value has any theoretical significance is debatable;

however, it is of interest that the best nucleophiles thus far observed (i.e. L-tryptophanamide and methyl L-tryptophanate) have rate constants which fall within this range. The observation of enhanced reactivity with nucleophiles containing three peptide units is consistent with the results of Schechter and Berger (1967) who found evidence for three sub-sites in the region from the reactive sulfhydryl group to the carboxyl terminus of the site.

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