Inhibition of α-Chymotrypsin by D-Tryptophan Amide Covalently Bound to Macromolecular Carriers: Specific, Steric, and Electrostatic Effects†

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ABSTRACT: Inhibitors for α-chymotrypsin of the general formula Gly_n-D-Trp-NH_2, where n = 0–4, as well as their corresponding *N*-acetyl derivatives were prepared. Water-soluble branched polyanionic derivatives of the inhibitors were prepared by coupling the free peptide amides to the backbone of an ethylene–maleic acid copolymer via their amino-terminal group. Additionally, branched polycationic and neutral derivatives of the inhibitors were prepared by condensation of the free carboxylic groups of the polyanionic derivatives with 1,2-diaminoethane and 2-aminoethanol, respectively. The inhibitory properties of the different branched polymers were compared with those of the corresponding *N*-acetyl oligopeptides. At high ionic strength $\Gamma/2$ = 1.0, where electrostatic interactions are largely suppressed, the distance of the inhibitory ligand from the polymer backbone is the important parameter which determines inhibition efficiency. On increasing the number of glycine residues of the side chain, efficiency of inhibition increases progressively. Thus, whereas the branched polymers in which n = 0 show no inhibitory activity, polymers with n = 3 or 4 resemble in their inhibition efficiency the corresponding low molecular weight oligopeptides. The ionic strength of the medium is also an important parameter which determines the efficiency of the polymeric inhibitors. A decrease in the ionic strength from $\Gamma/2$ = 1.0 to $\Gamma/2$ = 0.1, at pH 8.0, resulted in a 90-fold increase in the effectiveness of the polyanionic inhibitors relative to that of the analogous peptides. In contrast, inhibition of acetylchymotrypsin by the polyanionic inhibitors or of α-chymotrypsin by polycationic inhibitors, at pH 8.0, decreases with decreasing the ionic strength. The electrostatic potential of the polyanionic inhibitors was estimated by spectrophotometric titrations of analogous branched polymers in which a 3-nitrotyrosine ionizable chromophoric probe replaces D-tryptophan amide. The apparent pK_a values of the Tyr($3NO_2$) probe in the macromolecules, when compared with the pK_a value of the Tyr($3NO_2$) chromophore in Ac-Tyr-($3NO_2$), provide a quantitative measure of the electric field around the polyelectrolyte backbone. The values of the electrostatic potential thus obtained were found to account for the electrostatic effects observed on inhibition. Inhibition by the polyanionic inhibitors, while fully competitive at high ionic strength, resembles the partially competitive type of inhibition at low ionic strength. A model proposed to explain this behavior suggests two modes of binding of the enzyme to the polyanionic inhibitor at low ionic strength: one, which is specific in nature, involves the blocking of the active site of the enzyme by inhibitory groups of the macromolecule and is subject to electrostatic interactions; the other, nonspecific mode, does not prevent interaction between the active site and small molecules but prevents interactions of the active site with the inhibitor attached to a polyanionic carrier.

Enzyme inhibitors or substrates covalently bound to macromolecular carriers find wide application in biochemical studies, i.e., in the isolation and purification of the corresponding enzymes. Although numerous examples of such affinity purification procedures have been described during the last decade (cf. Cuatrecasas & Anfinsen, 1971; Jakoby & Wilchek, 1974; Flanagan & Barondes, 1975), little attention has been devoted to the detailed mechanism by which the polymer-bound ligands interact with the enzymes. Ideally, the polymer carrier is inert and interaction of the ligand with the enzyme is governed by the intrinsic affinity between the two. In practice, however, it has long been observed that the carrier might interfere sterically with the specific interaction (Cuatrecasas et al., 1968; Cuatrecasas, 1970; Blumberg & Katchalski, 1970), or exert its effects by means of ionic and hydrophobic forces (Blumberg et al., 1970, 1972; Dudai et al., 1972; Shaltiel & Er-el, 1973; Hofstee, 1973; Wilchek & Miron, 1976).

To delineate some of the factors involved in the interaction of an enzyme with a polymer-bound inhibitor, we have synthesized a series of water-soluble branched polymers in which inhibitory side chains for α-chymotrypsin are attached covalently to a well-defined synthetic polymeric backbone. The branched polymers prepared in this study consist of a poly(ethylene–maleic acid) backbone and oligoglycine-D-Trp-NH_2 inhibitory side chains, EMA-Gly_n-D-Trp-NH_2[1] (n = 0–4) (see Figure 1). Modification of the carboxylic groups in these polymers by condensation with 2-aminoethanol or 1,2-diaminoethane yielded neutral EMA-HE-Gly_n-D-Trp-NH_2 and polycationic EMA -AE-Gly_n-D-Trp-NH_2 polymeric derivatives, respectively (Figure 1). The inhibitory properties of the different branched polymers interacting with α-chymotrypsin were examined and compared with the inhibitory properties of the low molecular weight peptide inhibitors, Ac-Gly_n-D-Trp-NH_2. The results obtained demonstrate the importance

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[1] Abbreviations used: EMA-Gly_n-D-Trp-NH_2, branched polyanionic polymers in which poly(ethylene–maleic acid) is the backbone and the side chains are (glycyl)$_n$-D-tryptophan amide groups, where n is the number of glycyl residues; the side chains are linked to the backbone by amide bonds formed between the carboxyls of the backbone and the α-amino group of the peptide; the branched polymers synthesized contain, on the average, one peptide side chain per five ethylene–maleic acid repeating units of the backbone. EMA-Gly_n-Tyr($3NO_2$) are branched polymers similar to the above in which the residue terminating the side chains is 3-nitrotyrosine instead of D-tryptophanamide. EMA-HE-Gly_n-D-Trp-NH_2, neutral derivatives of EMA-Gly_n-D-Trp-NH_2 in which the free carboxyls of the backbone were condensed with the amino group of 2-aminoethanol; EMA-AE-Gly_n-D-Trp-NH_2, polycationic derivatives of EMA-Gly_n-D-Trp-NH_2 in which the free carboxyls of the backbone were condensed with one of the amino groups of 1,2-diaminoethane; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DMF, *N*,*N*-dimethylformamide. All other abbreviations are according to IUPAC–IUB rules; see, for example (1972) *J. Biol. Chem. 247*, 977.

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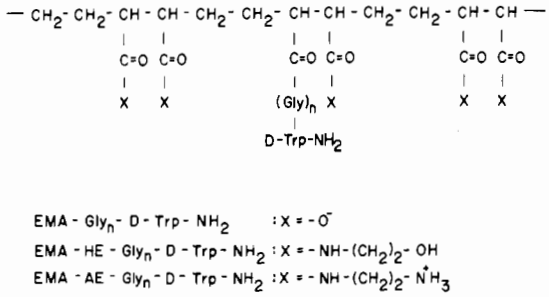


FIGURE 1: Structure of the branched polymeric inhibitors synthesized.

of steric and electrostatic effects of the polymer backbone in determining the efficiency and the mode of inhibition of the enzyme by the inhibitory macromolecules.

Materials and Methods

Enzymes. α-Chymotrypsin, 3× crystallized, lyophilized, and salt free (lot CDI 7KA), was obtained from Worthington Biochemical Corp. The enzyme was used without further purification. Stock solutions, 0.2–1.0 mg/mL, were made up in 1 mM HCl and were used within 12 h after dissolution. The normality of the enzyme, as determined by active-site titration (Schonbaum et al., 1961), was found to correspond to 80% of the total protein based on $A_{280}^{1\%} = 18.9$ (Faller & Sturtevant, 1966), mol wt 25 000 (Wilcox et al., 1957). Acetylchymotrypsin, obtained by acetylation of α-chymotrypsin with acetic anhydride, containing 10 mol of acetyl groups per mol of enzyme, was a gift from Dr. L. Goldstein. Electrophoresis on cellulose–acetate paper at pH 6.8 (Microzone Model R-100, Beckman) revealed that the derivative is negatively charged, in contrast to α-chymotrypsin which is positively charged under the same conditions.

Polymer. A linear copolymer of ethylene and maleic anhydride (molar residual ratio 1:1), DX-840-31, was a gift from Monsanto Chemical Co., Inorganic Chemical Div. A 1% solution in DMF at 25 °C yielded a specific viscosity of $\eta_{sp} = 1.0$ corresponding, according to the manufacturer, to an average molecular weight of 60 000. The polymer undergoes partial hydrolysis as a result of aspiration of moist air, but over 90% of the hydrolyzed groups could be converted back to the anhydride on heating to 110 °C for 24 h in vacuo. Complete hydrolysis of the maleic anhydride residues and the formation of a water-soluble ethylene–maleic acid copolymer could be effected by stirring in water at room temperature for 24 h.

Substrates. The following substrates were used: Ac-Tyr-OEt, Suc-Phe-OMe, Suc-Gly_2-Phe-(*p*-NO_2)anilide, and Suc-Gly_3-Phe-(*p*-NO_2)anilide.

Inhibitors. The low and high molecular weight inhibitors were: Ac-Gly_n-D-Trp-NH_2 (n = 0–4), EMA-Gly_n-D-Trp-NH_2 (n = 0–4), EMA-HE-Gly_n-D-Trp-NH_2 (n = 0, 1, 4) and EMA-AE-Gly_4-D-Trp-NH_2.

3-Nitrotyrosine Derivatives. The spectrophotometric characteristics of the following compounds were determined: Ac-Tyr($3NO_2$), Suc-Tyr($3NO_2$), and EMA-Gly_n-Tyr($3NO_2$) (n = 0, 2, 4, 6).

The preparation, analyses, and properties of the substrates, the inhibitors, and the Tyr($3NO_2$) derivatives are given in the supplementary section (see paragraph concerning supplementary material at the end of this paper).

Determination of the Concentration of the Compounds Synthesized. Concentration of D-tryptophan amide derivatives was determined by measuring the absorbance at 280 nm, using $\epsilon_{280} = 5520$ M^{-1} cm^{-1} for the tryptophan chromophore (Edelhoch et al., 1968). Concentration of Tyr($3NO_2$) derivatives was determined from the absorbance at 428 nm, measured at pH 10, using $\epsilon_{428} = 4100$ M^{-1} cm^{-1} for the *o*-nitrophenolate chromophore (Sokolovsky et al., 1966). Concentration of *p*-nitroanilide substrates was obtained from the absorbance at 313 nm, using a measured value of $\epsilon_{313} = 12800$ M^{-1} cm^{-1}. Concentration of ester substrates was derived from the alkaline uptake measured in the pH-stat during total hydrolysis by α-chymotrypsin. The concentrations of the samples, determined by the above methods, agree within 5% with the values calculated on the basis of the molecular weight, or elementary analyses, of the compounds.

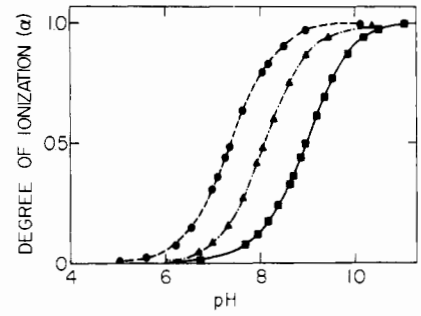


FIGURE 2: Spectrophotometric titrations of the branched polymeric derivative, EMA-Tyr($3NO_2$), at ionic strength (●) 1.0, (▲) 0.1, and (■) 0.01. The titration was carried out by adding NaOH to a solution of the polymer, 2×10^{-4} M in Tyr($3NO_2$) groups, adjusted to the appropriate ionic strength with NaCl, and monitoring the pH and the change in absorbance at 428 nm due to ionization of the Tyr-($3NO_2$) chromophore.

Spectrophotometric titrations of Tyr(3NO₂) derivatives were performed in a system consisting of a Radiometer Model 26 pH meter and a Zeiss PMQ 11 spectrophotometer. Twenty-milliliter samples of Tyr($3NO_2$) derivative, $\sim 2 \times 10^{-4}$ M in Tyr($3NO_2$) groups, were magnetically stirred in a water-jacketed cell thermostated at 25 °C and 1–5 μL aliquots of 1 N NaOH were added by means of a syringe microburet. After each addition of NaOH the pH was measured, a sample was transferred to a cuvette placed in the spectrophotometer and the absorbance at 428 nm recorded. The titration was terminated when raising the pH resulted in no further change of the absorbance.

Electrostatic Characterization of the Branched Polymers. Detailed information regarding the electrostatic character of the branched inhibitors EMA-Gly_n-D-Trp-NH_2 was achieved by spectrophotometric titrations of the analogous branched polymers EMA-Gly_n-Tyr($3NO_2$). These polymers are very similar in structure to the polyanionic inhibitors and differ from them in that the chromophoric Tyr($3NO_2$) probe replaces D-Trp-NH_2 as the residue terminating the side chains. Figure 2 shows typical spectrophotometric titrations of one such polymer, EMA-Tyr($3NO_2$), at different ionic strength values. On decreasing the ionic strength from 1.0 to 0.01, the spectrophotometric titration curves are shifted toward more alkaline pH values by about 1.5 pH units. The shape of the curves, however, resembles the titration curve of a compound with a single ionizable group. This behavior is due to the fact that most of the ionization of the 3-nitrotyrosine chromophore occurs at a pH range where the carboxylic groups of the polymer backbone are largely ionized and there is only slight variation in the degree of ionization of the poly(ethylene–maleic acid) backbone during the titration of the chromophore probe.[2] The apparent pK_a values of the chromophore in the

[2] Potentiometric titrations of the polymer backbone poly(ethylene–maleic acid) reveal two apparent ionization constants. The respective values of the apparent pK_1 and pK_2 at ionic strength 1.0, 0.1, and 0.01 were found to be 3.5 and 6.2, 3.9 and 6.9, and 4.7 and 7.9. It can be seen that, at ionic strength 1.0, 0.1, and 0.01, 75% of the carboxylic groups of the backbone are already ionized at pH 6.2, 6.9, and 7.9, respectively.

Table I: pK_a Values of the $Tyr(3NO_2)$ Chromophore at Half-Ionization and the Electrostatic Potential of Branched Polymeric Derivatives $EMA\text{-}Gly_n\text{-}Tyr(3NO_2)$ as a Function of the Ionic Strength and of the Distance of the Chromophore from the Polymer Backbone[a]

	$\Gamma/2 = 1.0$		$\Gamma/2 = 0.1$		$\Gamma/2 = 0.01$	
$Tyr(3NO_2)$ derivative	pK_a at $\alpha = 0.5$	ψ (mV)	pK_a at $\alpha = 0.5$	ψ (mV)	pK_a at $\alpha = 0.5$	ψ (mV)
$EMA\text{-}Tyr(3NO_2)$	7.35	21	8.1	51	9.0[b]	93
$EMA\text{-}Gly_2\text{-}Tyr(3NO_2)$	7.25	15	7.85	36	8.6	69
$EMA\text{-}Gly_4\text{-}Tyr(3NO_2)$	7.15	9	7.7	27	8.4	57
$EMA\text{-}Gly_6\text{-}Tyr(3NO_2)$	7.10	6	7.6	21	8.3	51
$Ac\text{-}Tyr(3NO_2)$	7.0		7.25		7.45[b]	
$Suc\text{-}Tyr(3NO_2)$	7.0		7.4		7.55	

[a] pK_a values at half-ionization were determined from the spectrophotometric titrations as described in the text. [b] pK_a values of the derivatives $EMA\text{-}Tyr(3NO_2)\text{-}NH_2$ and $Ac\text{-}Tyr(3NO_2)\text{-}NH_2$, in which the carboxylic group of $Tyr(3NO_2)$ is blocked by an amide, are 8.65 and 7.05, respectively. The difference between the pK_a values of these two derivatives, 1.6, is similar to that of the analogous derivatives with a free carboxylic group, 1.55.

different branched polymers and low molecular weight derivatives at half-ionization of the chromophore, as determined by the spectrophotometric titration method, are given in Table I. The difference between the pK_a values of the chromophore in the macromolecules $EMA\text{-}Gly_n\text{-}Tyr(3NO_2)$ (n = 0, 2, 4, 6) and in the low molecular weight derivative $Ac\text{-}Tyr(3NO_2)$, ΔpK_a, provides a quantitative measure of the electrostatic potential surrounding the polyelectrolyte backbone, according to eq 1 where e is the charge of an electron, k is the Boltzmann

$$\Delta pK_a = 0.43e\psi/kT \qquad (1)$$

constant, and T is the absolute temperature.

From the values of ψ in Table I, it can readily be seen that at ionic strength $\Gamma/2 = 1.0$ the electrostatic potential is rather small. In fact, a part of this small potential could be due to an effect of the medium, provided by the polymer backbone. At ionic strength 0.1 the electrostatic potential for the derivatives $EMA\text{-}Tyr(3NO_2)$ and $EMA\text{-}Gly_4\text{-}Tyr(3NO_2)$ is larger by 30 and 18 mV, respectively, than at ionic strength $\Gamma/2 = 1.0$. The long-range character of the electrostatic field around the polyelectrolyte backbone is best demonstrated by the fact that even for the longest side chain polymer $EMA\text{-}Gly_6\text{-}Tyr(3NO_2)$ the potential at ionic strength 0.01 is larger by 45 mV than that at ionic strength 1.0.

Rate Measurements of Enzymic Hydrolysis. Initial rates of enzymic hydrolysis of the ester substrates Suc-Phe-OMe and Ac-Tyr-OEt were determined in the pH-stat using the Radiometer titrator TTT-lc and titrigraph SBR-2c assembly. Reactions were run in a water-jacketed cell thermostated at 25 °C in a volume of 5–7 mL. The titrant was 0.02 N KOH. Nitrogen was blown over the surface of the reaction mixture to exclude CO_2.

Rates of hydrolysis of the *p*-nitroanilide substrates $Suc\text{-}Gly_2\text{-}Phe\text{-}(p\text{-}NO_2)$anilide and $Suc\text{-}Gly_3\text{-}Phe\text{-}(p\text{-}NO_2)$anilide were determined spectrophotometrically in a Gilford 2000 instrument, by following the increase in absorbance at 400 nm using ϵ_{400} = 11 700 for *p*-nitroaniline. Reactions were run at 25 °C in 1-cm pathlength quartz cuvettes, in a total volume of 2.5 mL.

Evaluation of the Apparent Inhibition Constants K_i. In all experiments the total concentration of α-chymotrypsin, $[E_0]$ (2×10^{-8} to 1.0×10^{-7} M), was well below the concentration of the low molecular weight ester or *p*-nitroanilide substrate [S] and the low molecular weight tryptophan containing inhibitor, or the concentration of the branched inhibitor [I], as expressed in molar concentration of the tryptophan residues terminating the various side chains. At the initial steps of the reaction one can thus assume that [S] and [I] equal the total concentrations of the substrate and the inhibitor. Initial velocities at varying [S] and in the absence and presence of fixed [I] were plotted in the usual double-reciprocal manner. In all cases the double-reciprocal plots of v^{-1} vs. $[S]^{-1}$ were linear and intersected on the ordinate. K_i was calculated from the intersection points on the abscissa of the curves obtained in the absence and presence of the inhibitor by using eq 2 which holds for competitive inhibition.

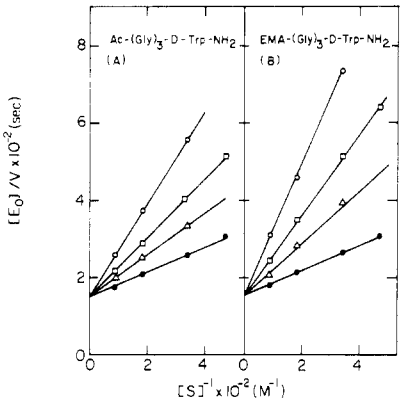


FIGURE 3: Inhibition of α-chymotrypsin-catalyzed hydrolysis of Suc-Phe-OMe by peptide and branched polymeric inhibitors at pH 8.0, $\Gamma/2$ = 1.0, 25 °C. Rates of hydrolysis of the substrate in the absence and presence of the inhibitors were measured in the pH-stat in 1 M KCl using 2×10^{-8} M enzyme. (A) Inhibition by $Ac\text{-}Gly_3\text{-}D\text{-}Trp\text{-}NH_2$: (●) no inhibitor, (△) 0.5 mM, (□) 1.0 mM, and (○) 2.0 mM inhibitor. (B) Inhibition by $EMA\text{-}Gly_3\text{-}D\text{-}Trp\text{-}NH_2$: (●) no inhibitor, (△) 0.63 mM, (□) 1.25 mM and (○) 2.5 mM inhibitor.

$$v_i = \frac{k[E_0][S]}{[S] + K_m(1 + [I]/K_i)} \qquad (2)$$

The apparent inhibition constant K_i could also be derived from experiments in which the concentration of the substrate was kept constant and that of inhibitor varied. The plotting of $(v/v_i - 1)(1 + [S]/K_m)$ vs. [I] where v and v_i are the rates in the absence and presence of the inhibitor, respectively, yielded K_i by using eq 3 and taking for K_m the value derived from the double-reciprocal plot.

$$(v/v_i - 1)(1 + [S]/K_m) = [I]/K_i \qquad (3)$$

When the above relationship was not linear over the whole range of inhibitor concentration [I], K_i was calculated from the slope at the initial portion of the graph (see eq 6, Discussion).

Results

Effect of the Distance of the Inhibitory Ligand from the Backbone on the Inhibitory Efficiency of the Branched Polymers. The branched polymers synthesized, EMA-

Table II: Inhibition Constants for Peptides and Branched Polymers Inhibiting the Activity of α-Chymotrypsin at pH 8.0, Γ/2 = 1.0, 25 °C[a]

inhibitor	no. of glycyl residues (n)	[I] range (mM)	K_i (mM)
Ac-$(Gly)_n$-D-Trp-NH_2	0	1.0–4.0	1.45
	1	0.5–2.1	0.50
	2	0.5–2.1	0.66
	3	0.5–2.0	0.73
	4	0.5–2.0	0.73
EMA-$(Gly)_n$-D-Trp-NH_2	0	1.0–10.0	>200[b]
	1	1.5–5.8	2.56
	2	0.5–1.9	1.00
	3	0.6–2.5	0.59
	4	0.5–2.2	0.69

[a] Inhibition was determined in the pH-stat, using 2×10^{-8} M enzyme and the substrate Suc-Phe-OMe in the concentration range 2.1–10 mM. The values of K_i were calculated from the double-reciprocal plots in the absence and presence of fixed [I] in the range indicated. [b] Minimal value estimated.

Table III: Inhibition Constants for Neutral, Anionic, and Cationic Branched Polymeric Derivatives Inhibiting the Activity of α-Chymotrypsin at pH 8.0, Γ/2 = 1.0, 25 °C[a]

branched polymer	type of polymer backbone	[I] (mM)	K_i (mM)
EMA-HE-D-Trp-NH_2	neutral	2.0	>50[b,c]
EMA-D-Trp-NH_2	anionic	10.0	>200[b,c]
EMA-HE-Gly-D-Trp-NH_2	neutral	3.3	4.0[b]
EMA-Gly-D-Trp-NH_2	anionic	2.5	2.56[b]
EMA-HE-Gly_4-D-Trp-NH_2	neutral	0.7	0.85[d]
EMA-AE-Gly_4-D-Trp-NH_2	cationic	0.9	0.78[d]
EMA-Gly_4-D-Trp-NH_2	anionic	1.6	0.69[b,d]

[a] Inhibition constants were calculated from double-reciprocal plots in the absence and presence of the inhibitor. [b] Inhibition was determined in the pH-stat in 1 M KCl, using Suc-Phe-OMe as the substrate. [c] Minimal values estimated. [d] Inhibition was determined spectrophotometrically in 50 mM Tris-HCl + KCl, Γ/2 = 1.0, using Suc-Gly_3-Phe-(p-NO_2)anilide as the substrate. The succinyl N-blocking group was introduced into the p-nitroanilide substrate in order to make it water soluble.

Gly_n-D-Trp-NH_2 (Figure 1), consist of oligoglycine-D-tryptophan amide side chains attached to a poly(ethylenemaleic acid) backbone. Thus, at neutral and alkaline pH they show characteristic polyelectrolyte behavior. To suppress electrostatic effects during the interaction of the polymers with α-chymotrypsin, the inhibition experiments were carried out at high ionic strength, Γ/2 = 1.0. Typical inhibitions by the polymeric inhibitor EMA-Gly_3-D-Trp-NH_2 and by the corresponding low molecular weight peptide Ac-Gly_3-D-Trp-NH_2 are shown in Figure 3. The Lineweaver–Burk plots derived in the absence of an inhibitor and in the presence of varying concentrations of inhibitors, as expressed in molar concentration of D-Trp-NH_2, were found to display all of the characteristics of competitive inhibition; the curves are linear, they intersect on the ordinate, and their slopes increase linearly on increasing inhibitor concentration. A similar kinetic behavior was observed with the other low molecular weight and high molecular weight branched inhibitors tested.

Inhibition constants for the entire series of peptide inhibitors Ac-Gly_n-D-Trp-NH_2 and the corresponding polymer derivatives EMA-Gly_n-D-Trp-NH_2 of n = 0–4 at pH 8.0 and Γ/2 = 1.0 are given in Table II. The values of K_i for all of the low molecular weight peptide inhibitors are rather similar (0.5–1.45 mM). The D-Trp-NH_2 inhibitory ligand thus seems to play a predominant role in the inhibition of the enzyme, whereas the glycyl residues of the peptide chain contribute only slightly to inhibition.

The values of K_i for the branched polymeric derivatives, on the other hand, vary markedly with the length of the side chain. EMA-D-Trp-NH_2, the derivative in which the ligand is bound directly to the poly(ethylene–maleic acid) backbone, fails to inhibit the enzyme even at a concentration of 10^{-2} M. A minimal value for this derivative, K_i >200 mM, was estimated. EMA-Gly-D-Trp-NH_2, the derivative with one glycyl residue in the side chain, inhibits α-chymotrypsin slightly, K_i = 2.56 mM, whereas the branched polymer containing two glycyl residues in the side chain approaches in its inhibitory effect the efficiency of its analogous peptide, K_i = 1.0 mM compared with 0.66 mM. Inhibition by the branched polymers with three or four glycines in the side chain is as efficient as that observed for the corresponding peptides (Table II). Apparently, the polymer backbone diminishes markedly the inhibition efficiency of the D-Trp-NH_2 ligand when the latter is closely attached to the polymer backbone. However, the effect of the backbone is completely abolished when three or four glycyl residues are interposed between the backbone and the ligand.

Effect of the Chemical Nature of the Backbone. Since the inhibitors EMA-Gly_n-D-Trp-NH_2 are polyanionic, it was of interest to investigate the inhibitory effect of analogous polycationic and neutral polymers. To attain this goal, the free carboxylic groups of the backbone were modified chemically by condensation with 2-aminoethanol or 1,2-diaminoethane yielding respectively neutral hydroxyethylcarboxamido(EMA-HE-Gly_n-D-Trp-NH_2) and positively charged aminoethylcarboxamido(EMA-AE-Gly_n-D-Trp-NH_2) branched polymeric derivatives (Figure 1) (Hoare & Koshland, 1967).

Like the anionic derivatives, the neutral derivatives with no glycines or with one glycine in the side chain do not inhibit the enzyme or inhibit it only slightly (Table III). Inhibition by the neutral or positively charged derivatives with four glycyl residues at pH 8.0 and Γ/2 = 1.0 is virtually as efficient as the inhibition by the corresponding anionic inhibitor (Table III). Since at high ionic strength the efficiency of inhibition increases with the distance of the ligand from the backbone irrespective of its charge, it appears that at high ionic strength the interfering effects of the backbone are predominantly steric.

Effect of the Electrostatic Character of the Backbone of the Branched Polymer Inhibitors. The charges of the backbone of the branched inhibitors allow detailed examination of the effect of electrostatic interactions on inhibition by controlling the ionic strength of the ambient medium. To differentiate between electrostatic effects and steric interference, the long side chain derivatives with three or four glycines were selected for study. The ionic strength dependence of the inhibition of α-chymotrypsin by the inhibitors EMA-Gly_3-D-Trp-NH_2 (Table IV) and EMA-Gly_4-D-Trp-NH_2 (Table V) was studied at pH 8.0 in the range Γ/2 = 0.1–2.0. For comparison the inhibition of the enzyme by the low molecular weight peptide Ac-Gly_4-D-Trp-NH_2 and the binding to the enzyme of the substrate employed, Suc-Gly_3-Phe-(p-NO_2)anilide, were also examined (Table V). It was found that the values of K_i and K_m for the respective low molecular weight inhibitor and substrate decrease gradually, though slightly, as the ionic strength increases from 0.1 to 2.0 (Figure 4). The multifunctional branched polymer inhibitors, in contradistinction to the monovalent peptide inhibitor, show an extremely high inhibitory effect at low ionic strength (Γ/2 = 0.1) (Figure 4). The inhibitory effect decreases on in-

Table IV: Ionic Strength Dependence of the Inhibition of α-Chymotrypsin by the Polyanionic Inhibitor EMA-Gly$_4$-D-Trp-NH$_2$ at pH 8.0, 25 °C[a]

ionic strength	[S] (mM)	[I] (mM)	K_m (mM)	K_i (mM)
0.105	0.4–1.5	0.03	1.50[b]	0.015[b]
	1.0	0.01–0.5		0.014[c]
0.210	0.4–1.5	0.18	1.09[b]	0.113[b]
	1.0	0.072–1.8		0.104[c]
1.00	0.4–1.5	1.44	0.70[b]	0.59[b]
	1.1	0.44–6.6		0.63[c]

[a] Inhibition was determined spectrophotometrically using the substrate Suc-Gly$_2$-Phe-(*p*-NO$_2$)anilide and 1.0×10^{-7} M enzyme. Experiments were performed in 50 mM of Tris-HCl + KCl added to the ionic strength specified. [b] Determined from the double-reciprocal plots. [c] Determined from the initial slope of $(v/v_i - 1)\cdot(1 + [S]/K_m)$ vs. [I] as described under Materials and Methods.

Table V: Ionic Strength Dependence of the Inhibition of α-Chymotrypsin by the Polyanionic Inhibitor EMA-Gly$_4$-D-Trp-NH$_2$ and by the Peptide Analogue Ac-Gly$_4$-D-Trp-NH$_2$ at pH 8.0, 25 °C[a]

ionic strength	K_m (mM)	[I] (mM)		K_i (mM)	
		peptide	polymer	peptide	polymer
2.0	0.26	1.8	1.8	0.56	0.40
1.5	0.38	0.6	0.7	0.67	0.63
1.0	0.57	1.6	1.6	0.74	0.69
0.55	0.67	0.8	0.6	0.83	0.53
0.27	0.83	0.8	0.3	1.05	0.28
0.21	0.91		0.32		0.14
0.11	1.00	0.7	0.02	1.25	0.021
0.105	1.00		0.02		0.018
0.10	1.00	0.7	0.05	1.25	0.014
0.16[b]	0.83		0.15		0.062
0.10[b]	0.95		0.05		0.014

[a] Inhibition was determined spectrophotometrically using the substrate Suc-Gly$_3$-Phe-(*p*-NO$_2$)anilide and 4×10^{-8} M enzyme, except when indicated. Experiments were performed in 50 mM Tris-HCl + KCl added to the ionic strength specified. K_m and K_i were derived from the double-reciprocal plots. [b] These experiments were performed in the pH-stat using Ac-Tyr-OEt as the substrate.

creasing the ionic strength and reaches an inhibitory capacity similar to that of the corresponding low molecular weight compound at $\Gamma/2 = 1.0$–2.0. The high inhibitory efficiency of the branched polymer at low ionic strength is obviously due to the electrostatic interaction between the negatively charged polyelectrolyte inhibitor and the positively charged enzyme, at the pH value employed, pH 8.0.

Effects of pH, Charge of the Enzyme, and Charge of the Backbone. In view of the above, it was of interest to test the inhibition of α-chymotrypsin by the negatively charged polymeric inhibitor while changing the net charge of the enzyme. This could be attained by varying the pH of the reaction mixture. Indeed, reducing the pH from 8.0 to 7.2 ($\Gamma/2 = 0.1$), which increases the positive net charge of the enzyme by 1–2 units (Marini & Wunsch, 1963), increases inhibition efficiency threefold, whereas raising the pH from 8.0 to 8.8 ($\Gamma/2 = 0.1$) decreases it threefold (Table VI). Even at pH 8.8, which is close to the isoelectric point of the enzyme, inhibition by the polymer inhibitor at low ionic strenth is more efficient than that of the corresponding low molecular weight peptide.

In accord with the above, it was found that inhibition of

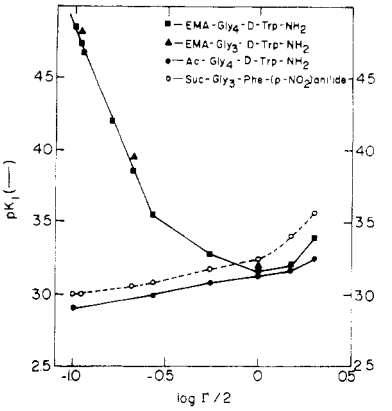


FIGURE 4: Ionic strength dependence of the inhibition constants of peptide and polymeric inhibitors acting on α-chymotrypsin. The variation of the Michaelis constant with ionic strength of the substrate Suc-Gly$_3$-Phe-(*p*-NO$_2$)anilide is also included. (■) K_i for the polymeric inhibitor EMA-Gly$_4$-D-Trp-NH$_2$. (▲) K_i for EMA-Gly$_3$-D-Trp-NH$_2$. (●) K_i for the peptide inhibitor Ac-Gly$_4$-D-Trp-NH$_2$. (○) K_m for the substrate Suc-Gly$_3$-Phe-(*p*-NO$_2$)anilide. Experimental details as in Tables IV and V.

Table VI: pH Dependence of the Inhibition of α-Chymotrypsin by the Polyanionic Inhibitor EMA-Gly$_4$-D-Trp-NH$_2$ and by the Peptide Analogue Ac-Gly$_4$-D-Trp-NH$_2$ at $\Gamma/2 = 0.1$, 25 °C[a]

pH	K_m (mM)	[I] (mM)		K_i (mM)	
		peptide	polymer	peptide	polymer
7.2	1.0	0.60	0.0085	1.25	0.0048
8.0	1.0	0.70	0.05	1.25	0.014
8.8	1.25	0.65	0.12	1.40	0.042

[a] Inhibition was determined spectrophotometrically using the substrate Suc-Gly$_3$-Phe-(*p*-NO$_2$)anilide and 4×10^{-8} M enzyme. Experiments were performed in 50 mM Tris-HCl + KCl, $\Gamma/2 = 0.1$. K_m and K_i were determined from the double-reciprocal plots.

α-chymotrypsin by the positively charged polymer–inhibitor EMA-AE-Gly$_4$-D-Trp-NH$_2$ at pH 8.0 decreases >10-fold as the ionic strength decreases from 1.0 to 0.1. Under similar conditions the inhibition of the negatively charged acetyl-chymotrypsin by the polyanionic inhibitors investigated was found to decrease on lowering the ionic strength.

Mode of Interaction of α-Chymotrypsin with Branched Polyanionic Inhibitors at Low Ionic Strength. As was noted above (Figure 3, Table II), inhibition of α-chymotrypsin by the polyanionic inhibitors is competitive at high ionic strength ($\Gamma/2 = 1.0$). This is demonstrated further in the inhibition of α-chymotrypsin-catalyzed hydrolysis of Suc-Gly$_2$-Phe-(*p*-NO$_2$)anilide by EMA-Gly$_3$-D-Trp-NH$_2$, where the double-reciprocal plots (Figure 5A) and the plot of $(v/v_i - 1)(1 + [S]/K_m)$ vs. [I] (Figure 5A′) are both characteristic of competitive inhibition. On lowering the ionic strength, the efficiency of the polyanionic inhibitors increases as a result of electrostatic interaction (Figure 4) but in addition inhibition ceases to be purely competive and acquires a partial competitive character (Figures 5B, C, B′, C′); inhibition of the enzyme in response to an increase in [I], as expressed by the plot $(v/v_i - 1)(1 + [S]/K_m)$ vs. [I], is no longer linear (Figures 5B′, C′). This implies that some of the enzyme activity cannot be inhibited even by an excess of the inhibitor. To account for this behavior, a scheme has been proposed which assumes two modes of binding of the enzyme to the polyelectrolyte inhibitor at low ionic strength (see Discussion).

Discussion

The results presented above show that the branched po-

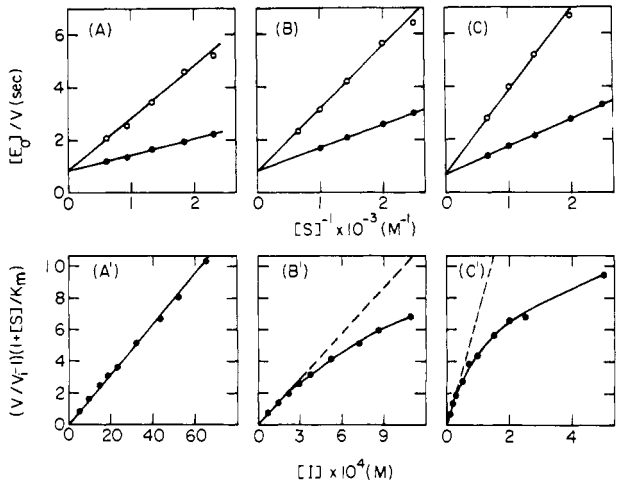


FIGURE 5: Effect of ionic strength on the mode of inhibition of α-chymotrypsin catalyzed hydrolysis of Suc-Gly_2-Phe-(p-NO_2)anilide by the polymeric inhibitor EMA-Gly_3-D-Trp-NH_2. Upper panels: Lineweaver–Burk plots in the absence (●) and presence (○) of the inhibitor. (A) Ionic strength 1.0, 1.44 mM inhibitor. (B) Ionic strength 0.21, 0.19 mM inhibitor. (C) Ionic strength 0.1, 0.03 mM inhibitor. Lower panels: Dependence of $(v/v_i - 1)(1 + [S]/K_m)$ on [I]. (A') Ionic strength 1.0, K_m = 0.7 mM, [S] = 1.08 mM. (B') Ionic strength 0.21, K_m = 1.09 mM, [S] = 1.0 mM. (C') Ionic strength 0.1, K_m = 1.5 mM, [S] = 1.0 mM. Enzyme concentration in all experiments was $\sim 10^{-7}$ M.

lyanionic derivatives EMA-Gly_n-D-Trp-NH_2 (n = 1–4) (Figure 1), at high ionic strength, inhibit α-chymotrypsin competitively similarly to the inhibition of the enzyme by the corresponding low molecular weight competitive inhibitors Ac-Gly_n-D-Trp-NH_2 (Figure 3). The effect of the polymer backbone on the inhibitory efficiency of the branched polymers, as expressed by their K_i values relative to the K_i values of the corresponding peptides, depends on the distance of the inhibitory group from the polymer backbone (Table II). The branched polymer EMA-D-Trp-NH_2, in which the D-Trp-NH_2 inhibitory ligand is bound directly to the polymer backbone, is at least 150-fold weaker an inhibitor than the corresponding low molecular weight inhibitor Ac-D-Trp-NH_2. The branched polymer EMA-Gly-Trp-NH_2 is fivefold weaker an inhibitor than Ac-Gly-D-Trp-NH_2. In contradistinction, EMA-Gly_3-D-Trp-NH_2 and EMA-Gly_4-D-Trp-NH_2 are as effective inhibitors as Ac-Gly_3-D-Trp-NH_2 and Ac-Gly_4-D-Trp-NH_2. This similarity in the inhibitory efficiency of the latter two branched polymers and their corresponding peptides shows that the macromolecular structure of the water-soluble polymeric inhibitors has no perturbing effect on the interaction with the enzyme, provided that the inhibitory ligands are sufficiently remote from the backbone. Furthermore, for α-chymotrypsin, an enzyme with only one active site per molecule, the polyvalency of the multifunctional branched inhibitors does not increase their efficiency above the level attained by the corresponding monovalent peptides.

The perturbing effect of the polymer structure in the derivatives with short side chains, at a high ionic strength, seems to result from steric interference of the polymer backbone. Indeed, such an interference was also found for the branched polymers with a neutral or a positively charged backbone (Figure 1, Table III). In these polymers it was also found that, when the side chains are relatively long, e.g., containing four glycine residues, the multifunctional inhibitors are devoid of steric interference irrespective of the structure of the backbone (Table III).

We have also examined the interaction at pH 8.0 and Γ/2 = 1.0 of α-chymotrypsin with polymer-bound substrates, EMA-Gly_n-Phe-OMe and EMA-Gly_n-Phe-(p-NO_2)anilide (n = 1, 2, 3, and 4), similar in structure to the inhibitors EMA-Gly_n-D-Trp-NH_2 and differing from them in that a Phe-OMe or a Phe-(p-NO_2)anilide hydrolyzable group terminates the side chains. It was found that the derivatives with no glycines and with one or two glycines at their side chains cannot be hydrolyzed by α-chymotrypsin, or are hydrolyzed rather poorly, whereas the derivatives with three or four glycines interposed between the backbone and the phenylalanyl residue are hydrolyzed completely and as efficiently as the corresponding low molecular weight substrates (Blumberg, unpublished results).

The above results are best viewed in terms of the structure of the active center of α-chymotrypsin. The enzyme has an extended active center that can accommodate a sequence of five amino acid residues, two residues on each side of its hydrophobic primary specificity site (Blow, 1974). The interference of the polymer backbone in preventing a free interaction of the inhibitory D-Trp-NH_2 or the hydrolyzable groups with the primary specificity site of the enzyme is apparent in the polymers possessing two or less glycyl residues in their side chains. The interference is abolished in derivatives with longer side chains.

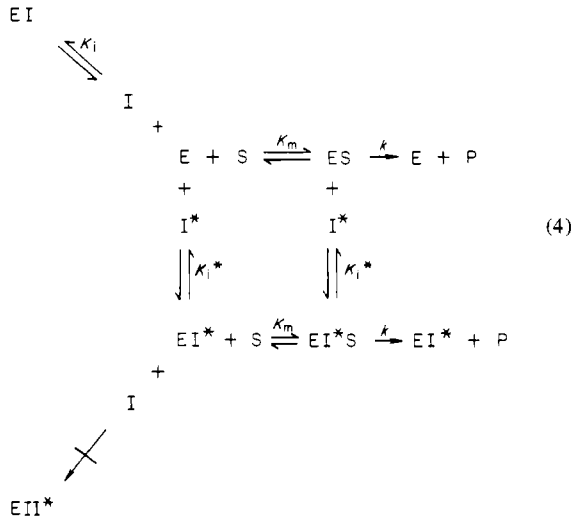
The polymers EMA-Gly_n-D-Trp-NH_2 (n = 1–4) are polyanionic, whereas α-chymotrypsin, with an isoelectric point of ~9.1 (Marini & Wunsch, 1963; Marini & Martin, 1971), is positively charged at the pH at which most of the experiments were carried out, pH 8.0. The lowering of the ionic strength from 1.0 to 0.1 thus results in an overall electrostatic interaction which increases the strength of binding of the enzyme to the inhibitory groups over and above the intrinsic affinity between the two (Tables IV and V; Figure 4). Similarly, the increase in the positive charge of the enzyme by lowering the pH from 8.8 to 7.2, at ionic strength 0.1, also results in stronger binding (Table VI). On the other hand, the strength of interaction between a polyanionic inhibitor and acetylchymotrypsin, which is negatively charged, or between α-chymotrypsin and a polycationic inhibitor decreases on decreasing the ionic strength.

Information regarding the electrostatic potential, ψ, prevailing within the domain of the polyelectrolyte branched inhibitors EMA-Gly_n-D-Trp-NH_2 was derived from spectrophotometric titrations of the analogous branched polymers EMA-Gly_n-Tyr(3NO_2), carried out at different ionic strengths for polymers with side chains of varying length (n = 0, 2, 4, and 6) (Table I). It is because of this potential that the net positively charged α-chymotrypsin is electrostatically attracted to the multifunctional polyanionic inhibitor within the pH range of 7.2–8.8. A precise calculation of the magnitude of these forces requires, however, information as to the conformation of the polyanionic inhibitor once combined with the enzyme, as well as the charge distribution on the enzyme within the complex formed. As such information is not available, no attempt has been made to calculate the electrostatic forces prevailing between the enzyme and the charged polyvalent inhibitors at the different pH and ionic strengths employed. It is of interest to note, however, that a rough estimate for the change of the inhibition constant, K_i, for EMA-Gly_4-D-Trp-NH_2 at Γ/2 = 0.1 on lowering the pH from 8.8, at which α-chymotrypsin has a positive net charge of ~1, to 7.2, at which the enzyme carries a net charge of ~+4 (Marini & Martin, 1971), using the expression $\log [K_i(\text{pH } 8.8)/K_i(\text{pH } 7.2)] = 0.43\Delta z e\psi/kT$, gives for the ratio of K_i(pH 8.8)/K_i(pH 7.2) a value of 8, assuming Δz = 3 and ψ = 18 mV. The value of ψ was derived from the difference in the electrostatic potential of EMA-Gly_4-Tyr(3NO_2) at Γ/2 = 0.1

and $\Gamma/2 = 1.0$, the latter being used as reference (see Table I). The calculated ratio for K_i(pH 8.8)/K_i(pH 7.2) of 8 is essentially the same as the experimental value derived from the data given in Table VI.

While studying the mode of interaction of α-chymotrypsin with branched polyanionic inhibitors at low ionic strength, it was found that some of the enzyme activity could not be inhibited even by an excess of the inhibitor (see Figure 5). To account for this behavior, a model is hereby proposed in which two modes of binding of the enzyme to the polyelectrolyte inhibitor are assumed: a specific mode of binding in which the active site of the enzyme is blocked by the inhibitory groups of the branched polymers, and a nonspecific mode of binding which leads to an α-chymotrypsin–polymer complex in which the active site of the enzyme is free to interact with the low molecular weight substrate, but is excluded from the inhibitory groups of the polymeric inhibitor.

The model can be depicted as shown in eq 4.



(4)

In this model I denotes the inhibiting D-tryptophan amide groups and I* the nonspecific binding sites for α-chymotrypsin. Assuming q nonspecific binding sites per D-tryptophan amide residue, one obtaines $[I^*] = q[I]$. EI* denotes the enzyme bound nonspecifically to the polymer backbone, whereas EI denotes the enzyme bound to the specific inhibitor groups, i.e., the D-tryptophan amide groups. It should be noted that in the above model it has been assumed that EI* does not react with I. This assumption stems from the fact that all the inhibitory D-tryptophan amide goups are attached covalently to the polyanionic polymer and an interaction, at low ionic strength, between the negatively charged polyelectrolyte EMA-Gly_n-D-Trp-NH_2 and enzyme bound nonspecifically to similar negatively charged polymer molecules is very unlikely.

The above model yields for the rate of substrate hydrolysis in the presence of the multifunctional inhibitor, v_i, the expression shown in eq 5, where $\beta = [EI^*]/[EI] = q(1/K_i^*)/(1/K_i)$. The ratio v/v_i between the rates of reaction in the absence and presence of inhibitor is thus given by eq 6.

$$v_i = \frac{k[E_0][S]}{[S] + K_m\left[1 + \frac{[I]/K_i}{1 + \beta[I]/K_i}\right]} \quad (5)$$

$$(v/v_i - 1)(1 + [S]/K_m) = \frac{[I]/K_i}{1 + \beta[I]/K_i} \quad (6)$$

It can be seen (eq 5 and 6) that in the absence of nonspecific binding, $\beta = 0$, the inhibition is competitive and eq 5 and 6 reduce to eq 2 and 3, respectively. For $\beta > 0$, the right side of eq 6 approaches the limiting value $1/\beta$ at high inhibitor concentration, and some of the enzyme activity cannot be inhibited.

An analysis, by means of eq 6, of the rates of hydrolysis of Suc-Gly_2-Phe-(p-NO_2)anilide by α-chymotrypsin at pH 8.0 in the presence of the multifunctional inhibitor EMA-Gly_3-D-Try NH_2 (see Figure 5) yields for β the values 0, 0.03, and 0.07 at the ionic strengths 1.0, 0.21, and 0.1, respectively, where the values of K_i were calculated from the initial slopes of the curves given in Figures 5B′ and 5C′. Thus, β increases as the ionic strength decreases. It may be expected that the value of β should also depend on the intrinsic affinity of the polymer-bound ligand to the enzyme, and on its distance from the backbone, as well as on the properties of the polymer backbone. Additionally, increasing the amount of polymer backbone relative to the inhibitory side chains should result in an increase in the amount of enzyme bound nonspecifically. Indeed, it was found that the extent of inhibition of the enzyme by high concentrations of EMA-Gly_3-D-Trp-NH_2 (0.35 mM) at $\Gamma/2 = 0.1$, pH 8.0, is reduced markedly upon adding the polymer backbone poly(ethylene–maleic acid). Apparently the added polymer binds some of the enzyme and thereby increases the proportion of enzyme bound nonspecifically relative to the amount of enzyme bound specifically.

The present work differentiates between the factors involved in the interaction of α-chymotrypsin with synthetic branched polymers carrying inhibitory D-Trp-NH_2 moieties at their side chains. While yielding specific information concerning the system investigated, the conclusions drawn are also pertinent to other protein–macromolecule interactions particularly to the complexes formed in affinity chromatography between polymer–inhibitor conjugates and the corresponding specific proteins. The results demonstrate how the interaction of a ligand with an enzyme is sterically hindered when the ligand is closely attached to a polymer backbone, and that the interaction is complete if the ligand is sufficiently remote; the optimal distance requisite for free interaction is related to the structure of the active center of the specific protein.

Our results show that substantial electrostatic interactions, either attraction or repulsion, contribute to the specific interaction between charged macromolecules and proteins even under physiological ionic strength conditions ($\Gamma/2 = 0.1$–0.2). It may seem that the polymers used in this study possess an unusually high charge density compared with biopolymers or with synthetic polymers in common biochemical use. However, α-chymotrypsin is only moderately charged compared with many other enzymes and proteins and hence the high charge density of the polymers is compensated by the low electrostatic charge of the enzyme. Additionally, we have limited our study to an ionic strength range of 2.0–0.1, whereas many studies employ lower ionic strength values. It seems to us that electrostatic interactions play an important role in many of the affinity chromatographic procedures reported in the literature, especially in cases where adsorption of a protein to an affinity resin and its desorption are carried out, each at a different ionic strength or pH value (Blumberg et al., 1970; Jost et al., 1974).

We have also shown that the charges of the backbone not only affect binding of the enzyme to the ligand but may also cause nonspecific binding where the enzyme binds to the backbone while its active site is not blocked by a ligand. The magnitude of this nonspecific binding, relative to the specific one, is increased on lowering ionic strength and is dependent on the particular resin, the intrinsic affinity of the ligand to the enzyme, the distance of the ligand from the backbone and

the relative amount of ligand and backbone in the conjugate. Such nonspecific binding of contaminating proteins is the most serious handicap in affinity chromatography.

Finally, the polymeric inhibitors prepared in this study are hydrophilic in nature and thus the interactions studied are predominantly specific and electrostatic. Interactions of proteins with polymers carrying hydrocarbon side chains or backbone are complicated by hydrophobic forces that may operate in conjunction with specific and electrostatic forces or even become a dominating factor (Blumberg et al., 1972; Shaltiel & Er-el, 1973; Hofstee, 1973; Wilchek & Miron, 1976).

Acknowledgments

The authors are indebted to Sara Rogozinsky and Raul Heller for carrying out the elemental analyses of the compounds synthesized.

Supplementary Material Available

Preparation, analyses, and properties of the substrates, inhibitors, and Try($3NO_2$) derivatives, including four tables (12 pages). Ordering information is given on any current masthead page.

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