Enzymatic Attack on Side Chains of Synthetic Polymers

CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF SPECIFIC SUBSTRATE GROUPS ATTACHED TO ACRYLAMIDE OR ACRYLIC ACID CO-POLYMERS*

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Three vinyl monomers, M 1, M 3, and M 5, in which L-phenylalanine p-nitroanilide was acylated with CH₂=CHCONH(CH₃)₂CO— (n = 1, 3, 5) were synthesized. They were co-polymerized with a large excess of acrylamide (co-polymers PAm-1, PAm-3, and PAm-5) and with a large excess of acrylic acid (co-polymers PAC-1, PAC-3, and PAC-5). In addition, M-5 was co-polymerized with acrylamide containing 2.8 mol % of the hydrophobic monomer N-acrylyl-1-naphthylamine (co-polymer PAm-5N). The rates of the chymotrypsin-catalyzed hydrolysis of the nitroanilide groups of M-5 and the various co-polymers were determined over a range of pH. For some of the systems data were also obtained over a range of substrate concentrations to derive values for $V_{max}$ and $K_m$. Results obtained with PAm-5 were found to be independent of the chain length of the co-polymer. At pH 7, 25° and with $2.7 \times 10^{-4}$ M enzyme, $V_{max}$ values for M-5, PAm-5, PAm-5N, and PAC-5 were $5.5, 5.5, 10$, and $3.6 \times 10^{-4}$ m/s, while $K_m$ values were $8.5, 16.5, 10$, and $2.2 \times 10^{-4}$, respectively. With PAC-5, the pH activity profile was shifted to higher acidities as compared to the profiles obtained with M-5 and PAm-5. The susceptibility of the co-polymers to chymotrypsin attack decreases sharply with a decreasing spacing of the L-phenylalanine $p$-nitroanilide residue from the backbone of the polymer chains.

Enzymes which have high molecular weight chain molecules as their specific substrates may be divided into two categories. The first comprises those which catalyze the scission of a bond in the chain backbone. Typical examples are the proteolytic enzymes, nuclease, and amylases. The second category of enzymes catalyze a transformation in the side chain of a linear macromolecule. This group is exemplified by enzymes catalyzing the methylation of natural polynucleotides (1), pectin-methylesterase (2), and enzymes catalyzing the hydrolysis of the sulfates of various polysaccharides (3). Even though a bond in the side chain is being attacked in these cases, the nature of the polymer backbone plays an important part in determining the enzyme specificity.

In the present investigation we have explored enzymatic attack on a sensitive bond in the side chain of a synthetic polymer where no specific interaction would be expected between the enzyme and the polymer chain backbone. We wished to determine how the characteristics of enzymatic catalysis are affected by the spacing of the sensitive bond from the chain backbone and by energetic interactions (due to Coulombic forces or to hydrophobic bonding) between the enzyme and the polymer. The process studied involved the chymotrypsin-catalyzed hydrolysis of L-phenylalanine $p$-nitroanilide residues at the end of the polymer side chains. Specifically, chymotrypsin substrates were prepared from monomers of the type

$$\text{CH}_2=\text{CHCONH(CH}_3)_2\text{CO— (n = 1, 3, 5)}$$

with $n = 1, 3$, or 5 (designated as M-1, M-3, and M-5) which were co-polymerized with a large excess of acrylamide (yielding co-polymers PAm-1, PAm-3, and PAm-5) or acrylic acid (yielding co-polymers PAC-1, PAC-3, and PAC-5). In addition, we tested the terpolymer PAm-5N prepared from acrylamide, M-5, and the hydrophobic monomer N-acrylyl-1-naphthylamine.

MATERIALS AND METHODS

Preparation of Substrates—To prepare monomer M-5 the following procedure was performed. Acylation of 6-aminocaproic acid with acryl chloride under Schotten-Baumann conditions gave a 65$\%$ yield of N-acrylyl-6-aminocaproic acid (m.p. 87-88°) (4). A solution containing 4.7 g of this material in 400 ml of dry tetrahydrofuran was cooled to $-20^\circ$ and 3.4 g of isobutyl chloroformate with 3.5 of N-methylmorpholine were added. After 20 min, 7.1 g of L-phenylalanine $p$-nitroanilide (m.p. 160-160°, literature (4) 160-161°) and another 2.5 of N-methylmorpholine were added. The mixture was kept for 2 hours at $-20^\circ$ and allowed to warm to room temperature overnight. After addition of 400 ml of ethyl acetate, the mixture was washed with saturated NaHCO₃.

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the substrate concentration \(8.95 \times 10^{-3} \text{ M}\). Studies involving M-5 as buffer solutions; unless otherwise stated, the ionic strength was 0.1 and nine p-nitroanilide residues. Kinetic runs were carried out at 25°C in expressed as the spectroscopically determined molarity of L-phenylalanine.

Adding the substrate solutions gave final chymotrypsin concentrations of \(2.7 \times 10^{-2} \text{ M}\).

Characterization of Co-polymers—The p-nitroanilide content of the co-polymers was obtained from the optical density at 315 and 350 nm, respectively, using the molar extinction coefficients of 5.66 \( \times 10^{3} \) and 9.1 \( \times 10^{3} \) found for monomer M-5. To determine the content of \(\alpha\)-acylaminoacrylamide residues, the acetyl derivative with molar extinction coefficients of 5.66 \( \times 10^{3} \) and 9.1 \( \times 10^{3} \) at 292 and 315 nm, respectively, was used as a standard.

Since the composition of co-polymers obtained at low conversion was very similar to the composition of the monomer mixture, it could be assumed that the co-monomer units were distributed at random in the polymer chain. This result was also expected since the substituents of the acrylamide were too far from the reactive vinyl group to affect its polymerization characteristics.

The intrinsic viscosity-molecular weight relations found for polyacrylamide (5) and poly(acrylic acid) (6) were assumed to be applicable to the corresponding co-polymers. Results obtained are listed in Table I.

Enzyme—Bovine pancreatic \(\alpha\)-chymotrypsin (three times recrystallized, salt-free, sterile, Lot 4787) was purchased from Schwarz/Mann Co. The enzyme concentration (milligrams per ml) was obtained by multiplying the absorbance at 280 nm by 0.486 (7) and a molecular weight of 25,000 was used to obtain the molar concentration. Adding 0.20 ml of a stock solution containing 10.72 mg of enzyme/ml to 3 ml of the substrate solutions gave final chymotrypsin concentrations of \(2.7 \times 10^{-3} \text{ M}\).

Kinetic Studies—The initial substrate concentrations \(S_i\) were expressed as the spectroscopically determined molarities of L-phenylalanine p-nitroanilide residues. Kinetic runs were carried out at 25°C in buffer solutions; unless otherwise stated, the ionic strength was 0.1 and the substrate concentration \(5.35 \times 10^{-4} \text{ M}\). Studies involving M-5 as the substrate were carried out in solutions containing 12 volume % methanol, since this compound was too insoluble in pure water. The release of p-nitroaniline was followed with a Cary 14 spectrophotometer by the absorbance at 410 nm, using a molar extinction coefficient of 8.44 \( \times 10^{3}\).

RESULTS AND DISCUSSION

The mechanism of chymotrypsin-catalyzed reactions may be represented (8) with some simplification by

\[
\text{Ch} - \text{OH} + \text{R} \text{C} = \text{O} \xrightarrow{k_1} \text{[Ch-OH-RC]} \xrightarrow{k_2} \text{[Ch-OCR]} \xrightarrow{k_3} \text{[Ch-OH-RC]}
\]

Here the designation of the enzyme by \(\text{Ch} - \text{OH}\) emphasizes the high reactivity of the hydroxyl of \(\text{Ser}_{195}\) which is acylated by the substrate to an acyl enzyme intermediate. With substrates such as N-acetyl L-phenylalaninamide, the enzyme acylation is the rate-determining step (9) and the same may be assumed to be the case with the p-nitroanilides used in the present study.

The pH dependence of \(k_r\) is determined by the requirement that \(H_{195}\) (with \(pK \approx 7\)) be in its basic form, while the formation of the enzyme-substrate association complex takes place only when the \(\alpha\)-amino group of \(\text{Ile}_{64}\) (\(pK \approx 8.5\)) is protonated (10).

Since the polymeric substrate exists in solution as a randomly coiled chain, it was considered possible that only groups on the "outside" of the molecular coil will be readily accessible to the enzyme, while those located in the "interior" of the coil at any one time might be shielded from enzymatic attack. If this reasoning is correct, then enzymatic catalysis of reactions of groups appended to polymer chains should become less efficient as the chainlength of the polymer is extended, placing an increasing fraction of the susceptible groups into the interior of the molecular coil. We carried out experiments on two preparations of \(\text{PAm-5}\) with molecular weights estimated as \(1.2 \times 10^{6}\) and \(2.5 \times 10^{6}\) but found no significant difference in their reactivities. This result is similar to that which we obtained earlier (11) in the study of reactions involving two randomly coiled polymeric species carrying reactive and catalytic chain substituents, respectively, with the rate-determining step characterized by a high energy of activation. On the other hand, in studies of kinetics of DNA renaturation, where the nucleation of the double helix is rate-determining, the rate constant for the nucleation was found to decrease with the chainlength of the polynucleotide as expected from the shielding effect discussed above (12).

The chymotrypsin-catalyzed hydrolysis rates of monomer M-5 and of its co-polymers with acrylamide (\(\text{PAm-5}\)), acrylic acid (\(\text{PAC-5}\)), and with acrylamide containing 2.8 mol % of \(N\)-acryloyl-1-naphthylamine (\(\text{PAm-5N}\) ) are plotted in Fig. 1 as a function of pH. Conventional Lineweaver-Burk plots (13) of the reciprocal reaction rate against the reciprocal substrate concentration for the four systems at pH 7 are shown in Fig. 2 and the \(V_{max}\) and \(K_m\) values extracted from the data are listed in Table II. The following points may be made:

1. The \(V_{max}\) values for monomer M-5 and co-polymer PAm-5 are identical. Thus, with this spacing of the sensitive bond from the chain backbone in this co-polymer, the enzyme acylation step does not appear to be impeded by the polymer chain. The 9-fold higher value of the \(K_m\) characterizing the co-polymer compared to the monomer contains a small contribution from a change in the solvent medium, since M-5
could only be held in aqueous solution by addition of 12 volume % methanol. When the co-polymer was studied in the same mixed solvents, $K_m$ was increased by 8%. The remaining difference in the $K_m$ values for monomer and co-polymer can be understood as a consequence of the need of segmental diffusion of the co-polymer to bring the bonds susceptible to enzyme attack to the surface of the molecular coil; diffusion of the co-polymer as a whole does not appear to be a limiting factor, since co-polymers differing by a factor of 5 in their chainlength reacted at identical rates. We have also tested the effect of the addition of polyacrylamide ($\eta = 0.85$ dl/g) on the appearance of the Lineweaver-Burk plot for PAm-5. This produced no change in $V_{max}$; $K_m$ was increased by about 30% at a polyacrylamide concentration of 2.4 g/liter, but changed no further when the concentration of the homopolymer was doubled. On the whole, we may then conclude that attaching a substrate group to a polyacrylamide chain has little effect on its susceptibility to enzyme attack provided the susceptible bond is sufficiently far from the chain backbone.

When the substrate group is attached to a poly(acrylic acid) chain in co-polymer PAC-5, the pH activity curve is shifted to lower pH values. This effect could be due to two causes. First, at pH values below 8.3, the isoelectric point of chymotrypsin (14), the cationic enzyme would be attracted to the anionic polymer and this would be expected to reduce $K_m$. Second, the high local hydrogen ion concentration in the immediate vicinity of the anionic polymer would tend to favor the protonation of His$_2^+$ and thus reduce $V_{max}$. (This would be analogous to the shift in the pH activity curves observed with enzymes immobilized in an ionized cross-linked resin (13).)

The data listed for co-polymer PAC-5 in Table II seem to illustrate both these effects. However, we found that a change in ionic strength from 0.06 to 0.3 had no significant effect on the reaction rate at pH 6.

3. The incorporation of the naphthyl residues into co-polymer PAm-5N increased substantially the susceptibility of the substrate groups in the co-polymer side chains to chymotrypsin attack, in spite of the rather low concentration of these hydrophobic groups along the chain molecules. Surprisingly, this effect was due to an increase in $V_{max}$. Erlanger et al. (16) have described allosteric activation of chymotrypsin by an azobenzene derivative and a similar mechanism may be involved in our case.

When we decrease the spacing of the sensitive bond from the polymer backbone below that which exists in the M-5 co-polymers, the susceptibility to enzymatic attack is sharply reduced. This effect is illustrated in Fig. 3. As may be seen, the decrease in the susceptibility to enzyme action in passing from M-5 to M-3 co-polymers is much more precipitous in the case of the acrylic acid co-polymers than with acrylamide co-polymers. Unfortunately, Lineweaver-Burk plots of data obtained with M-5 and M-3 co-polymers have a slope which is so steep that any estimate of the intercept has a prohibitively large error; thus it is not possible to analyze the dependence of $V_{max}$ and $K_m$ on the spacing of the phenylalanine p-nitroanilide residue from the polymer chain backbone. We may note that the difficulty with which enzymes attack substrates attached too close to a polymer backbone is analogous to the need for an "anchoring arm" spacing a competitive inhibitor from a cross-linked polymer network in the affinity chromatography of enzymes (17, 18).

Studies of the type we are reporting may be useful in providing background material for the design of substances of pharmacological interest. Batz et al. (19) have reported methods for the synthesis of polymeric drugs and they point out that these are of two types, i.e. those which appear to have a physiological effect without degradation of the polymer chain and those in which an active small molecule must be detached from the polymer before it can perform its function. In the latter case, the sustained release of a drug from an inactive precursor may lead to prolonged activity; evidence for phenomena of this kind has been reported from a number of laboratories (20-25) and the general field has been summarized by Donaruma (26). We should like to draw special attention to the report by Jatzkewitz (20) that mescaline attached to a pyrrolidine-acrylic acid carrier through a glycylleucyl spacer is effective in experimental animals. Characteristically, no mescaline release could only be observed when the mice were injected with the co-polymer.
carrying the drug directly attached to the chain backbone. These results carry the strong implication that the action of an enzyme on the group by which the mescaline is attached to the polymer depends strongly on the spacing of the sensitive bond from the polymer chain backbone. Nevertheless, it is important to confirm this interpretation by in vitro studies. This purpose of the present investigation has previously been the subject of an unpublished investigation by Blumberg (27). He used as his parent polymer an alternating ethylene-maleic anhydride co-polymer and condensed about 10% of the anhydride groups with a polyglycyl-3-nitrotyrosyl ester. It was found, in analogy with our results, that a tetraglycyl spacer was required before the susceptibility of the polymer to chymotrypsin attack was comparable to that of an analogous low molecular weight substrate.

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