Binding Properties of Oligomeric α-Chymotrypsin*

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ABSTRACT: α-Chymotrypsin is known to aggregate with loss of catalytic activity. Equilibrium dialysis and temperature-jump relaxation methods have been used to study the binding of a competitive inhibitor, proflavin, to the enzyme over a concentration range in which the percentage of monomer decreases from 96% to less than 30%. All measurements were made at pH 6.2 and 22.5° in 0.2 M ionic strength phosphate buffer. The dissociation constants for dimeric and trimeric α-chymotrypsin reported by Rao and Kegeles for these experimental conditions were used to deduce the availability of active sites in oligomeric forms of the enzyme. The simplest explanation of our results is that only monomeric α-chymotrypsin binds proflavin. The measured second-order rate constant for binding of proflavin to the enzyme is then $(4.1 \pm 0.1) \times 10^7$ M^{-1} sec^{-1}, and the rate constant for decomposition of the enzyme–proflavin complex is $(1.7 \pm 0.7) \times 10^3$ sec^{-1}. The dissociation constant for the monomer–proflavin complex is $(3.8 \pm 0.7) \times 10^{-5}$ M.

The association of α-CT[1] was among the first reacting systems studied by ultracentrifugation (Schwert, 1949). Numerous subsequent measurements of sedimentation velocities, light scattering, viscosities, diffusion rates, fluorescence depolarization, and enthalpies of dilution (Schwert and Kaufman, 1951; Smith *et al.*, 1951; Frenkel, 1952; Steiner, 1954; Massey *et al.*, 1955; Egan *et al.*, 1957; Tinoco, 1957; Rao and Kegeles, 1958; Morimoto and Kegeles, 1967; Shiao and Sturtevant, 1969), as well as gel filtration (Winzor and Scheraga, 1963, 1964), provide unequivocal evidence that α-CT oligomerizes in solution. At pH 6.2 and 20–25° in phosphate buffer of 0.2 M ionic strength, Rao and Kegeles (1958) concluded that a rapid equilibrium exists among monomeric, dimeric, and trimeric forms of the enzyme.

α-CT monomers are also associated in the crystalline enzyme (Blow *et al.*, 1964; Matthews *et al.*, 1967; Sigler *et al.*, 1968; Blow, 1969). The crystal is constructed of parallel chains of molecules. Adjacent molecules within a chain are related by 180° rotation without translation, so that like regions of neighboring molecules are in contact. Two types of paired interactions about local twofold symmetry axes A and B alternate along the chains.

The specific activity of α-CT toward a number of model substrates has been found to decrease in the concentration range where the enzyme associates (Booman and Niemann, 1957; Martin and Niemann, 1958; Bender and Zerner, 1962; Kezdy and Bender, 1962; Inagami and Sturtevant, 1965). Crystalline α-CT also exhibits impaired catalytic activity (Kallos, 1964; Sigler *et al.*, 1968). Experiments designed to explain why oligomer formation results in reduced activity have yielded equivocal results.

Neither of the inactive precursors of CT, CTogen-A nor CTogen-B, associates significantly in solution (Schwert, 1949, 1951; Smith *et al.*, 1951; Smith and Brown, 1952; Frenkel, 1952; Gladner and Neurath, 1954; Massey *et al.*, 1955; Neurath and Dreyer, 1955) suggesting that the active site of the enzyme is directly involved in oligomer formation. Kezdy and Bender (1962) have presented evidence that the dimer is a covalent species in which the active serine (serine-195) of one molecule is acylated by a second enzyme molecule. Photooxidized α-CT in which one histidine (histidine-57) residue is destroyed with loss of activity does not dimerize (McClaren and Finkelstein, 1950; Egan *et al.*, 1957). In apparent contradiction to the preceding results, DIP-CT and AcCT, in which serine-195 is acylated, do dimerize (Schwert and Kaufman, 1951; Smith and Brown, 1952; Massey *et al.*, 1955; Neurath and Dreyer, 1955; Winzor and Scheraga, 1963; Morimoto and Kegeles, 1967). Most X-ray crystallographic observations have been made on serine-195 tosylated α-CT.

Several efforts have been made to correlate rate data with the aggregation behavior of α-CT. Using the constancy of the kinetically determined dimer dissociation constant for different enzyme and substrate concentrations as their criterion, Martin and Niemann (1958) concluded that dimeric α-CT can bind substrate, but that only the substrate complex with monomeric enzyme decomposes to give reaction products at a significant rate. Faller (1964) compared acylation rates (Bender and Zerner, 1962; Kezdy and Bender, 1962) to the distribution of α-CT among oligomeric forms calculated using measured dissociation constants for dimeric and trimeric enzyme (Steiner, 1954; Rao and Kegeles, 1958). Although the unavailability of rate and association data for the same experimental conditions necessitated interpolation, the comparison supported the view that only monomeric enzyme is acylated.

Using the same approach, Inagami and Sturtevant (1965) reached a different conclusion. They made stopped-flow measurements of the acylation rate at high enzyme concentrations under the experimental conditions used by Rao and Kegeles (1958) in their sedimentation study. Inagami and Sturtevant concluded that oligomeric forms of α-CT are not completely inactive, but that their acylation and/or binding

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[1] The abbreviations used in the text are Pro for proflavin, CT for chymotrypsin, and CTogen for chymotrypsinogen. The usual prefixes α, β, γ, δ, and π are used to denote the different forms of the active enzyme, and the suffixes A and B are used to distinguish the two forms of the zymogen. DIP-CT and AcCT denote the diisopropylphosphoryl- and the acetyl-enzymes, respectively.

properties are impaired in oligomers. Sarfare *et al.* (1966) have presented evidence that it is the reactivity of α-CT which is reduced in oligomers. They studied the sedimentation behavior of the enzyme in the presence of a competitive inhibitor, β-phenylpropionate. Since there was no shift toward low molecular weight material, they reasoned that binding is independent of oligomerization. Neither the number of available binding sites nor the affinity of an active site for a competitive inhibitor is reduced when α-CT associates.

In this paper we report equilibrium dialysis and temperature-jump relaxation studies of competitive inhibitor binding in high concentrations of α-CT. All measurements were made under the experimental conditions used by Rao and Kegeles (1958), so that the distribution of the enzyme among monomeric, dimeric, and trimeric forms could be calculated. The experiments were designed to test the hypothesis (Sarfare *et al.*, 1966) that neither access to the active center, nor its binding properties are affected by the oligomerization of α-CT in solution.

Experimental Section

Materials. Three-times-recrystallized bovine α-CT was purchased from Worthington Biochemical Corporation (lots CDI-6JF, 7KD, and 8HF) and from Mann Research Laboratories (lot CDI-U1020). Sephadex G-25 purified α-CT (Yapel *et al.*, 1966) was purchased from Worthington Biochemical Corp. (lot CDS-9JB). Since experimentally indistinguishable results were obtained using the three preparations, Worthington three-times-recrystallized enzyme was used in most of the reported experiments. The enzyme concentration was measured spectrophotometrically using an absorptivity of 5.1×10^4 M^{-1} cm^{-1} at 280 nm (Dixon and Neurath, 1957; Hartley, 1964).

Proflavin (3,6-diaminoacridine) dihydrochloride dihydrate was purchased from Mann Research Laboratories and used without further purification (Glazer, 1965). We chose to study Pro binding to the enzyme for three reasons. First, Pro competitively inhibits α-CT (Wallace *et al.*, 1963; Bernhard *et al.*, 1966). Second, a red shift in the Pro spectrum results from association with the enzyme (Glazer, 1965; Bernhard *et al.*, 1966), and third, at low pH a single relaxation effect accompanies binding (Havsteen, 1967). Absorbance measurements were made with a Cary Model 14 spectrophotometer. The absorptivities found for Pro in pH 6.2, 0.2 M ionic strength phosphate buffer were $(2.58 \pm 0.07) \times 10^4$ M^{-1} cm^{-1} at 460 nm and $(3.80 \pm 0.08) \times 10^4$ M^{-1} cm^{-1} at 444 nm. The latter is in good agreement with the value reported by Glazer, using 318.2 as the molecular weight of the dihydrate. The Pro concentration used in the experiments reported, 2.55×10^{-5} M, was well below the concentration at which the dye itself begins to aggregate (Haugen and Melhuish, 1964; Glazer, 1965; Shiao and Sturtevant, 1969).

The pH 6.2, 0.2 M ionic strength phosphate buffer was prepared from J. T. Baker Analyzed reagent grade salts (0.029 M disodium hydrogen phosphate + 0.114 M sodium dihydrogen phosphate). pH measurements were made with a Leeds and Northrup expanded scale pH meter.

Kinetic Measurements. A temperature-jump instrument has been constructed. The single-beam photometric system employs quartz optics and is mounted on a Gaertner Scientific Corp. double-rod optical bench. Light from a 75-W xenon lamp, supplied by a PEK low ripple power supply, is made monochromatic by a Bausch and Lomb ultraviolet-visible, high-intensity, grating monochromator. An RCA 1P28 photomultiplier in an emitter-follower circuit is used to detect changes in light intensity. The photomultiplier is supplied by a Keithly Instruments Model 240 regulated high-voltage supply. Data are recorded on a Tektronix 561A oscilloscope with Type 1A6 and 2B67 plug-in units, using a Polaroid Land camera. The measurement cell and capacitor housing constructions are based on designs kindly supplied by Dr. L. DeMaeyer of the Max Planck Institute for Physical Chemistry in Göttingen. The temperature in the cell is controlled by a Lauda TK3OH constant-temperature bath. High voltage is supplied by an oil-insulated Kilovolt Corp. KVP 60-10 power pack with a high-voltage disconnect solenoid which isolates the load and disconnects the primary power after charging to a preset voltage. The 0.05-μF capacitor is discharged through the cell by a variable-spark gap which also triggers a single oscilloscope sweep. The measured heating time constant was 5 μsec, which is in good agreement with the value calculated for the measured resistance of the cell containing 0.2 M ionic strength phosphate buffer. The actual temperature jump in the experiments reported was 7.5 ± 0.2°. It was measured for a high-voltage discharge of 32 kV using an indicator, *p*-nitrophenol, of known standard enthalpy change. Since the initial temperature, measured by inserting a thermistor into the cell, was 15.0 ± 0.5°, the reported rate constants refer to 22.5 ± 0.7°. The spectral shift at 460 nm when Pro binds to the enzyme was used to monitor the time course of the reaction.

Equilibrium dialysis experiments were conducted in the same buffer used in the temperature-jump studies except for the addition of 0.001 M sodium nitride to retard bacterial growth. Plexiglas cells were constructed. Visking dialysis tubing was prepared by washing repeatedly in distilled water over a steam bath and rinsing thoroughly with phosphate buffer. The temperature was maintained constant at 22.5 ± 1.0° and the cells were agitated gently in a Precision Scientific Co. shaker bath. Nonspecific adsorption of Pro to the membrane was measured in experiments in the absence of protein. Equilibrium was monitored spectrophotometrically at 460 nm. Periodic assays permitted appropriate correction for the slight autolysis which occurred at the higher enzyme concentrations during the approximately 15 hr required for equilibration (Weiner and Koshland, 1965; Kumar and Hein, 1970).

Results

The mean values of the dissociation constants for the α-CT–Pro complex and the measured relaxation times for binding of inhibitor to the enzyme are given in Tables I and II. The standard deviations in the measurements are shown by the vertical bars in Figures 1, 2, 4, 5, and 6.

Discussion

If inhibitor binding and oligomerization are independent phenomena, as Sarfare *et al.* (1966) concluded, then the dissociation constant for the enzyme–inhibitor complex

$$K_I \equiv \frac{(\bar{E})(\bar{I})}{(\bar{EI})} \tag{1}$$

should be independent of the total enzyme concentration. In eq 1 $(\bar{E})$ and $(\bar{EI})$ are the equilibrium concentrations of free and occupied binding sites whether they are on mono-

TABLE I: Dissociation Constants of the α-Chymotrypsin–Proflavin Complex.[a]

Total Enzyme Concn (M × 10⁴), $(E)_0$	Dissocn Constants[b] (M × 10⁵), $\bar{K}_I$
1.02	4.73
1.85	5.66
3.47	7.60
4.17	7.75
5.36	9.02
6.12	8.53
7.13	9.50
8.38	8.91
9.12	10.00
10.25	11.25
11.73	11.59
13.57	12.85
14.91	12.51

[a] At pH 6.2 and 22.5° in 0.2 M ionic strength phosphate buffer; $(I)_0 = 2.55 \times 10^{-5}$ M. [b] Dissociation constant for the enzyme–inhibitor complex. Each reported value is the mean of at least three independent measurements. A total of 45 measurements were made. The standard deviations of the measured values are shown by the vertical bars in Figure 1.

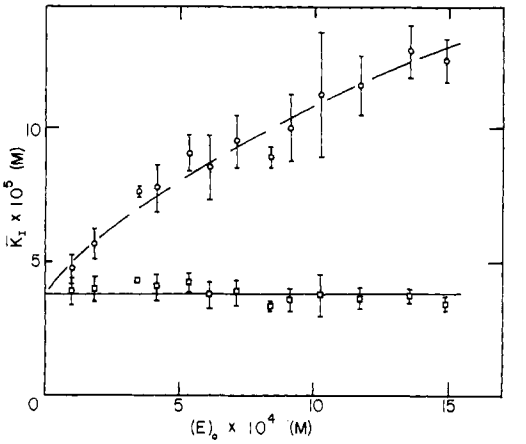

FIGURE 1: The mean dissociation constants for the α-CT–Pro complex calculated from equilibrium dialysis measurements at pH 6.2 and 22.5° in 0.2 M ionic strength phosphate buffer are shown by open circles. The vertical bars give the standard deviations of the measurements. The open squares are the values of $\bar{K}_{MI}$ calculated using eq 25 which was derived by assuming that only monomeric enzyme binds the inhibitor. The vertical bars give the standard deviations in the calculated values of K_{MI}.

meric or on oligomeric forms of the enzyme. $(\bar{I})$ is the concentration of free inhibitor. The dissociation constants for the α-CT–Pro complex calculated from equilibrium dialysis measurements are shown plotted (open circles) against the total enzyme concentration $(E)_0$ in Figure 1. The affinity of the enzyme for Pro appears to decrease when it aggregates. The dissociation constant for Pro from monomeric α-CT

$$K_{MI} \equiv \frac{(\bar{M})(\bar{I})}{(\overline{MI})} \qquad (2)$$

(M) can be found by extrapolating to infinite dilution. While precise extrapolation is not possible, justification will be given below for setting $K_{MI} = 3.8 \times 10^{-5}$ M. This value is in satisfactory agreement with previously reported values of the α-CT–Pro dissociation constant (Table III).

If α-CT binds inhibitor independently of its self-association, then complex formation involves a bimolecular reaction between free enzyme binding sites and free inhibitor and the

$$E + I \underset{k_d}{\overset{k_f}{\rightleftharpoons}} EI \qquad (3)$$

reciprocal of the relaxation time would be expected to vary directly with the sum of the equilibrium concentrations of unbound Pro and unoccupied sites.

$$1/\tau = k_f[(\bar{E}) + (\bar{I})] + k_d \qquad (4)$$

k_f is the rate constant for formation of the enzyme–inhibitor complex, and k_d is the rate constant for its decomposition. The ratio of k_d to k_f is the equilibrium dissociation constant (K_I) for the complex. The concentration of occupied sites

TABLE II: Relaxation Times for the Binding of Proflavin to α-Chymotrypsin.[a]

Total Enzyme Concn (M × 10⁴), $(E)_0$	Relaxation Times[b] (μsec), $\bar{\tau}$
0.20	350
0.37	302
0.57	258
0.74	235
0.89	224
1.21	176
1.60	158
1.78	144
2.71	116
3.13	115
3.52	97
4.17	97
4.62	92
5.11	91
5.84	87
7.16	76
8.36	68
9.06	71
10.83	62

[a] At pH 6.2 and 22.5° in 0.2 M ionic strength phosphate buffer; $(I)_0 = 2.55 \times 10^{-5}$ M. [b] Each reported relaxation time is the mean of at least three independent measurements. A total of 74 measurements were made. The standard deviations in the measured values are indicated by the vertical bars in Figures 2, 4, 5, and 6.

at equilibrium $(\overline{EI})$ can be evaluated from the quadratic equation

$$(\overline{EI})^2 - [(E)_0 + (I)_0 + K_I](\overline{EI}) + (E)_0(I)_0 = 0 \qquad (5)$$

if K_I has been independently measured. Since binding has

TABLE III: Comparison of the Measured Dissociation Constant for the α-Chymotrypsin–Proflavin Complex to Previously Reported Values.

Dissocn Constant ($M \times 10^5$), K_{MI}	Method	Exptl Conditions	Reference
3.8 ± 0.7[a]	Equilibrium dialysis	pH 6.2, 0.2 M ionic strength, phosphate, 22.5°	This study
4.2 ± 1.5[a]	Relaxation kinetics	pH 6.2, 0.2 M ionic strength, phosphate, 22.5°	This study
13 ± 4	Competitive[b] inhibition	pH 7.9, 0.1 M NaCl, 25°	Wallace *et al.* (1963)
2.5 ± 0.2	Equilibrium dialysis	pH 7.0, 0.05 M phosphate, 2°	Weiner and Koshland (1965)
2.2 ± 0.5	Spectrophotometric titration	pH 7.6, 0.02 M phosphate	Glazer (1965)
3.9 ± 0.3	Spectrophotometric titration	pH 8.0, 0.1 M ionic strength Tris	Bernhard *et al.* (1966)
3.7 ± 0.3	Competitive[c] inhibition	pH 8.0, 0.1 M ionic strength Tris	Bernhard *et al.* (1966)
6	Not given	pH 6.32, 0.1 M ionic strength phosphate, 25°	Bernhard *et al.* (1966)
31 ± 1	Spectrophotometric titration	pH 6.77	Havsteen (1967)
4.4 ± 0.4	Spectrophotometric titration	pH 6.0, 0.1 M phosphate, 0.39 M ionic strength, 24°	Brandt *et al.* (1967)

[a] The kinetic and thermodynamic determinations reported in this study are not completely independent, since the latter was used to calculate the concentrations of free monomer and free inhibitor from eq 20. [b] Competitive inhibition of *N*-acetyl-L-valine methyl ester. [c] Competitive inhibition of *N*-acetyl-L-tyrosine ethyl ester.

been assumed to be independent of association, K_I must equal K_{MI}. The concentrations of free inhibitor and unoccupied sites can then be calculated from the conservation equations

$$(E)_0 = (E) + (\bar{E}\bar{I}) \quad (6)$$

$$(I)_0 = (I) + (\bar{E}\bar{I}) \quad (7)$$

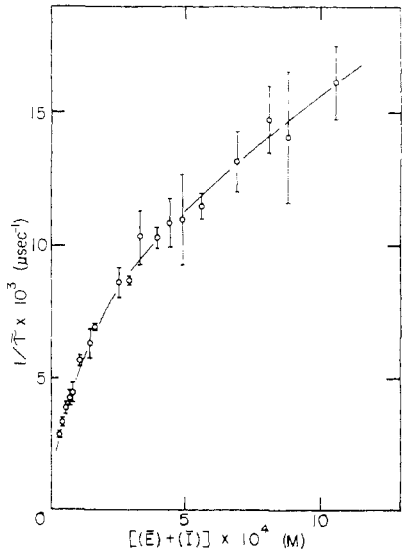

FIGURE 2: Reciprocal mean relaxation times for the binding of Pro to α-CT at pH 6.2 and 22.5° in 0.2 M ionic strength phosphate buffer plotted against the sum of the equilibrium concentrations of free inhibitor and of unoccupied binding sites, regardless whether the site is on a monomer or on an oligomer of the enzyme. The vertical bars give the standard deviations of the measured reciprocal relaxation times.

The measured relaxation times are shown plotted according to eq 4 in Figure 2. It is evident that the results of this study are inconsistent with the view that the ability of α-CT to bind molecules at the active site is independent of its tendency to associate.

Either the number of available binding sites is reduced when α-CT aggregates, or the affinity of a given site for the inhibitor is reduced in oligomeric forms of the enzyme. Both effects might be occurring simultaneously. The first possibility could be tested if the distribution of the enzyme among monomeric and oligomeric forms of the enzyme were known. That distribution, at pH 6.2 and 20–25° in 0.2 M ionic strength phosphate buffer, can be calculated by solving the cubic equation (Uspensky, 1948)

$$(\bar{M})^3 + (^2/_3)K_T(\bar{M})^2 + (^1/_3)K_DK_T(\bar{M}) - (^1/_3)K_DK_T(E)_0 = 0 \quad (8)$$

in which K_D and K_T are the dissociation constants for dimeric (D) and trimeric (T) α-CT measured by Rao and Kegeles (1958).[2]

$$D \rightleftharpoons 2M \quad K_D \equiv \frac{(M)^2}{(D)} = 9.6 \times 10^{-4}\ M \quad (9)$$

$$T \rightleftharpoons M + D \quad K_T \equiv \frac{(M)(D)}{(T)} = 2.9 \times 10^{-4}\ M \quad (10)$$

[2] The quoted values of K_D and K_T are for a monomer molecular weight of 23,000. Rao and Kegeles (1958) arrived at that value by extrapolating a plot of weight-average molecular weight *vs.* enzyme concentration to infinite dilution. It was used in subsequent calculations to obtain a linear plot from whose intercept and slope K_D and K_T were evaluated. Consequently, the original data would be necessary to redetermine K_D and K_T for a monomer molecular weight of 25,000. A control calculation showed that a 9% change in K_D and K_T would not alter the conclusion of the present study.

TABLE IV: Possibilities for Oligomer Formation.[a]

Case	No. of Sites on Dimer	No. of Sites on Trimer	Correlation[b] Coeff	F-Ratio[b] Test Statistic
I	0	0	0.924847	425.727
II	0	1	0.91849	388.431
III	1	0	0.922876	413.503
IV	1	1	0.915714	373.908
V	1	2		
VI	2	1		
VII	2	2		
VIII	2	3	0.903313	319.249

[a] Only the availability of sites has been considered. The binding properties of a site are assumed identical whether on monomeric, dimeric, or trimeric forms of the enzyme. [b] The number of degrees of freedom is 72. For a discussion of regression analysis, see, for example, Rickmers and Todd (1967).

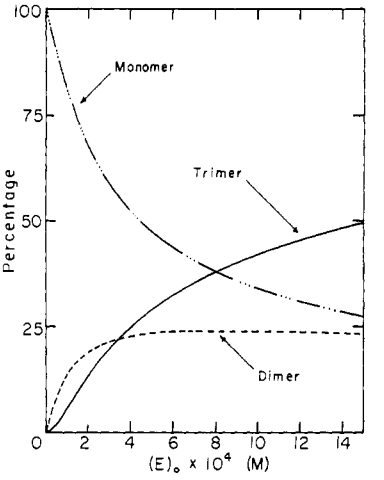

FIGURE 3: The percentage distribution of α-CT among monomeric, dimeric, and trimeric forms of the enzyme at pH 6.2 and 20–25° in 0.2 M ionic strength phosphate buffer is shown.

The percentage distribution of monomers, dimers, and trimers over the range of enzyme concentrations used in this study is shown in Figure 3. This distribution varies only slightly with temperature (Steiner, 1954; Aune and Timasheff, 1970). However it will be perturbed by Pro, since binding of this inhibitor and oligomerization of the enzyme are related. The total number of free binding sites will depend on the number of available sites in each oligomeric form of the enzyme. The possibilities are summarized in Table IV. Case VIII has already been rejected. Of those that remain, cases I and II seem most probable.

Of the two types of symmetrical interactions which alternate along the chains of α-CT molecules in the crystalline enzyme, the better developed interaction about dyad axis A brings the active centers of adjacent molecules close together. In serine-195 tosylated α-CT, which forms monoclinic crystals with two molecules per asymmetric unit identical with those formed by the native enzyme, the sulfonyl groups of adjacent molecules are only 11.9 Å apart (Sigler *et al.*, 1966). Their proximity suggests interactions between the following pairs of residues near the active sites of adjacent molecules: (a) the α-NH_3^+ of alanine-149 with the β-COO^- of aspartate-64, (b) the α-COO^- of tyrosine-146 with peptide bond 57–58, (c) the phenol of tyrosine-146 with the imidazole of histidine-57, and (d) the side chains of the methionine-192 residues in the paired molecules. The phenolic ring of tyrosine-146 in one molecule is inserted "below" the imidazole ring of histidine-57 in the adjacent molecule. The alternative pairwise interaction about dyad axis B places the following groups on adjacent molecules close together: (a) the amide group of asparagine-204 and the amide group of glutamine 240 and (b) the hydroxyl of serine-125 and the β-COO^- of aspartate-128. It is the interaction about dyad A involving the active centers of adjacent molecules which determines the crystal structure of α-CT, because δ-CT, which does not have free tyrosine-146 and alanine-149 termini, forms tetragonal crystals (actually a mixture of π-, δ-, and γ-CT) with just one molecule in the asymmetric unit (Wright *et al.*, 1968). The pairwise interaction between tyrosine-146 of one molecule and histidine-57 of its neighbor is absent in crystals of the δ family.

A priori the modes of dimerization in crystals and in aqueous solution need not be related, but two experiments suggest that the same groups are involved in solution and about dyad A in the crystal. First, carboxypeptidase treated DIP–CT, from which the C-terminal residue of the B chain, tyrosine-146, has been cleaved, does not dimerize (Gladner and Neurath, 1954). Second, the dependence of α-CT dimerization on pH (Egan *et al.*, 1957) is consistent with electrostatic attractions between pairs of anionic and cationic groups on the monomeric subunits with pK values of 3.3 and 8.5 and a repulsion between a pair of anionic groups with pK = 5.0 (Timasheff, 1969). Since there is no evidence for an ion-pair interaction about dyad B in the crystal, Timasheff tentatively identified the former groups as the β-COO^- of aspartate-64 (or alternatively the α-COO^- of tyrosine-146) and the α-NH_3^+ of alanine-149. More recently the important ion-pair attraction has been identified with the α-COO^- of tyrosine-146 and the imidazole ring of histidine-57 (Birktoft *et al.*, 1969; Aune and Timasheff, 1970).

Assuming that dimerization in solution does involve the pairwise interaction of groups in or near the active centers of the monomer subunits in a way which precludes simultaneous binding of a competitive inhibitor, a trimer might have one or no available binding sites. The former would result if the third molecule were added to the dimer by an interaction similar to that observed about dyad axis B in the crystalline enzyme. The binding mechanism corresponding to case II is

$$\begin{array}{ccccc} M & \rightleftharpoons & D & \rightleftharpoons & T \\ + & & & & + \\ I & & & & I \\ \Updownarrow & & & & \Updownarrow \\ MI & + & D & \rightleftharpoons & TI \end{array} \qquad (11)$$

The dissociation constants for the dimeric and trimeric forms of the enzyme are given by eq 9 and 10.[3] Since only the availability of binding sites and not modification of their binding properties is being considered, at equilibrium

$$K_{TI} \equiv \frac{(\bar{T})(\bar{I})}{(\bar{TI})} = K_{MI} = 3.8 \times 10^{-5}\ M \qquad (12)$$

[3] Since association is assumed to occur independently of inhibitor binding, (MI)(D)/(TI) = (M)(D)/(T) = K_T.

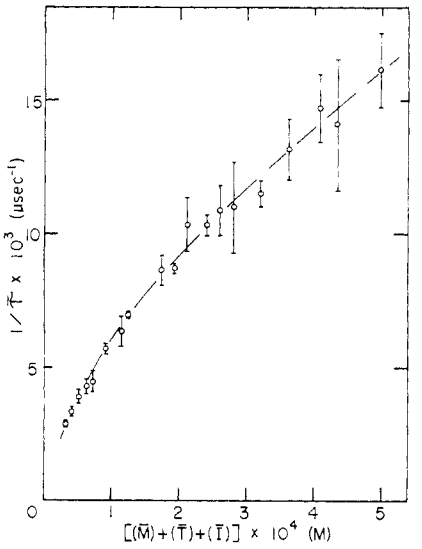

FIGURE 4: The reciprocal relaxation times are shown plotted for case II in which monomers and trimers are each assumed to possess one equivalent binding site.

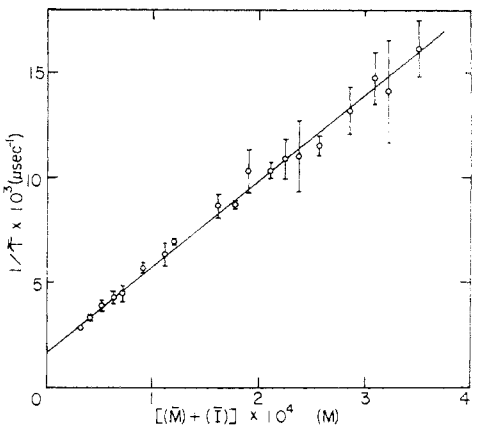

FIGURE 5: The reciprocal relaxation times are shown plotted for case I in which it is assumed that only monomers can bind inhibitor. The rate constants for formation, $k_f = 4.1 \times 10^7$ M^{1-} sec,$^{-1}$ and decomposition, $k_d = 1.7 \times 10^3$ sec^{-1}, of the α-CT–Pro complex are given by the slope and y intercept, respectively, of the least-squares line shown drawn through the points.

The conservation equations for inhibitor and enzyme are

$$(I)_0 = (\bar{I}) + (\bar{MI}) + (\bar{TI}) \quad (13)$$

$$(E)_0 = (\bar{M}) + (\bar{MI}) + 2(\bar{D}) + 3[(\bar{T}) + (\bar{TI})] \quad (14)$$

If oligomerization is slow compared to the rate at which inhibitor binds, this mechanism predicts that the reciprocal of the relaxation time for binding will vary linearly with the sum of the concentrations of free monomer, trimer, and inhibitor

$$1/\tau = k_f[(\bar{M}) + (\bar{T}) + (\bar{I})] + k_d \quad (15)$$

The quantity in brackets can be calculated because eq 2, 9, 10, 12, 13, and 14 provide six equations in the six unknowns $(\bar{M})$, $(\bar{D})$, $(\bar{T})$, $(\bar{MI})$, $(\bar{TI})$, and $(\bar{I})$. Equations 2, 12, and 13 were solved for the concentrations of the enzyme–inhibitor complexes

$$(\bar{MI}) = \frac{(I)_0(\bar{M})}{K_{MI} + (\bar{M}) + (\bar{T})} \quad (16)$$

$$(\bar{TI}) = \frac{(I)_0(\bar{T})}{K_{MI} + (\bar{M}) + (\bar{T})} \quad (17)$$

Substituting eq 9, 10, 16, and 17 into eq 14 and rearranging gives the sixth-degree equation

$$(\bar{M})^6 + (^2/_3)K_T(\bar{M})^5 + (^4/_3)K_DK_T(\bar{M})^4 + K_DK_T[(^2/_3)K_T + K_{MI} + (I)_0 - (^1/_3)(E)_0](\bar{M})^3 + (^1/_3)K_DK_T^2(2K_{MI} + K_D)(\bar{M})^2 + (^1/_3)(K_DK_T)^2[K_{MI} + (I)_0 - (E)_0](\bar{M}) - (^1/_3)(K_DK_T)^2K_{MI}(E)_0 = 0 \quad (18)$$

Equation 18 was solved by evaluating the left-hand side for possible values of $(\bar{M})$ and graphing to find the solution. $(\bar{T})$ and $(\bar{I})$ were then found from eq 9, 10, 16, 17, and 13. Computations were performed on a General Electric 625 computer accessed through time sharing. The data are shown plotted according to eq 15 in Figure 4.

At high enzyme concentrations the measured relaxation times are longer than they would be if both monomers and trimers possessed equivalent binding sites. We therefore assumed that only monomers can bind a competitive inhibitor. Case I predicts that the reciprocal of the relaxation time will vary directly with the sum of the free monomer and inhibitor concentrations.

$$1/\tau = k_f[(\bar{M}) + (\bar{I})] + k_d \quad (19)$$

Solving, as in the previous case, gives the quartic equation

$$(\bar{M})^4 + [(^2/_3)K_T + K_{MI}](\bar{M})^3 + (^1/_3)K_T(K_D + 2K_{MI})(\bar{M})^2 + (^1/_3)K_DK_T[K_{MI} + (I)_0 - (E)_0](\bar{M}) - (^1/_3)K_DK_TK_{MI}(E)_0 = 0 \quad (20)$$

with

$$(I)_0 = (\bar{I}) + (\bar{MI}) \quad (21)$$

$$(E)_0 = (\bar{M}) + (\bar{MI}) + 2(\bar{D}) + 3(\bar{T}) \quad (22)$$

and

$$(\bar{MI}) = \frac{(I)_0(\bar{M})}{K_{MI} + (\bar{M})} \quad (23)$$

Equation 20 was solved exactly by Ferrari's method (Uspensky, 1948). The result of plotting $1/\bar{\tau}$ *vs.* $[(\bar{M}) + (\bar{I})]$ is shown in Figure 5. Within experimental error the plot is linear. The line drawn through the points was found by least squaring all the experimental points. The slope gives the bimolecular rate constant for formation of the monomeric α-CT–Pro complex, $k_f = (4.1 \pm 0.1) \times 10^7$ M^{-1} sec^{-1}. The rate constant for decomposition of the α-CT–Pro complex, $k_d = (1.7 \pm 0.7) \times 10^3$ sec^{-1}, is found from the intercept with the $1/\bar{\tau}$ axis. Their ratio (k_d/k_f) is the dissociation constant for the α-CT monomer–Pro complex, $K_{MI} = (4.2 \pm 1.5) \times 10^{-5}$ M.

The values of the monomer–inhibitor dissociation constant (K_{MI}) corresponding to the measured values of the enzyme–inhibitor dissociation constant (K_I) can be calculated for case I. The constancy of K_{MI} with varying $(E)_0$ provides a test of the compatibility of the equilibrium dialysis data with the assumption that only monomers of α-CT can bind Pro. The conservation equation for inhibitor in case I (eq 21) becomes

$$(I)_0 = (\bar{I}) + (\overline{MI}) + (I)_m \quad (24)$$

in the equilibrium dialysis experiments. The additional term, $(I)_m$, is the amount of inhibitor adsorbed on the semipermeable membrane, expressed as a concentration. It is measured in experiments in the absence of protein. $(\bar{I})$ is found by measuring the absorbance on the non-protein side of the membrane at equilibrium, permitting $(\overline{MI})$ to be evaluated from eq 24. Combining eq 2, 9, and 10 with eq 22 gives

$$K_{MI}^3 + (^2/_3)K_T\frac{(\bar{I})}{(\overline{MI})}K_{MI}^2 + (^1/_3)K_DK_T\left[\frac{(\bar{I})}{(\overline{MI})}\right]^2K_{MI} + (^1/_3)K_DK_T\left[\frac{(\bar{I})}{(\overline{MI})}\right]^2\left[(\bar{I}) - \frac{(\bar{I})}{(\overline{MI})}(E)_0\right] = 0 \quad (25)$$

which was solved for K_{MI}. The average values of K_{MI} calculated from the equilibrium dialysis data are shown plotted against $(E)_0$ by the open squares in Figure 1. Using the values of K_{MI} calculated for all the experimental data at every enzyme concentration to evaluate the mean and standard deviation gives $K_{MI} = (3.8 \pm 0.7) \times 10^{-5}$ M. This value equals the kinetically determined value of K_{MI} within experimental error (Table III). The thermodynamic and kinetic values of K_{MI} are not completely independent, since the former was used to calculate the sum of the concentrations of free monomer and free inhibitor in eq 19. However, the kinetic value does not depend critically on the choice of K_{MI}, because only the abscissa values in Figure 5 corresponding to low total enzyme concentrations depend significantly on the value of K_{MI} used in eq 20.

Two other possible mechanisms for oligomer formation were checked. If the most important interactions in solution were similar to those observed about dyad B in crystalline α-CT and if trimerization involved interactions similar to those about dyad A, case VI would result. The concentration of free monomer cannot readily be calculated for this mechanism, but case IV, in which monomers, dimers, and trimers each possess one equivalent binding site, was solved. Finally, case III was solved, because it will give a nearly linear relationship between the measured reciprocal relaxation times and the concentrations of reactants that it predicts. The polynomial expressions for cases IV and III are

$$(\bar{M})^6 + (^5/_3)K_T(\bar{M})^5 + (^2/_3)K_T(2K_D + K_T)(\bar{M})^4 + K_DK_T[K_T + K_{MI} + (I)_0 - (^1/_3)(E)_0](\bar{M})^3 + (^1/_3)K_DK_T^2[K_D + 2K_{MI} + 2(I)_0 - (E)_0](\bar{M})^2 + (^1/_3)(K_DK_T)^2[K_{MI} + (I)_0 - (E)_0](\bar{M}) - (^1/_3)(K_DK_T)^2K_{MI}(E)_0 = 0 \quad (26)$$

and

$$(\bar{M})^5 + [K_D + (^2/_3)K_T](\bar{M})^4 + K_D(K_{MI} + K_T)(\bar{M})^3 + (^1/_3)K_DK_T[2K_{MI} + 2(I)_0 + K_D - (E)_0](\bar{M})^2 + (^1/_3)K_DK_T[K_{MI} + (I)_0 - (E)_0](\bar{M}) - (^1/_3)K_D^2K_TK_{MI}(E)_0 = 0 \quad (27)$$

respectively.

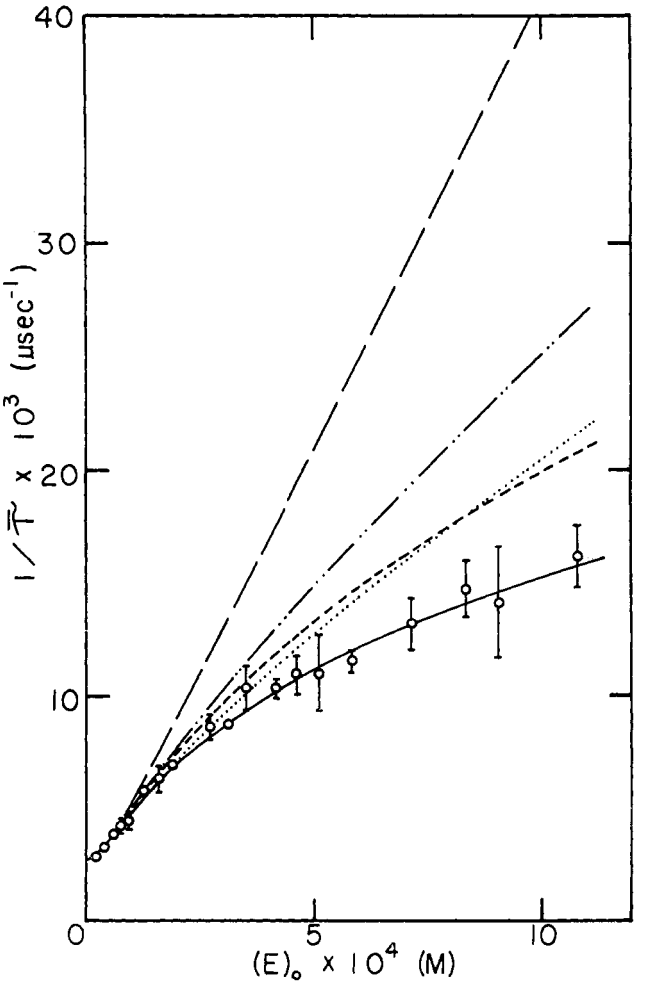

FIGURE 6: The measured reciprocal relaxation times are shown superimposed on theoretical curves calculated by assuming that only the availability of binding sites is affected when α-CT associates. The method used to calculate the theoretical curves is explained in the text. (——) Case I, only monomers bind Pro; (· · · ·) case II, monomers and trimers each have one, equivalent binding site; (----) case III, monomers and dimers each possess one binding site; (—· ·—) case IV, monomers, dimers, and trimers each have one free site; (— —) case VIII, monomers possess one, dimers two, and trimers three equivalent sites.

The correlation coefficients and F ratios for linear regression of the measured reciprocal relaxation times on the sum of the concentrations of free inhibitor and unoccupied binding sites predicted by each of the five mechanisms tested are given in Table IV. Case I gives the best fit to the experimental data. This is illustrated more clearly by Figure 6 in which the mean reciprocal relaxation times and their standard deviations are shown plotted on a family of theoretical curves for the mechanisms considered. The theoretical reciprocal relaxation times were calculated using the values of the rate constants, $k_f = 4.1 \times 10^7$ M^{-1} sec^{-1} and $k_d = 1.7 \times 10^3$ sec^{-1}, found for case I.

The simplest explanation of our results is that, under the experimental conditions used, only monomeric α-CT binds Pro. Both the equilibrium dialysis data and the relaxation kinetic data are consistent with this interpretation. The values of the monomer–proflavin dissociation constant found by the two semiindependent methods are experimentally equivalent. The rate constants evaluated for formation and decomposition of the enzyme–inhibitor complex are typical

of those previously reported for enzyme–substrate interactions (Eigen and Hammes, 1963). Our interpretation is consistent with X-ray crystallographic results which show that the best-developed interaction in crystalline α-CT involves groups in or near the active center (Sigler *et al.*, 1968) and with recent evidence that the same groups are responsible for the predominant interaction in solution (Timasheff, 1969; Aune and Timasheff, 1970). A plausible interpretation of the failure of the highest molecular weight aggregates to bind Pro is that they are largely tetramers rather than trimers (Massey *et al.*, 1955; Rao and Kegeles, 1958; Winzor and Scheraga, 1963). Finally, Shiao and Sturtevant (1969) have reached the same conclusion from calorimetric measurements of the enthalpy of binding of Pro to α-CT at pH 7.8 and 25° in 0.05 M phosphate buffer, 0.2 M in KCl.

While this investigation was in progress, the results of crystallographic studies of inhibitor binding to the active site of α-CT were published (Steitz *et al.*, 1970). The 2.5-Å difference map between *N*-formyl-L-tryptophan: α-CT and native enzyme shows that the hydrophobic pocket can easily accommodate indole. It is therefore likely that the discrepancy between our results and those of Sarfare *et al.* (1966) is a consequence of the difference in inhibitor size. Presumably β-phenylpropionate, but not the larger Pro molecule, can be accommodated at the active site without impairing oligomerization. Projection studies suggest that the hydrophobic pocket of crystalline α-CT is not deep enough to accommodate the aromatic ring of β-(*p*-iodophenyl)propionate (Steitz *et al.*, 1970). Neet and Brydon (1970) have concluded that large acyl groups such as cinnamoyl and phenylmethylsulfonyl affect the association of chemically modified α-CT. Of course, the physiological substrates for α-CT are still larger than Pro. It will be shown elsewhere (L. D. Faller, submitted for publication) that the kinetic data of Inagami and Sturtevant (1965) for the acylation of α-CT by *N*-acetyl-L-tyrosine-*p*-nitroanilide is consistent with the conclusion that only monomeric enzyme binds that model substrate.

In addition to noting that different competitive inhibitors may affect oligomerization differently, two additional reservations should be explicitly stated. First, the association of α-CT depends dramatically on pH and ionic strength. Several authors have suggested that the nature of the dimer interaction may depend on the experimental conditions (Kezdy and Bender, 1962; Shiao and Sturtevant, 1969). Second, the reported experiments do not absolutely preclude the possibility that Pro can bind to oligomers of α-CT. The assumption that Pro binds to oligomers, but with a different dissociation constant for each form, would introduce additional parameters into the derived polynomial expressions for monomer concentration and into the expressions for the reciprocal relaxation time. The experimental data could presumably be satisfactorily fitted by the proper choice of those parameters.

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Kinetic Studies of Tryptophan Synthetase. Interaction of L-Serine, Indole, and Tryptophan with the Native Enzyme*

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ABSTRACT: The synthesis of L-tryptophan from indole and L-serine, as catalyzed by bacterial tryptophan synthetase ($\alpha_2\beta_2$), has been studied with steady-state and rapid reaction techniques. Initial velocity measurements of the reaction have been made utilizing the absorption difference between indole and tryptophan at 289 nm. The results are consistent with both a compulsory sequence of substrate addition, and a random, rapid equilibration between enzyme and substrates. Dissociation constants and/or Michaelis constants for serine and indole were obtained. Temperature-jump, stopped-flow, and combined stopped-flow–temperature-jump measurements were made on solutions containing β_2 protein and an excess of α protein combined with indole, L-serine, L-tryptophan, and indole plus L-serine. The results are compared to data from similar studies in which β_2 protein alone was the catalyst.

The results obtained indicate that (1) L-serine binds much more tightly to $\alpha_2\beta_2$ than to β_2 protein, (2) the binding of L-tryptophan is too fast to measure for the holoenzyme, (3) the enzyme isomerization previously observed for β_2 protein probably does not occur with $\alpha_2\beta_2$, and (4) only the single first-order (rate-limiting) process observed previously can be detected on interaction of the enzyme–serine complex with indole. The mechanism of action of tryptophan synthetase in catalyzing this reaction is discussed in terms of the results obtained.

Tryptophan synthetase isolated from *Escherichia coli* is a multisubunit enzyme consisting of two different subunits (Yanofsky, 1960). The fully associated form, $\alpha_2\beta_2$, where α and β represent different protein chains, is dissociable into two α subunits and a β_2 dimer (Wilson and Crawford, 1964; Hathaway *et al.*, 1969). The overall reaction catalyzed by the intact enzyme is

$$\text{InGP} + \text{Ser} \underset{\text{PLP}}{\overset{\alpha_2\beta_2}{\rightleftharpoons}} \text{Trp} + \text{GP} \qquad (1)$$

which is the sum of reactions catalyzed by the individual subunits

$$\text{InGP} \overset{\alpha}{\rightleftharpoons} \text{In} + \text{GP} \qquad (2)$$

$$\text{In} + \text{Ser} \underset{\text{PLP}}{\overset{\beta_2}{\rightleftharpoons}} \text{Trp} \qquad (3)$$

Here InGP is indole-3-glycerol phosphate, Ser is L-serine, GP is glyceraldehyde 3-phosphate, and PLP is pyridoxal 5′-phosphate. Each subunit has the ability to markedly increase the reaction rate for the process catalyzed by the other protein (Wilson and Crawford, 1965; Hatanaka *et al.*, 1962).

This work represents the second part of a study of the synthesis of tryptophan from L-serine and indole. Steady-state, difference spectral, and rapid reaction techniques were used to examine this reaction in the presence of the fully active tryptophan synthetase molecule ($\alpha_2\beta_2$) and the results obtained are compared to those obtained for catalysis by the β_2 protein alone (Faeder and Hammes, 1970). A model is postulated for the role of α protein in the mechanism of action of the tryptophan synthetase system.

Experimental Section

Materials. The β_2 protein of tryptophan synthetase was purified from the A2/F′A2 mutant of *E. coli* K-12, while α protein was prepared from the B-8 mutant. All preparative procedures were done at 2–5°. Both strains were kindly supplied to us by Dr. C. Yanofsky and Dr. L. Soll. Some of the bacteria were grown by Mr. A. Tannahill of the Department of Chemical Engineering, Cornell University, and some were supplied by the New England Enzyme Center. The procedure used for preparing β_2 protein has been described elsewhere (Wilson and Crawford, 1965; Faeder and Hammes, 1970). The β_2 protein was prepared for experiments by dialyzing against 0.1 M potassium phosphate buffer containing 1×10^{-5} M pyridoxal 5′-phosphate (pH 7.8). The α protein was prepared by a scaled-up version of the method of Henning

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