Preferential Binding of Competitive Inhibitors to the Monomeric Form of α-Chymotrypsin*

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ABSTRACT: The binding of β-phenylpropionic and phenylpropiolic acids to α-chymotrypsin has been studied under a variety of conditions of pH and ionic strength in order to establish the relative binding affinities of monomeric and polymeric forms of the enzyme for substrate analogs. Combination of ligand-binding results, obtained by frontal gel chromatography on Sephadex G-25, and molecular weight measurements in the presence and absence of the inhibitors has allowed the qualitative conclusion to be made that the single binding site on monomeric α-chymotrypsin is partially obscured in the formation of higher polymers. Results of studies in phosphate (I = 0.28, pH 7.8) have been interpreted quantitatively in terms of a monomer–dimer acceptor system, the association constant for the interaction of phenylpropiolate with the two binding sites on the dimeric form of α-chymotrypsin being approximately four-fifths of the corresponding value for the single site on monomeric enzyme. These results, pertaining to the inhibitor-binding properties of α-chymotrypsin *in solution* correlate well with published X-ray crystallographic data, which indicate that the approach of substrate analogs to the active sites of dimeric α-chymotrypsin is subject to steric hindrance.

The coexistence of monomeric and polymeric forms of α-chymotrypsin in solution is well established, the nature and extent of this reversible association being dependent on the pH and ionic strength of the environment (*e.g.*, Schwert, 1949; Steiner, 1954; Massey *et al.*, 1955; Egan *et al.*, 1957; Nichol and Bethune, 1963; Winzor and Scheraga, 1963, 1964; Shiao and Sturtevant, 1969; Aune and Timasheff, 1971; Aune *et al.*, 1971). Timasheff (1969) attempted to explain the pH dependence of dimerization of the enzyme in terms of electrostatic interactions between specific groups on the interacting molecules and suggested that His-57, part of the active site (Schoellmann and Shaw, 1963), might be involved either directly or indirectly in the dimerization process. Subsequently, direct involvement has been postulated on the basis of sedimentation equilibrium results in the pH range 2.5–5.5, which were interpreted in terms of an interaction between the imidazole group of His-57 and the α-carboxyl of Tyr-146 (Aune and Timasheff, 1971).

From kinetic studies with acetyl-L-valine methyl ester and methyl hippurate as substrates, Martin and Niemann (1958) concluded that the dimeric form of the enzyme was less active than its monomeric counterpart–their results best fitted a model in which dimer could bind but not hydrolyze substrate. Inagami and Sturtevant (1965) pointed out that such a reaction scheme was incompatible with their kinetic data for the hydrolysis of acetyl-L-tyrosine *p*-nitroanilide in phosphate (I = 0.2, pH 6.2), but agreed with the basic conclusion of Martin and Niemann (1958) that polymeric forms of the enzyme exhibited less activity than monomeric α-chymotrypsin. Thus the kinetic evidence suggests that polymer formation *either* involves participation of the active site (or part thereof), *or* impedes the binding and/or breakdown of substrate. In either event, chemical modification of the active site could be expected to change the polymerization behavior of the enzyme.

One of the first such reports concerned the photooxidation of His-57 to yield a nonpolymerizing derivative (Egan *et al.*, 1957). Subsequently, in a more detailed study of active-site modification upon the sedimentation velocity behavior of α-chymotrypsin, Neet and Brydon (1970) have observed that the extent of interference with the polymerization reaction is dependent largely on the size of the modifying group. For example, enzyme association is prevented at pH 4 by attachment of the relatively cumbersome phenylmethanesulfonyl or cinnamoyl groups onto Ser-195, but is not affected by attachment of the smaller acetyl residue; the similarity between the polymerization behavior of α-chymotrypsin and its acetyl derivative has also been established by molecular weight estimations (Morimoto and Kegeles, 1967). Partial overlap between the active-site region and the site of polymerization provides the most direct explanation of these observations but indirect mechanisms involving conformational changes cannot be excluded.

The binding of competitive inhibitors to α-chymotrypsin possibly represents a situation bearing closer resemblance to that prevailing in the enzyme–substrate system, but such studies have yielded diametrically opposed viewpoints. Whereas Sarfare *et al.* (1966) have employed molecular weight estimations to establish that the binding of β-phenylpropionate proceeds independently of enzyme polymerization, studies of inhibitor binding by flow microcalorimetry had led Shiao and Sturtevant (1969) to conclude that dimeric α-chymotrypsin possesses little, if any, affinity for indole, *N*-acetyl-D-tryptophan, or proflavine. From a study of the binding of proflavin to α-chymotrypsin by equilibrium dialysis and temperature-jump relaxation methods, Faller and LaFond (1971) also favored a model in which inhibitor bound exclusively to monomeric enzyme, but did not consider the possibility that

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TABLE 1: Summary of Buffers Used in This Study.

Nominal Buffer Composition[a]	pH	I[b]
0.029 M Na_2HPO_4–0.114 M NaH_2PO_4	6.10	0.201
0.0272 M Na_2HPO_4–0.1048 M NaH_2PO_4–0.0200 M NaPhpl	6.11	0.206
0.0903 M Na_2HPO_4–0.0097 M NaH_2PO_4	7.80	0.281
0.0857 M Na_2HPO_4–0.0092 M NaH_2PO_4–0.0200 M NaPhpl	7.80	0.286
0.020 M NaCl–0.030 M NaV–0.008 M HV	8.45	0.050
0.020 M NaPhpl–0.030 M NaV–0.008 M HV	8.45	0.050
0.020 M NaPhpn–0.030 M NaV–0.008 M HV	8.45	0.050

[a] Phpl = phenylpropiolate; Phpn = β-phenylpropionate; V = diethylbarbiturate. [b] Calculated assuming complete ionization according to the stoichiometric formulae; values cited for phosphate buffers must therefore be regarded as approximate.

polymeric forms of α-chymotrypsin might possess binding sites with modified affinities for ligand.

In seeking to clarify the situation with respect to the possible interplay between reversible inhibitor binding and enzyme polymerization, the present study investigates the binding of β-phenylpropionate and phenylpropiolate ions to α-chymotrypsin. The comparison of the relative affinities of these inhibitors is of interest, since the structure of phenylpropiolate ($C_6H_5C{\equiv}CCOO^-$) is far more rigid and inflexible than that of β-phenylpropionate ($C_6H_5CH_2CH_2COO^-$) because of the triple bond. As well as direct binding studies by frontal gel chromatography (Nichol and Winzor, 1964; Cooper and Wood, 1968; Nichol *et al.*, 1971), results showing the effects of inhibitor binding on the extent of association of the enzyme are presented.

Experimental Section

Materials. Salt-free, lyophilized α-chymotrypsin was obtained from Sigma Chemical Co., St. Louis, Mo., the two competitive inhibitors, *viz.*, β-phenylpropionic and phenylpropiolic acids, being supplied by Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England; these materials were used as received. Glass-distilled water was used in the preparation of all buffers, their nominal compositions, determined by weight, being summarized in Table I. Buffers containing the enzyme inhibitors at a total concentration of 0.02 M were carefully adjusted to match as closely as possible the corresponding inhibitor-free buffers in terms of pH and ionic strength. The procedure involved trial titrations of the mixtures with standardized sodium hydroxide to obtain the required pH values and adjustment of buffer salt constituents to yield the desired ionic strength. Solutions of enzyme and of the inhibitors were prepared by direct dissolution into the appropriate buffer, concentrations being checked spectrophotometrically. Values of 2.50×10^2 and 1.47×10^4 were used for the molar absorptivities of 250 nm of β-phenylpropionate and phenylpropiolate, respectively, the extinction coefficient ($E_{1\,cm}^{1\%}$) of α-chymotrypsin at 280 nm being taken as 20.1 (Egan *et al.*, 1957).

Binding Studies. The binding of phenylpropiolate to α-chymotrypsin was determined by frontal gel chromatography (Nichol and Winzor, 1964; Cooper and Wood, 1968; Nichol *et al.*, 1971) on a 1.0 × 12.7 cm column of Sephadex G-25 equilibrated with the appropriate buffer. Temperature control was effected by circulating water from a constant-temperature bath through a water jacket surrounding the column. Reaction mixtures and eluting buffers were also thermostated prior to introduction onto the column. The size of each eluate fraction, collected on a time basis, was determined by reweighing the previously tared tubes. In all experiments the loading volume was 20 ml, and the flow rate was adjusted to 60 ml/hr in experiments performed at 20° and 20 ml/hr in those at 4°. This procedure ensured the elution of all solute from the column within time intervals of 1 and 3 hr at the higher and lower temperatures, respectively.

As a preliminary to the studies of enzyme–inhibitor mixtures, the frontal gel chromatographic behavior of phenylpropiolate alone was examined at 20° and also at 4°. At either temperature no concentration dependence of elution volume was observed in the range 50–250 μM, suggesting that the ligand does not self-interact: values of 1.8 and 1.9 were obtained for K_D, the distribution coefficient (Gelotte, 1960), at the higher and lower temperatures, respectively. Despite the fact that chromatographic migration of phenylpropiolate is not solely due to liquid–liquid partition, the interactions governing its migration are reversible, since (a) the concentration of inhibitor in the plateau region equalled the applied concentration in each experiment, (b) the advancing and trailing sides yielded identical estimates of K_D, and (c) all of the solute applied to the column was detected in the eluate.

Although binding experiments were completed in time intervals considerably shorter than those pertaining to a conventional equilibrium dialysis experiment, their duration was still sufficient for enzyme autolysis to pose a problem. Since the enzyme and enzyme–inhibitor complexes are excluded from the gel phase of Sephadex G-25, their velocities of migration down the column are identical, whereupon the trailing side of the elution profile should contain a plateau region extending from the void volume to the elution volume of inhibitor (Gilbert and Jenkins, 1959; Nichol and Winzor, 1964; Nichol and Ogston, 1965a). However, in these experiments the absorbancy readings decreased steadily rather than exhibiting the expected plateau (Figure 1), due to the contamination of this region with small peptide fragments. By assuming that no spectral shifts were associated with the joint presence of inhibitor (I) and fragments (F), eq 1a and 1b could be applied.

$$A_{250} = m_I(\epsilon_I)_{250} + c_F(E_F)_{250} \quad (1a)$$

$$A_{280} = m_I(\epsilon_I)_{280} + c_F(E_F)_{280} \quad (1b)$$

In these expressions A denotes the measured absorbancy in a 1-cm cell, m_I, and ϵ_I refer to the molar concentration and molar absorptivity, respectively, of inhibitor, while c_F and E_F represent the weight concentration (g/100 ml) and extinction coefficient ($E_{1\,cm}^{1\%}$), respectively, of the peptide fragments. The following values were found to apply: $(\epsilon_I)_{250} = 1.47 \times 10^4$, $(\epsilon_I)_{280} = 3.85 \times 10^3$, $(E_F)_{250} = 5.6$, and $(E_F)_{280} = 12.6$. The latter determinations involved measurement of the absorbancies at the two wavelengths of the diffusate from a dialysis experiment in which α-chymotrypsin (30 g/l.) was dialyzed against phosphate buffer (pH 7.9, $I = 0.03$) for 2 hr at 20°, conditions which were found to favor autolysis. The corresponding concentrations of the dialysable fragments were determined refractometrically and checked by evaporation and drying to constant weight over P_2O_5, corrections being

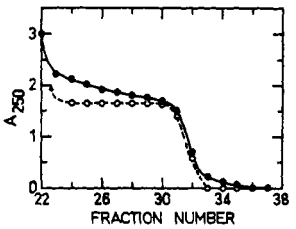

FIGURE 1: Elution profile (trailing side) obtained in frontal gel chromatography on Sephadex G-25 of a mixture containing α-chymotrypsin (10 g/l.) and phenylpropiolate (0.025 g/l.) in phosphate buffer (pH 6.1, I = 0.2). (●) Actual absorbance readings of individual fractions; (○) corresponding values corrected for protein autolysis (see text).

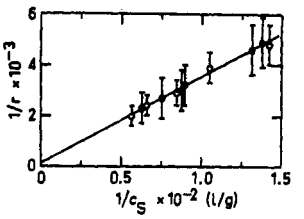

FIGURE 2: Double-reciprocal plot of binding data obtained with the α-chymotrypsin–phenylpropiolate system in phosphate buffer (pH 6.1, I = 0.2). (●) 20 and (○) 4°. Error bars denote the maximum error (not the standard error of the mean) arising from scatter of corrected absorbance readings in the inhibitor plateau region of the elution profiles (Figure 1).

made for the weight of buffer salts determined by evaporating the same volume of buffer alone. On application of eq 1a and 1b to obtain corrected values for the concentration of unbound inhibitor, the predicted plateau region was indeed observed (open circles in Figure 1), and was therefore taken as the equilibrium concentration of unbound inhibitor, c_S (Nichol and Winzor, 1964; Cooper and Wood, 1968; Nichol *et al.*, 1971). The binding function, r, was then determined as the weight of ligand bound per gram of acceptor by combining this value with those of the total inhibitor and total enzyme concentrations.

Differential Gel Chromatography. To a 1.8 × 15.7 cm column of Sephadex G-75, thermostated at 20° and equilibrated with phosphate (I = 0.28, pH 7.80), was added 50 ml of α-chymotrypsin (5 g/l.) dissolved in phosphate–0.02 M phenylpropiolate buffer (I = 0.28, pH 7.80). Eluate from the column, the flow rate of which was maintained at 17.9 ml/hr, was monitored by the biuret procedure, a wavelength of 350 nm being used for the colorimetric analysis to increase its sensitivity. A more detailed description of this differential procedure has been published elsewhere (Baghurst *et al.*, 1971).

Sedimentation. Ultracentrifugal experiments were performed in a Spinco Model E ultracentrifuge using the schlieren optical system. The resulting photographic records were measured on a Nikon two-dimensional comparator, fitted with a projection screen and accurate to 2 μm. A value of 0.736 was used for the partial specific volume of α-chymotrypsin (Schwert and Kaufman, 1951).

In order to minimize the effects of autolysis, each solution of α-chymotrypsin (with or without inhibitor) was prepared directly by weight and used immediately without prior dialysis. Molecular weights were estimated either by the Klainer and Kegeles (1955) adaptation of the Archibald (1947) method, or by the very rapid sedimentation equilibrium procedure (Yphantis, 1960). In the approach-to-equilibrium experiments, performed at 7447 rpm, measurements were confined to the air–solution meniscus, apparent weight-average molecular weights being obtained from each of four exposures taken at equal intervals throughout the 60-min duration of an individual run. These values were then extrapolated to zero time to yield the reported results, the accuracy of which is believed to be ±5%. The use of very short columns for equilibrium sedimentation represents a compromise between the need to restrict the length of time required for attainment of equilibrium and the desire to take advantage of the greater accuracy afforded by such data. An uncertainty of ±2% is believed to apply to these molecular weight estimates, each of which represents the mean of the value obtained by combining dc/dx at the column midpoint with the initial enzyme concentration and that calculated from the slope of log c *vs.* x^2 at the column midpoint. A speed of 11,272 rpm was used in the sedimentation equilibrium experiments, the duration of which was approximately 2 hrs. Doubling of the length of time available for autolysis did not seem to affect unduly the results obtained, which were indistinguishable from those determined by the Archibald method. From measurements of the amount of dialysable material liberated, it has been estimated that autolysis would have accounted for approximately 3–5% of the α-chymotrypsin by the end of an ultracentrifuge experiment.

Results

Studies at pH 6.1, I = 0.20. Figure 2 summarizes binding data obtained with phenylpropiolate at 4 and 20°, the concentration of α-chymotrypsin being fixed at 10 g/l. in each mixture. No obvious curvature of the double-reciprocal plot is observed, the line shown in Figure 2 representing that obtained by least-squares calculations. Combination of the ordinate intercept with values of 146 and 25,000 for the molecular weights of inhibitor and monomeric enzyme, respectively, yields an estimate of 0.94 for p, the number of binding sites on monomeric enzyme (from eq 9 of Nichol *et al.*, 1967), the association constant, K_A, for the interaction of phenylpropiolate with this single binding site being 680 M^{-1} (obtained from the slope of the plot). Within experimental error the same line describes the data at both temperatures, a result which signifies that complex formation between enzyme and inhibitor is accompanied by essentially zero enthalpy change. The same thermodynamic situation pertains to polymerization of α-chymotrypsin (Steiner, 1954). The final point to be noted from Figure 2 is that the linearity of the double-reciprocal plot is seemingly in accord with the conclusion of Sarfare *et al.* (1966) that a competitive inhibitor binds with equal affinity to the various oligomers of α-chymotrypsin. However, since earlier calculations (Winzor *et al.*, 1968) had shown that investigations of molecular size were likely to provide more sensitive methods for detecting preferential binding in polymerizing acceptor systems, molecular weight studies were accordingly undertaken at this stage.

The dependence of the apparent weight-average molecular weight (M_w) of α-chymotrypsin upon protein concentration ($\bar{\nu}_p$) in this environment has been studied by Rao and Kegeles (1958), the current enzyme preparation yielding values in good agreement with their results. For example, from an approach-to-equilibrium run on a 14.5-g/l. solution of α-chymotrypsin, M_w was estimated to be 42,000, a value in good agreement

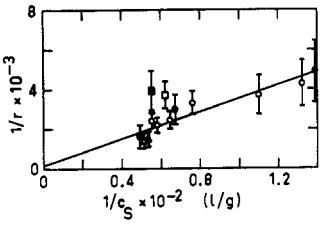

FIGURE 3: Double-reciprocal plot of binding data obtained with the α-chymotrypsin–phenylpropiolate system in phosphate buffer (pH 7.8, $I = 0.28$). (○) $\bar{c}_p = 10$ g/l., $T = 4°$; (●) $\bar{c}_p = 10$ g/l., $T = 20°$; (△, ▲) $\bar{c}_p = 5$ g/l. and $T = 4$ and 20°, respectively; (□, ■) $\bar{c}_p = 20$ g/l. and $T = 4$ and 20°, respectively. The straight line represents the theoretical relationship for $p = 1$ and $K_A = 680$ M^{-1}. Error bars denote maximum error, as in Figure 2.

with that of 43,500 inferred from Figure 1 of Rao and Kegeles (1958). However, whereas the introduction of 0.04 M β-phenylpropionate had no effect on the molecular weight of the system (Sarfare *et al.*, 1966), pronounced decreases in M_w were observed when phenylpropiolate (0.02 M) was used as the competitive inhibitor. In an experiment with 9.5 g/l. of α-chymotrypsin M_w was estimated to be 30,000 in the presence of phenylpropiolate, compared to a value of 40,000 (Rao and Kegeles, 1958) in its absence. The discrepancy persisted in experiments employing other concentrations of enzyme, as is evident from the following comparisons (values in parentheses are again those of Rao and Kegeles (1958) for the enzyme alone at the same protein concentration): $\bar{c}_p = 14$ g/l., $M_w = 31{,}000$ (43,000); $\bar{c}_p = 18$ g/l., $M_w = 32{,}000$ (46,000). This depression of M_w by the addition of inhibitor cannot be attributed to autolysis, the effect of which would be to cause underestimation of the molecular weight in the *absence* of inhibitor. Thus, in contrast with the findings of Sarfare *et al.* (1966), who used β-phenylpropionate as inhibitor, the present molecular weight studies provide evidence which favors the postulate of preferential binding of phenylpropiolate to the lower oligomers of α-chymotrypsin under these conditions, where monomer, dimer, and trimer are believed to coexist (Rao and Kegeles, 1958).

Studies at pH 7.8 I = 0.28. Binding data obtained with phenylpropiolate under these conditions are shown in Figure 3, values of 1 and approximately 680 M^{-1} being estimated for p and K_A, respectively, from experiments in which the enzyme

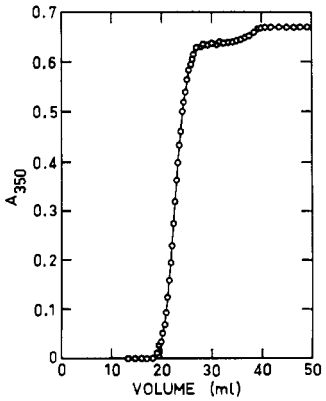

FIGURE 4: Elution profile resulting from application of 50 ml of α-chymotrypsin (5 g/l.) in phosphate–00.2 M phenylpropiolate buffer ($I = 0.28$, pH 7.8) to a 40-ml column of Sephadex G-75 equilibrated with phosphate buffer of the same pH and ionic strength.

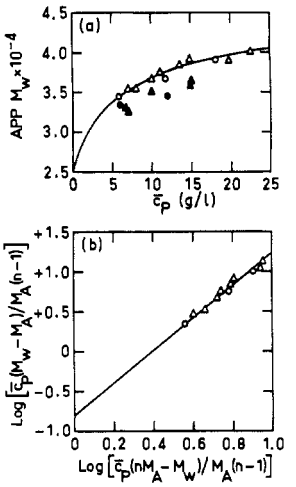

FIGURE 5: Apparent weight-average molecular weight data obtained with α-chymotrypsin in phosphate (pH 7.8, $I = 0.28$). (a) Concentration dependence of M_w for α-chymotrypsin alone (open symbols) and solutions 0.02 M with respect to phenylpropiolate (closed symbols), triangles and circles referring to experiments at 20 and 4°, respectively, (b) a subsidiary plot of the data for enzyme alone on the basis of a monomer–dimer equilibrium.

concentration, $\bar{c}_p$, was either 10 or 5 g/l. Although the close conformity of these experimental points with a straight line again seemingly negates the concept of preferential binding to monomeric α-chymotrypsin, it is noted that data from experiments employing twice the enzyme concentration (squares in Figure 3) do deviate from the line; moreover, the deviation is in the predicted direction (Figure 4 of Nichol *et al.*, 1967). Investigations of molecular size have established this point further.

Qualitative, but definitive, evidence for the preferential binding of phenylpropiolate to the lower oligomers of α-chymotrypsin was obtained by differential gel chromatography. Figure 4 presents the elution profile obtained on applying α-chymotrypsin (5 g/l.) in phosphate–0.02 M phenylpropiolate buffer (pH 7.8, $I = 0.28$) to a 40-ml column of Sephadex G-75 equilibrated with phosphate buffer having the same pH and ionic strength. Inspection of Figure 4 shows that the profile of protein concentration exhibits two distinct plateau regions, the initial elution of enzyme being at a concentration less than that applied. The boundary across which the enzyme concentration attains the applied value coincides with the appearance of phenylpropiolate in the eluate. Since no such effect is observed with α-chymotrypsin in either buffer system, it follows from considerations of mass conservation that the α-chymotrypsin must have migrated at a faster rate ahead of the inhibitor boundary than behind it. Application of the relevant moving-boundary equation (Johnston and Ogston, 1946; Nichol and Ogston, 1965b; Baghurst *et al.*, 1971) to these results yields an elution volume of 23.5 ml for α-chymotrypsin in the presence of 0.02 M phenylpropiolate, a value considerably in excess of the 22.7 ml observed in its absence. Such a difference in weight-average elution volume reflects the presence of a greater proportion of the more slowly migrating form(s) of acceptor in the enzyme solution containing inhibitor.

Quantitative information on the binding of phenylpropiolate to α-chymotrypsin oligomers under these conditions of pH and ionic strength has come from molecular weight studies. Figure 5a presents apparent weight-average molecular weight data obtained for enzyme alone at 20 and 4°. The

results are replotted in Figure 5b in a form suggested by eq 3 of Rao and Kegeles (1958), which refers specifically to a monomer–single higher polymer system ($nA \rightleftarrows A_n$). A value of 2 for n has been used, this being the only value for which the replotted data described a straight line of slope n, as required by the rearranged equation. From the ordinate intercept (log X') an estimate of 0.17 l./g is obtained for X', the association equilibrium constant describing the monomer–dimer equilibrium that prevails under these conditions of pH and ionic strength. Consistency of these values of n and X' with the experimental data is emphasized by employing them to calculate the theoretical dependence of molecular weight on protein concentration for such a system (the curve drawn in Figure 5a). Qualitatively, the effect of introducing 0.02 M phenylpropiolate into α-chymotrypsin solutions at 20 and 4° parallels that observed at pH 6.1 inasmuch as pronounced decreases in the weight-average molecular weight are again observed. In principle, such data should provide the additional information required for quantitative evaluation of the binding characteristics of dimeric α-chymotrypsin.

Consider a system in which monomeric acceptor A (molecular weight M_A), bearing p equivalent sites per molecule for ligand binding with association constant K_A, is in equilibrium with single higher polymer C (molecular weight nM_A), for which the corresponding ligand parameters are q and K_C. On addition of ligand such that its equilibrium concentration (molar scale) is m_S, the constituent concentrations (weight scale) of the two oligomeric acceptors are given by eq 2a and 2b (Klotz, 1946; Nichol *et al.*, 1967), where X' is the association constant for acceptor polymerization. Furthermore,

$$\bar{c}_A = c_A(1 + K_A m_S)^p \quad (2a)$$

$$\bar{c}_C = X'(c_A)^n (1 + K_C m_S)^q \quad (2b)$$

provided it may be assumed that ligand binding does not affect appreciably the molecular weight of either form of acceptor and that the concentration (gradient) distribution obtained after subtracting that of solvent (phosphate–inhibitor buffer) reflects only the macromolecular components of the system, evaluation of M_w, the weight-average molecular weight, yields the following expressions for $\bar{c}_A$ and $\bar{c}_c$, where $\bar{c}_p$ denotes the

$$\bar{c}_A = \bar{c}_p(M_w - nM_A)/M_A(1 - n) \quad (3a)$$

$$\bar{c}_C = \bar{c}_p - \bar{c}_A = \bar{c}_p(M_A - M_w)/M_A(1 - n) \quad (3b)$$

total acceptor concentration. Equations 2 and 3 may then be combined to yield

$$\frac{X'(1 + K_C m_S)^q}{(1 + K_A m_S)^{np}} = \frac{\bar{c}_p^{1-n}(M_A - M_w)M_A^{n-1}(1 - n)^{n-1}}{(M_w - nM_A)^n} \quad (4)$$

For the present sytem $n = 2$, $p = 1$, and $q = np = 2$, whereupon eq 4 becomes

$$(1 + K_C m_S)^2 = \frac{(M_w - M_A)M_A(1 + K_A m_S)^2}{X'\bar{c}_p(M_w - 2M_A)^2} \quad (5)$$

The value of X' has already been determined, while in instances where the total concentration of acceptor is sufficiently small compared to that of ligand, the latter may be equated with the equilibrium concentration of free S. Since an estimate of K_A (680 M^{-1}) has been obtained from the results shown in Figure 3, only K_C remains to be evaluated. Consideration of the binding sites on dimeric acceptor to be nonequivalent yields, by an analogous procedure, eq 6, in which K_{C_1} and K_{C_2}

$$(1 + K_{C_1} m_S)(1 + K_{C_2} m_S) = \frac{(M_w - M_A)M_A(1 + K_A m_S)^2}{X'\bar{c}_p(M_w - 2M_A)^2} \quad (6)$$

denote the binding constants for the two sites on C. Values of M_w pertaining to two values of m_S must now be obtained to provide sufficient simultaneous equations for the determination of the two constants describing the binding of ligand to C.

TABLE II: Molecular Weight Data for Evaluating the Binding of Phenylpropiolate to Dimeric α-Chymotrypsin at pH 7.8, $I = 0.28$.

Protein Concn ($\bar{c}_p$, g/l.)	Inhibitor Concn ($\bar{m}_S \sim m_S$, M)	Mol Wt ($M_w \times 10^{-4}$)	K_C (M^{-1}) (from eq 5)	$(1 + K_{C_1} m_S) \times (1 + K_{C_2} m_S)$ (from eq 6)	
6.80	0.02	3.30	510	128.2	129
9.80	0.02	3.46	520	129.3	
15.0	0.02	3.64	520	128.9	
8.02	0.01	3.39	520	38.44	38.2
9.61	0.01	3.47	520	38.61	
14.4	0.01	3.63	520	37.44	

Table II summarizes results obtained in order to evaluate the binding affinity of dimeric α-chymotrypsin for phenylpropiolate, the values of M_w listed in column 3 having been obtained by the very short-column procedure of Yphantis (1960). Column 4 records the estimates of K_C found on substituting the relevant values of $\bar{c}_p$, M_w, m_S, X' (0.17 l./g), and K_A (680 M^{-1}) in the eq 5. The constancy of these values establish the consistency of the data with a model in which the binding affinities of both binding sites on dimeric α-chymotrypsin are approximately four-fifths of K_A, the corresponding parameter for the one site on monomer. Although solution of eq 6 by combining results obtained with the two inhibitor concentrations leads to slightly different values of K_{C_1} and K_{C_2} (570 and 470 M^{-1}), the discrepancy between them is well with in experimental error. Certainly these values cannot be considered to refute the model in which $K_{C_1} = K_{C_2} = 520$ M^{-1}.

Studies at pH 8.45, I = 0.05. The concentration dependence of the apparent weight-average molecular weight of α-chymotrypsin alone under these conditions is shown in Figure 6a. These results support the mass migration data of earlier investigations (Massey *et al.*, 1955; Tinoco, 1957; Nichol and Bethune, 1963; Winzor and Scheraga, 1963), which indicate that at alkaline pH values and low ionic strengths polymeric species larger than trimer are formed. Although a monomer–hexamer system has been suggested (*e.g.*, Gilbert, 1955; Nichol and Bethune, 1963, Ackers and Thompson, 1965), the present results do not comply with such a model, as is evident from Figure 6b, the subsidiary plot analogous to that shown in Figure 5b. A line of the required slope ($n = 6$) has been drawn through the mean of the experimental data. Since all other values of n tested yielded equally poor correlation between theory and experiment, several oligomers must coexist under these conditions, whereupon no simple quantitative interpretation of the data is possible. For this reason, the line

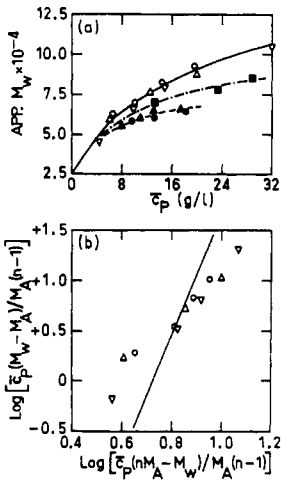

FIGURE 6: Apparent weight-average molecular weight data obtained with α-chymotrypsin in diethylbarbiturate buffer (I = 0.05, pH 8.45). (a) Concentration dependence of M_w for enzyme alone (open symbols) and in the presence of 0.02 M inhibitors (closed symbols); (△) 20, (○) 4, (▽) 20° (unpublished data of L. W. Nichol, L. L. Sia, and G. Kegeles); (▲, ●) enzyme plus 0.02 M phenylpropionate at 20 and 4°, respectively; (■) enzyme plus 0.02 M β-phenylpropionate (20°). (b) Subsidiary plot of data for enzyme alone on the basis of a monomer–hexamer equilibrium (see text).

drawn through the open symbols in Figure 6a has no theoretical significance, its sole function being to indicate the trend of the concentration dependence. In this connection it is noted that the enthalpy change for the formation of higher polymers, like that of dimer and trimer (Steiner, 1954), is essentially zero.

Solid symbols in Figure 6a summarize the effects on M_w of the addition of inhibitors to α-chymotrypsin under these conditions, the triangles and circles referring to experiments with 0.02 M phenylpropiolate at 20 and 4°, respectively, while the squares refer to α-chymotrypsin solutions containing 0.02 M β-phenylpropionate. Although the failure of the acceptor system to conform with the simple monomer–single higher polymer model precludes quantitative interpretation of these data, it is clear that both inhibitors bind preferentially to the lower oligomeric forms of α-chymotrypsin, the decrease in M_w being less pronounced in the case of β-phenylpropionate.

Discussion

The present studies of α-chymotrypsin in the absence of inhibitors substantiate and extend previous findings. Not only are the enthalpy changes involved in dimer and trimer formation essentially zero (Steiner, 1954), but those associated with formation of higher polymers are also of similar magnitude (Figure 6). In this connection, Aune *et al.* (1971) have reported that the dimerization reactions at pH 4.1 and 20° is accompanied by a very small but finite enthalpy change and further suggested that the enthalpy change of dimerization was temperature dependent in accordance with Kirchoff's law. Under conditions of relatively high ionic strength (I = 0.28) at pH 7.8 the present data (Figure 5) implicate the existence of an equilibrium mixture comprising monomer and dimer, the situation postulated by Shiao and Sturtevant (1969) for α-chymotrypsin under very similar conditions (I = 0.34, pH 7.8): indeed, there is excellent agreement between the present values of 0.17 l./g for the association equilibrium constant and that of 0.23 l./g inferred from heats of dilution. However, in view of results obtained at pH 6.1 (Rao and Kegeles, 1958), the possible coexistence of some trimer under the present conditions cannot be excluded, especially since no allowance has been made for any effects of nonideality (Adams, 1967).

Of greater importance is the detection of preferential binding of competitive inhibitors to the monomeric (or lower oligomeric) forms of α-chymotrypsin, irrespective of the nature of the oligomers present. This effect has been demonstrated by differential chromatography (Figure 4) and by decreases in the weight-average molecular weight of α-chymotrypsin on introduction of inhibitors into the system (Figures 5a and 6a). Moreover, the concept of preferential binding to monomer is not inconsistent with the binding data presented in Figure 2 and 3, where experimental error may well have masked any nonlinearity of the double-reciprocal plots. Under either set of conditions appreciable amounts of monomer exist in a 10-g/l. solution of α-chymotrypsin prior to the addition of inhibitor, whereupon the predicted deviation from linearity is extremely small.

In providing evidence that the single inhibitor-binding site on monomeric α-chymotrypsin is at least partially obscured in the formation of higher oligomers the present investigation confirms conclusions drawn from kinetic (Martin and Niemann, 1958; Inagami and Sturtevant, 1965), chemical modification (Egan *et al.*, 1957; Neet and Brydon, 1970), and inhibitor-binding (Shiao and Sturtevant, 1969; Faller and La-Fond, 1971) studies, but is at variance with those of Sarfare *et al.*, which were based on molecular weight measurements in the presence and absence of β-phenylpropionate. This seeming discrepancy will be considered later.

The factor responsible for the preferential binding of inhibitors to monomeric α-chymotrypsin could be (i) a loss of binding sites upon polymerization, (ii) a lowered affinity of polymer-binding sites for inhibitor, or (iii) a combination of both phenomena. A distinction between these three alternatives has been made on the basis of molecular weight measurements in the presence and absence of phenylpropiolate at pH 7.8, I = 0.28, conditions under which monomeric and dimeric forms of α-chymotrypsin are believed to coexist. The method of assessing quantitatively the binding characteristics of dimeric enzyme from the effect of ligand addition on M_w is similar to, but extends, that used in an earlier study of inhibitor binding to lysozyme (Sophianopoulos, 1969), where only the first alternative, *viz.*, possible loss of binding sites, was considered. From Table II the second alternative provides the most likely situation in the case of α-chymotrypsin, whereas the mechanism of dimerization proposed by Aune and Timasheff (1971) would, we feel, have favoured the first. On the other hand, the proposals are not necessarily incompatible since the dimerization reaction discussed by Aune and Timasheff (1971) involving the imidazole group of His-57 was maximal at pH 4.4 and considerably diminished at pH 5.5, whereas the polymerization reactions under study in this work were those operating in more alkaline media and thus conceivably could have proceeded by a different mechanism. In this connection, the conclusion that phenylpropiolate binds to both potential binding sites of dimeric α-chymotrypsin, but with reduced affinity compared to that of monomer, correlates well with X-ray crystallographic studies of enzyme–inhibitor complexes (Steitz *et al.*, 1969). The fact that attachment of inhibitor to one molecule of enzyme was accompanied by repositioning of part of the neighboring molecule implies steric hindrance to binding (and hence decreased binding affinity), while the retention of a local twofold axis of symmetry between adjacent α-chymotrypsin molecules implies

identical intrinsic association constants for the two inhibitor binding sites.

There remains the problem of resolving the apparent conflict between the present results with phenylpropiolate at pH 6.1 and those of Sarfare *et al.* (1966), who used β-phenylpropionate as inhibitor in experiments of otherwise identical design. One theoretical possibility is that β-phenylpropionate binds to α-chymotrypsin to a lesser extent than does phenylpropiolate: while the value of 180 M^{-1} for K_A (Neurath and Gladner, 1951) is less than that (680 M^{-1}) found for propiolate, calculations have shown that this factor alone would not have accounted for the difference in observed effects on M_w. The other theoretical possibility is that the two association constants K_A and K_C describing the binding of inhibitor to monomer and polymer(s), respectively, are more similar in magnitude for β-phenylpropionate than for phenylpropiolate, whereupon smaller decreases in M_w on inhibitor addition would be predicted for the former. To this end it is noted that the results shown in Figure 6 (for a monomer–higher polymers system) would be entirely consistent with this proposition. Furthermore, differences in the degree of steric hindrance to inhibitor binding by α-chymotrypsin polymers seems highly plausible for these two compounds, the triple bond in phenylpropiolate conveying rigidity to the structure in comparison with that of β-phenylpropionate.

In summary, this physicochemical investigation of inhibitor binding to α-chymotrypsin has provided further evidence for the preferential binding of inhibitors to the lower oligomeric forms of the enzyme. Under conditions where essentially only monomer and dimer coexist, molecular weight data obtained in the presence and absence of competitive inhibitor have yielded a quantitative assessment of the ligand-binding properties of the less active, dimeric species, with respect to both number and association constants of binding sites. The results are consistent with the postulate that approach of inhibitors to the active sites of α-chymotrypsin dimers is subject to steric hindrance, the situation deduced from X-ray crystallographic studies. This correlation between studies of the enzyme–inhibitor complex formation in the crystalline state and in solution thus supports the generally held contention that structures of proteins deduced from X-ray crystallography studies are pertinent to their solution properties.

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