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Evaluation of Equilibrium Constants for the Binding of *N*-Acetyl-L-tryptophan to Monomeric and Dimeric Forms of α-Chymotrypsin†

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ABSTRACT: The binding of *N*-acetyl-L-tryptophan to the monomeric and dimeric forms of α-chymotrypsin in $I = 0.2$ acetate–chloride buffer, pH 3.86, has been studied quantitatively. Equilibrium sedimentation studies in the absence of inhibitor yielded a dimerization constant of 3.5 L/g. This value was confirmed by frontal gel chromatography of the enzyme on Bio-Gel P-30, which was also used to establish that *N*-acetyl-L-tryptophan binds preferentially to monomeric enzyme. From kinetic studies of competitive inhibition with *N*-acetyl-L-tryptophan ethyl ester as substrate, an equilibrium constant of 1300 M^{-1} was determined for the binding of *N*-acetyl-L-tryptophan to monomeric α-chymotrypsin. An intrinsic binding constant of 250 M^{-1} for the corresponding interaction with dimeric enzyme was calculated on the basis of these results and binding data obtained with concentrated (18.5 g/L) α-chymotrypsin. The present results refute earlier claims for exclusive binding of competitive inhibitors to monomer and also those for equivalence of inhibitor binding to monomeric and dimeric forms of α-chymotrypsin.

The effect of covalent active-site-directed inhibitors on the macromolecular state of α-chymotrypsin has received considerable attention [e.g., Neet & Brydon (1970), Horbett & Teller (1973), and Gorbunoff et al. (1978)] because of the close proximity or, indeed, identity of the active site and the dimerization site (Steitz et al., 1969; Aune & Timasheff, 1971; Birktoft & Blow, 1972; Vandlen & Tulinski, 1973). These studies are in general agreement that (1) inhibitor binding leads to an increase in the proportion of monomeric enzyme and (2) the extent of the shift toward monomer depends at least in part on the bulkiness of the inhibitor group introduced into the enzyme molecule.

However, no such concord extends to studies of the relative affinities of monomeric and dimeric α-chymotrypsin species for competitive (noncovalent) inhibitors. Whereas Sarfare et al. (1966) concluded, on the basis of molecular weight studies, that the binding of β-phenylpropionate proceeds independently of enzyme polymerization, Shiao & Sturtevant (1969) considered that their studies of inhibitor binding by flow microcalorimetry were best described by a model in which dimeric enzyme possessed little, if any, affinity for indole, *N*-acetyltryptophan, or proflavin. A similar conclusion was reached by Faller & LaFond (1971), who studied the binding of proflavin to α-chymotrypsin by equilibrium dialysis and temperature-jump relaxation methods. An intermediate stand was taken by Nichol et al. (1972), who combined binding data and molecular weight measurements to show that phenylpropiolate and β-phenylpropionate bind to dimeric enzyme, but with decreased affinity. Subsequently, Gilleland & Bender (1976) have considered kinetic studies of proflavin binding in terms of exclusive binding to monomeric α-chymotrypsin, whereas the original Sarfare et al. model with equivalent binding to monomeric and dimeric enzyme has returned to favor as the result of NMR studies of the binding of *p*-fluorocinnamate (Gerig et al., 1977).

Because of the uncertainty created by these conflicting viewpoints, we have investigated the binding of *N*-acetyl-L-tryptophan to α-chymotrypsin in $I = 0.2$ acetate–chloride, pH 4, a medium in which (a) polymerization of the enzyme is restricted to a monomer–dimer equilibrium (Winzor & Scheraga, 1964; Winzor et al., 1967; Morimoto & Kegeles, 1967; Aune & Timasheff, 1971; Horbett & Teller, 1973, 1974; Gorbunoff et al., 1978) and (b) the problem of autolysis in equilibrium binding studies by frontal gel chromatography (Nichol et al., 1972) is minimal. A combination of equilibrium sedimentation, gel chromatographic, and enzyme kinetic studies has been used to evaluate the binding constants for the interactions of *N*-acetyl-L-tryptophan with monomeric and dimeric α-chymotrypsin.

Experimental Section

Materials. α-Chymotrypsin (3-times crystallized, freeze-dried, and salt-free) was obtained from Worthington Biochemical Corp., *N*-acetyl-L-tryptophan was from Sigma Chemical Co., and *N*-acetyl-L-tryptophan ethyl ester was from Vega-Fox Biochemicals. Other chemicals were of reagent grade, and glass-distilled water was used in the preparation of all buffers and solutions. Acetate–chloride buffer, pH 3.86, $I = 0.20$ (0.18 M sodium chloride and 0.02 M sodium acetate; pH adjusted with acetic acid), was used throughout this investigation.

Solutions of α-chymotrypsin were prepared by direct dissolution into the acetate–chloride buffer, and any autolysis fragments were removed by zonal gel chromatography of 2-mL aliquots on a column (2.4 × 17 cm) of Sephadex G-75 preequilibrated with the same buffer. A high protein concentration (~20 g/L) such that the α-chymotrypsin would be essentially dimeric was used to improve the separation of the enzyme from

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the slower migrating autolysis fragments. Material comprising the first two-thirds of the eluted zone was considered to represent reversibly dimerizing α-chymotrypsin; any large autolysis fragments also undergoing polymerization would, of course, have been included in the sample of α-chymotrypsin used for further study. Titration of active sites with *p*-nitrophenyl acetate (Kézdy & Bender, 1962) indicated that these solutions typically contained ~90% active enzyme. Concentrations of α-chymotrypsin were usually determined either spectrophotometrically at 280 nm on the basis of an extinction coefficient ($A_{1cm}^{1\%}$) of 20.1 (Egan et al., 1957) or refractometrically at 546 nm on the basis of a specific refractive increment of 1.86×10^{-4} L/g (Sarfare et al., 1966; Aune & Timasheff, 1971). However, measurement of enzyme concentrations in the presence of *N*-acetyl-L-tryptophan required the use of the biuret procedure (Gornall et al., 1949), which was calibrated with α-chymotrypsin as the standard protein; these estimations were shown to be unaffected by the presence of *N*-acetyl-L-tryptophan in the protein samples.

Solutions of *N*-acetyl-L-tryptophan and *N*-acetyl-L-tryptophan ethyl ester were prepared by weight and the concentrations confirmed spectrophotometrically at 300 nm by using molar absorptivities of 850 and 692 for the acid and ester, respectively, at pH 3.86; these values are based on a comparison of absorbance measurements at pH 3.86 and 6.98, plus the reported absorptivities at pH 6.98 (Zerner et al., 1964).

Equilibrium Sedimentation. Speeds in the range 7200–24 000 rpm were used for sedimentation equilibrium experiments in a Spinco Model E ultracentrifuge fitted with electronic speed control. The equilibrium distributions resulting from these experiments, conducted at 20 °C, were recorded as Rayleigh interferograms, which were measured on a Nikon two-dimensional comparator. Experiments at low (Van Holde & Baldwin, 1958) and high (Yphantis, 1964) angular velocities were analyzed in terms of the Ω(r) function (Milthorpe et al., 1975) using values of 25 000 for the monomeric molecular weight (Hartley, 1964), 0.736 mL/g for the partial specific volume of α-chymotrypsin (Schwert & Kaufman, 1951), and 1.0055 g/mL for the buffer density (Schwert & Kaufman, 1951).

Gel Chromatography on Bio-Gel P-30. Concentration dependence of the weight-average elution volume of α-chymotrypsin was determined by frontal gel chromatography (Winzor & Scheraga, 1963) on a column (1.1 × 51 cm) of Bio-Gel P-30 equilibrated with the acetate–chloride buffer. The flow rate of the column was maintained at 0.44 mL/min and the temperature at 20 °C. Solutions of α-chymotrypsin (30 mL, 0.03–1.78 g/L) were loaded onto the column, and the eluate was divided into approximately 1-mL fractions. The size of each fraction, collected on a time basis, was determined by reweighing the previously tared tubes, and its protein concentration was measured either spectrophotometrically at 280 nm or by the biuret method (Gornall et al., 1949). The weight-average elution volume was then obtained as the median bisector of the advancing elution profile.

Kinetic Studies. Kinetic measurements were carried out on a Varian Superscan 3 spectrophotometer in which the cell compartment was thermostatically maintained at 20 °C. Hydrolysis of *N*-acetyl-L-tryptophan ethyl ester in the presence and absence of the inhibitor *N*-acetyl-L-tryptophan was followed by measuring the increase in absorbance at 300 nm (Zerner et al., 1964). A cuvette containing 3 mL of acetate–chloride buffer, pH 3.86, was first allowed to equilibrate at 20 °C in the sample compartment of the spectrophotometer. A solution of the ester in acetonitrile (25 μL) was then added and recording of the absorbance at 300 nm commenced. After ~30 s the enzyme solution (50 μL) was added and recording recommenced. For the inhibition studies *N*-acetyl-L-tryptophan (5.03 or 2.23 mM) was dissolved in the acetate–chloride buffer used to fill the cuvette initially.

Initial velocities v were determined from the limiting slopes of experimental traces obtained with six substrate concentrations [S] in the range 0.04–0.67 mM and with an enzyme concentration of 3.5 μM. Results were analyzed by the method of Eisenthal & Cornish-Bowden (1974), except that the two simultaneous equations for each pair of (v, [S]) data were solved algebraically rather than geometrically to determine values of the maximal velocity v_m and Michaelis constant K_m. The former values, which showed no obvious departure from a normal distribution, were subjected to standard statistical analysis, and the mean was taken as v_m. Values of K_m appropriate to each substrate concentration were then redetermined with this value of v_m, and the resultant K_m values were subjected to statistical analysis; mean values ($\pm 2\ s_m$) are reported for v_m and K_m.

Binding Studies. The binding of *N*-acetyl-L-tryptophan to α-chymotrypsin was studied by frontal gel chromatography (Nichol & Winzor, 1964; Cooper & Wood, 1968; Nichol et al., 1971) on a column (0.9 × 5.5 cm) of Sephadex G-25 (fine) equilibrated with the $I = 0.2$ acetate–chloride buffer, pH 3.86, and thermostated at 20 °C; stock solutions of enzyme and inhibitor were also maintained at this temperature. Reaction mixtures (5 mL) containing α-chymotrypsin (18.5 g/L) and *N*-acetyl-L-tryptophan (0.14–7.2 mM) were applied to the column at a flow rate of 0.33 mL/min. Throughout the subsequent elution with acetate–chloride buffer the column effluent was monitored continuously at 300 nm. The equilibrium concentration of *N*-acetyl-L-tryptophan ([I]) was determined from the absorbance of the protein-free plateau region of the elution profile, and the magnitude of ν, the binding function (moles of ligand bound per base mole of enzyme), was calculated from this value and those of the total inhibitor and total enzyme concentrations.

Results

Evaluation of the Dimerization Constant for α-Chymotrypsin. Results of five equilibrium sedimentation experiments on α-chymotrypsin in $I = 0.2$ acetate–chloride, pH 3.86, are summarized in Figures 1 and 2. The former presents the Ω(r) analysis (Milthorpe et al., 1975) used to evaluate the thermodynamic activity of monomer, $a_A(r_F)$, associated with the selected reference total concentration, $\bar{c}(r_F)$, of 1.87 g/L; the ratio $a_A(r_F)/\bar{c}(r_F)$ is given by the ordinate intercept. Figure 2 presents a test of the resultant [$a_A(r)$, $\bar{c}(r)$] data for conformity with a simple monomer–dimer system. In this connection it should be noted that thermodynamic ideality has been assumed since concentrations and activities have been combined in the ordinate variable. As noted previously (Tellam et al., 1978), this approximation is likely to be reasonable for relatively small globular proteins over the limited concentration range (0.1–5 g/L) studied. Least-squares calculations on the experimental results shown in Figure 2 yield a slope of 1.95. Consequently, the data conform very well with the law of mass action, written in logarithmic form, for a monomer–dimer system. This result thus confirms earlier claims (Winzor & Scheraga, 1964; Winzor et al., 1967) that α-chymotrypsin undergoes reversible dimerization under these conditions. Furthermore, the mean value of 3.5 ± 0.1 L/g for the dimerization constant that is deduced from all of the [$a_A(r)$, $\bar{c}(r)$] data is in very good agreement with previous estimates of 3.8 (Winzor & Scheraga, 1964), 7 (Winzor et al., 1967),

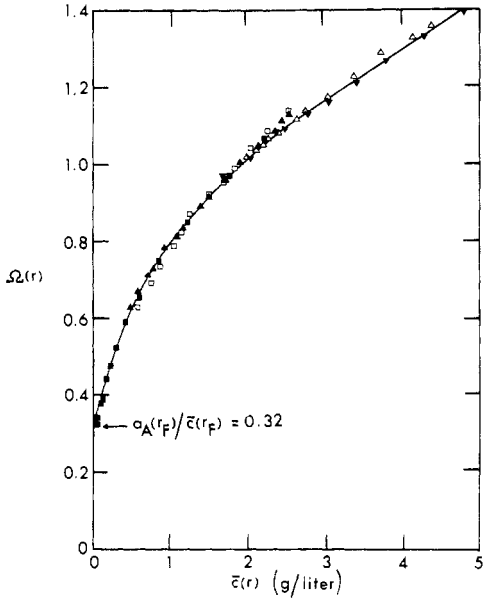


FIGURE 1: Analysis of equilibrium sedimentation experiments on α-chymotrypsin in $I = 0.2$ acetate–chloride, pH 3.86, by means of the Ω(r) analysis (Milthorpe et al., 1975): representative plot of the extrapolation involved in determining $a_A(r_F)$, the activity of monomer at reference total enzyme concentration $\bar{c}(r_F)$, which has been fixed at 1.87 g/L in each of five experiments.

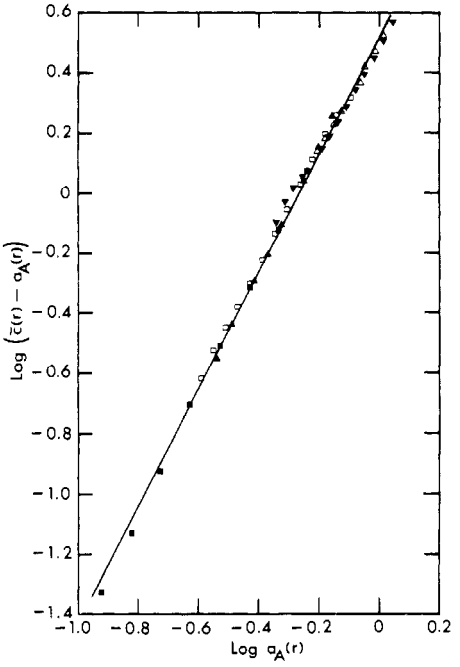


FIGURE 2: Test of the equilibrium sedimentation results obtained with α-chymotrypsin for conformity with a monomer–dimer system; experimental conditions and details were as in Figure 1.

and 2.2 L/g (Horbett & Teller, 1974). Finally, it is noted that inclusion of 0.81% acetonitrile (v/v) in the acetate–chloride buffer had no discernible effect on the sedimentation equilibrium behavior of the enzyme. The same equilibrium constant is therefore operative in the kinetic studies to be described.

Gel Chromatography on Bio-Gel P-30. Evidence for the preferential binding of *N*-acetyl-L-tryptophan to the monomeric form of α-chymotrypsin was obtained from frontal gel chromatographic studies using Bio-Gel P-30, a medium selected to exclude dimeric enzyme from the stationary phase of the column. The solid symbols in Figure 3 represent experimental weight-average elution volumes $\bar{V}_w$ of α-chymotrypsin in the

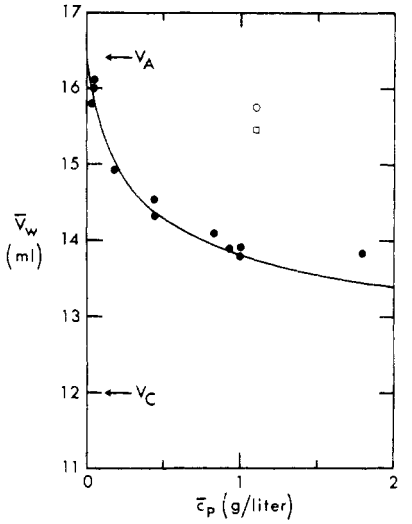
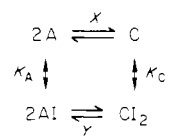


FIGURE 3: Concentration dependence of the weight-average elution volume for α-chymotrypsin on a 0.9 × 51 cm column of Bio-Gel P-30 equilibrated with $I = 0.2$ acetate–chloride, pH 3.86. Solid symbols denote experimental points, and the curve denotes the theoretical dependence based on a dimerization constant of 3.5 L/g. Open symbols refer to experiments with 9.17 (○) and 4.77 mM (□) *N*-acetyl-L-tryptophan included in the buffer.

Scheme I



concentration range 0.03–1.78 g/L, and the solid line represents the theoretical relationship for a monomer–dimer system with $X' = 3.5$ L/g (the value inferred from Figure 2), $V_C = 12.0$ mL, and $V_A = 16.4$ mL; the elution volume of dimer (V_C) was taken as the void volume of the column, and that of monomer (V_A) was obtained by extrapolating the experimental points to zero concentration. Agreement between theoretical and experimental $\bar{V}_w$ vs. $\bar{c}_P$ relationships is considered to be excellent, particularly in view of the fact that the deviation observed at the highest concentration undoubtedly reflects the failure of the theoretical curve to take into account the consequences of osmotic shrinkage of the gel phase (Edmond et al., 1968; Baghurst et al., 1975).

In the presence of *N*-acetyl-L-tryptophan, $\bar{V}_w$ is increased markedly (open symbols in Figure 3), the extent of the increase being related to the concentration of inhibitor used. These results refute the concept of equivalent binding to monomeric and dimeric α-chymotrypsin (Gerig et al., 1977) and support the previously held contention [e.g., Nichol et al. (1972)] that monomeric enzyme binds inhibitors preferentially. Horbett & Teller (1973) have also noted that *N*-acetyl-L-tryptophan strongly inhibits the dimerization of α-chymotrypsin but have made no quantitative assessment of the system.

From the quantitative viewpoint, complete characterization of the system was considered to require evaluation of the parameters X, K_A, and K_C shown in Scheme I. K_A denotes the binding constant for the interaction of *N*-acetyl-L-tryptophan (I) with the single active site on monomeric enzyme (A), and K_C denotes the intrinsic binding constant (Klotz, 1946) for interaction of the inhibitor with two equivalent and independent sites on the dimer (Nichol et al., 1972); X, the dimerization constant expressed on a molar scale, is readily calculated to be 44 000 M^{-1} from the corresponding weight-scale quantity ($X = X'M_A/2$, where M_A is the molecular

weight of the monomeric enzyme). The additional constant Y describing the dimerization of the complex AI is redundant since only three equilibrium constants are required to define uniquely the composition of the system; the possible choice of X, K_A, and Y (instead of X, K_A, and K_C) is considered later.

Evaluation of K_A from Kinetic Studies. The fact that X is so large has placed stringent demands on the method used to evaluate K_A since the protein concentration employed must be low enough for the enzyme to be predominantly monomeric; a concentration of less than 0.1 g/L was in fact required for the system to comprise more than 75% monomeric chymotrypsin. K_A was therefore determined from kinetic studies with *N*-acetyl-L-tryptophan as a competitive inhibitor of the enzyme-catalyzed hydrolysis of *N*-acetyl-L-tryptophan ethyl ester; the enzyme concentration of 3.5 μM (0.087 g/L) that was used represented a compromise between the need for the lowest possible enzyme concentration and for velocities that could be measured with reasonable precision.

Results of this kinetic study in I = 0.2 acetate–chloride buffer, pH 3.86, were first plotted in Lineweaver–Burk format to establish their conformity with the competitive inhibition of α-chymotrypsin by *N*-acetyl-L-tryptophan. Several points in relation to this study merit comment. (1) Because of the high absorbances of reaction mixtures containing 4.91 or 2.18 mM inhibitor, background absorbance was blanked out by including an identical concentration of inhibitor in the reference cuvette; addition of an aliquot of *N*-acetyl-L-tryptophan (calculated from the molar absorptivity reported above to give an absorbance increase ΔA of ~0.1) yielded a ΔA in good agreement with the calculated value (ΔA = 0.106 corresponds to hydrolysis of 0.67 mM substrate). (2) Although the results were plotted in the familiar double-reciprocal format, the best-fit descriptions of the system have been based on values of parameters deduced from an Eisenthal & Cornish-Bowden (1974) analysis of the results to avoid the bias of experimental error introduced by transforming a rectangular hyperbola into a linear plot. (3) The maximum velocity v_m is estimated to be 3.10 ± 0.04 M min^{-1}, which on combination with the enzyme concentration (3.5 μM) yields a k_{cat} of 0.148 ± 0.002 s^{-1}, a value in good agreement with those reported in Table IV of Bender et al. (1964). The estimate of (6.3 ± 0.7) × 10^{-5} M for K_m is also in agreement with their data. (4) Analysis of the results obtained in the presence of inhibitor yields an association equilibrium constant of 1300 ± 300 M^{-1} for the interaction of *N*-acetyl-L-tryptophan with α-chymotrypsin under these conditions. Although this value is larger than previous estimates of 100 and 500 M^{-1} from kinetic studies at pH 7.9 and 6.9, respectively (Foster & Niemann, 1955), the larger equilibrium constant presumably reflects (a) the coexistence of the acyl enzyme and the noncovalent complex formed by interaction of the enzyme with protonated *N*-acetyl-L-tryptophan (Kézdy et al., 1964) and (b) the greater interaction of the inhibitor anion with the protonated form of the active-site histidine. Indeed, enhanced interaction of this histidine with a carboxylate group has also been invoked to account for the pronounced increase in the extent of α-chymotrypsin dimerization that is observed in the acid pH range (Aune & Timasheff, 1971; Horbett & Teller, 1973, 1974). (5) The value of 1300 M^{-1} has been taken to represent a satisfactory approximation of the magnitude of K_A, even though only 80% of the enzyme would have been monomeric in the absence of added substrate and inhibitor (see below).

Binding Studies. Scatchard (1949) plots of gel chromatographically obtained binding data on the interaction of *N*-acetyl-L-tryptophan with a concentrated solution (18.5 g/L) of α-chymotrypsin in I = 0.2 acetate–chloride, pH 3.86, are shown as solid symbols in Figure 4. Open symbols refer to results inferred from reported values (Johnson & Knowles, 1966) of inhibition constants from experiments conducted at pH 3.45 (○) and 4.27 (□) with concentrated (25 g/L) solutions of enzyme. Clearly there is reasonable agreement about the extent of inhibitor binding, i.e., about the value of ν for a given free inhibitor concentration [I], but the earlier interpretation of data in terms of a single equilibrium constant (Johnson & Knowles, 1966) is untenable. The solid line in Figure 4 has been drawn with slope $-K_A$ (1300 M^{-1}) to indicate the theoretical plot for the binding of *N*-acetyl-L-tryptophan to monomeric α-chymotrypsin. This line would also describe the experimental data in the event that monomer and dimer possessed equal affinities for inhibitor (Gerig et al., 1977); clearly, it does not. Three curvilinear plots have been calculated on the basis of eq 1

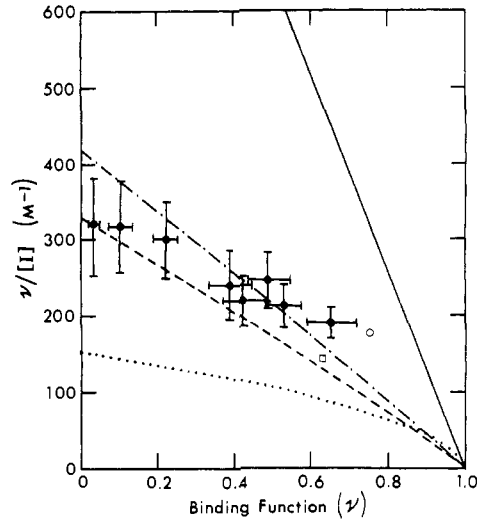


FIGURE 4: Scatchard plot of binding data for the interaction of *N*-acetyl-L-tryptophan with α-chymotrypsin in I = 0.2 acetate–chloride, pH 3.86. Closed symbols denote experimental results obtained with an enzyme concentration of 18.5 g/L, and the lines represent various attempts to fit the data by Scheme I with X = 44 000 M^{-1}, K_A = 1300 M^{-1}, and different dimeric affinities for the inhibitor: (—) K_C = 1300 M^{-1}; (···) K_C = 0; (-·-) K_C = 300 M^{-1}; (---) K_C = 200 M^{-1}. Open symbols denote points calculated from the data of Johnson & Knowles (1966) at pH 3.45 (○) and 4.27 (□).

$$\nu = \frac{K_A[A][I] + 2X[A]^2K_C[I](1 + K_C[I])}{[A](1 + K_A[I]) + 2X[A]^2(1 + K_C[I])^2} \quad (1)$$

in which [I] denotes the concentration of free inhibitor. [A], the concentration of free monomer, may readily be obtained for any given value of K_C (Nichol et al., 1967). The dotted line in Figure 4 represents the system with inactive dimer (K_C = 0), the situation favored by Shiao & Sturtevant (1969), Faller & LaFond (1971), and Gilleland & Bender (1976); again, the agreement with experiment is poor. A much better description of the experimental results is obtained by assigning a value of 200 (---) or 300 M^{-1} (---) to K_C. Preferential binding of *N*-acetyl-L-tryptophan to monomeric α-chymotrypsin is thus indicated by these binding data, the degree of preference being such that K_C is between $0.15K_A$ and $0.23K_A$. Furthermore, it will now be shown that the weight-average elution volume results reported in Figure 3 are in quantitative agreement with this finding.

In the presence of a concentration [I] of free *N*-acetyl-L-tryptophan, the constituent concentrations of the two oligomeric acceptors $\bar{c}_A$ and $\bar{c}_C$ (weight scale) are given by (Klotz, 1946; Nichol et al., 1967)

$$\bar{c}_A = c_A(1 + K_A[I]) \quad (2a)$$

$$\bar{c}_C = X'c_A^2(1 + K_C[I])^2 \quad (2b)$$

where $X' = 3.5$ L/g in the present context (Figure 2). Provided that the binding of inhibitor to either monomer or dimer does not affect its elution volume ($V_{AI} = V_A$; $V_{CI_2} = V_{CI} = V_C$), it is readily shown that

$$\bar{c}_A = \bar{c}_P(\bar{V}_w - V_C)/(V_A - V_C) \quad (3a)$$

$$\bar{c}_C = \bar{c}_P(V_A - \bar{V}_w)/(V_A - V_C) \quad (3b)$$

where $\bar{c}_P$ denotes the total protein concentration ($\bar{c}_P = \bar{c}_A + \bar{c}_C$). Combination and rearrangement of eq 2 and 3 then yield the expression

$$(1 + K_C[I])^2 = \frac{(V_A - \bar{V}_w)(V_A - V_C)(1 + K_A[I])^2}{X'\bar{c}_P(\bar{V}_w - V_C)^2} \quad (4)$$

which, on substitution of the value 1300 M^{-1} for K_A, allows the evaluation of K_C from a single ($\bar{V}_w$, [I]) experimental point. Application of eq 4 to the two such experimental points (both with $\bar{c}_P = 1.1$ g/L) shown in Figure 3 yields values of 210 and 240 M^{-1} for experiments with [I] = 9.17 (○) and 4.77 mM (□), respectively. A value of 250 M^{-1} for K_C is thus reasonably consistent not only with the direct binding data (Figure 4) but also with the observed displacement of the monomer–dimer equilibrium position caused by preferential binding of *N*-acetyl-L-tryptophan to monomeric α-chymotrypsin.

In the above interpretation of Figure 4 the inhibition constant deduced from the enzyme kinetic studies (1300 M^{-1}) has been taken as the equilibrium constant for the interaction between *N*-acetyltryptophan and monomeric α-chymotrypsin. Retrospective justification for the validity of this approximation can now be made. By assuming that the relationship $K_C = 0.19K_A$ (Figures 3 and 4) also applies to the interaction between enzyme and *N*-acetyltryptophan ethyl ester and that we can equate the Michaelis complex and the acyl enzyme in their effects on dimer formation, it can be calculated that over the substrate range used the percentage of monomeric enzyme would have varied between 89 ([S] = 0.04 mM) and 98% ([S] = 0.67 mM) in the absence of inhibitor and between 96 and 99% in the presence of 2.18 mM *N*-acetyl-L-tryptophan.

Discussion

The present study of the binding of *N*-acetyl-L-tryptophan to α-chymotrypsin substantiates our earlier finding (Nichol et al., 1972) that competitive inhibitors bind preferentially rather than exclusively to monomeric enzyme. On this occasion, however, the degree of preference is much greater ($K_C = 0.19K_A$ compared with $K_C = 0.75K_A$). Although changes in factors such as pH cannot be ruled out as a possible cause of this difference, a more likely explanation is that *N*-acetyl-L-tryptophan, being bulkier than the inhibitor phenylpropiolate that was used previously (Nichol et al., 1972), is subject to greater steric hindrance in its approach to the inhibitor binding site, which is in very close proximity to the dimerization site (Steitz et al., 1969). Indeed, some of the seeming conflict with earlier studies may also be attributable to differences in the sizes of inhibitors studied. For example, proflavin may well be sufficiently large for steric considerations to preclude binding to dimeric enzyme, the situation envisaged by Shiao & Sturtevant (1969), by Faller & LaFond (1971), and by Gilleland & Bender (1976). However, this explanation cannot be invoked to account for the finding, based on flow microcalorimetry studies, that indole and *N*-acetyltryptophan also bind exclusively to monomeric α-chymotrypsin (Shiao & Sturtevant, 1969). Nor does the size factor seem to explain the failure to detect preferential binding with smaller inhibitors than *N*-acetyl-L-tryptophan. The fact that formation of hydrocinnamoyl and cinnamoyl derivatives of α-chymotrypsin leads to discernible changes in the sedimentation equilibrium behavior of the enzyme (Horbett & Teller, 1973; Gorbunoff et al., 1978) implies that the results obtained with β-phenylpropionate (Sarfare et al., 1966) and *p*-fluorocinnamate (Gerig et al., 1977) signify insensitivity of the methods used rather than equivalence of binding to monomeric and polymeric species.

In our previous investigation of preferential binding of inhibitors to α-chymotrypsin at pH 7.8 (Nichol et al., 1972), the evidence for phenylpropiolate interacting with both binding sites of the dimeric enzyme was considered to be incompatible with the mechanism of dimerization proposed by Aune & Timasheff (1971) to operate at low pH because of the involvement of active-site His-57. Indeed, on this basis it was suggested that different modes of dimerization may pertain under the two sets of conditions. However, now that unequivocal evidence of preferential (not exclusive) binding has also been obtained under the acidic conditions, it seems more likely that the active-site region is sufficiently large for inhibitor binding and enzyme dimerization to occur concurrently in different areas within its bounds. Depending on size, this incorporation of inhibitor into the dimeric species is presumably associated with weakening or, indeed, disruption of some of the hydrogen bonds and salt bridges that emanate from the active-site region to stabilize dimeric α-chymotrypsin (Aune & Timasheff, 1971; Birktoft & Blow, 1972; Vandlen & Tulinski, 1973; Horbett & Teller, 1973; Gorbunoff et al., 1978).

In attempts to define the quaternary interactions responsible for the dimerization of α-chymotrypsin in solution, considerable emphasis has been placed on studies of the extent of dimerization observed with covalently modified derivatives of the enzyme (Horbett & Teller, 1973; Gorbunoff et al., 1978). By illustrating approaches for studying quantitatively the effect of noncovalent inhibitor binding on dimerization of the enzyme, the present investigation and also its predecessor (Nichol et al., 1972) could make an important contribution to this field by (1) expanding the choice of enzyme–inhibitor complexes available for study and (2) providing a means of probing the structure of α-chymotrypsin molecules that are enzymically active but undergoing competitive inhibition, a biologically relevant situation. Such studies of quaternary interactions could presumably involve interpretation of results in terms of the effect of inhibitor binding on dimerization (i.e., X, K_A, and Y in Scheme I) rather than the present interpretation in terms of the effect of dimerization on inhibitor binding (X, K_A, and K_C). Because of the redundancy of the fourth equilibrium constant in the cyclic array of chemical equilibria shown in Scheme I, either interpretation provides a valid thermodynamic description of the system (Nichol & Winzor, 1976).

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