Calorimetric Investigations of the Binding of Inhibitors to α-Chymotrypsin. I. The Enthalpy of Dilution of α-Chymotrypsin and of Proflavin, and the Enthalpy of Binding of Indole, *N*-Acetyl-D-tryptophan, and Proflavin to α-Chymotrypsin*

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ABSTRACT: A flow microcalorimeter has been employed to measure the enthalpies of binding of indole, *N*-acetyl-D-tryptophan, and proflavin to α-chymotrypsin at pH 7.8 and also the heats of dilution of α-chymotrypsin and proflavin at the same pH. The following points are discussed. (1) The studies of the heats of dilution of α-chymotrypsin as a function of enzyme concentration lead to the suggestion that monomer–dimer equilibrium of this enzyme exists at pH 7.8. The calculated thermodynamic functions for this equilibrium are consistent with the view that the dimeric forms of the enzyme can be identified as the enzyme–substrate intermediates preceding the autolysis reaction. (2) The heat effects due to the dilution of proflavin are shown to be consistent with the assumption that the self-association of this compound can be adequately represented by a series of equilibria involving dimer, trimer, and other higher polymers. The large negative enthalpy of polymerization (−4.0 kcal/mole) shows that the self-association of proflavin is accompanied by an unfavorable entropy change. (3) The apparent heats of binding of inhibitors to α-chymotrypsin were observed to be strongly dependent on enzyme concentration. These observations lead to the suggestion that the dimeric forms of the enzyme are incapable of binding inhibitors. (4) The enthalpies of binding of indole, *N*-acetyl-D-tryptophan, and proflavin to α-chymotrypsin were observed to be −15.2, −19.0, and −11.3 kcal/mole, respectively, at pH 7.8. On the basis of these numbers it is concluded that conformational changes in the enzyme are induced by the binding of inhibitors.

The binding of various inhibitors to α-CT[1] has been extensively investigated by various techniques. Niemann and his colleagues examined over 100 compounds, determining their inhibition constants toward the CT-catalyzed hydrolysis of acetyl-L-valine (Hein and Niemann, 1962; Wallace *et al.*, 1963). From those studies, useful information concerning the topography of the active site of α-CT was obtained. The comparison of binding constants of some charged and uncharged inhibitors as a function of pH (Foster and Niemann, 1955; Johnson and Knowles, 1966) has led to the suggestion that the active site of α-CT is negatively charged at neutral pH values where the molecule is enzymically active. This suggestion has recently received support from X-ray studies of α-CT (Blow *et al.*, 1969).

The binding of various substrates and inhibitors to α-CT produces an enhancement of the fluorescence of the enzyme (Sturtevant, 1962). The fluorescence increase takes place with first-order rate constants of about 1 sec^{-1} for widely different ligands, the rate constants being independent of ligand concentrations. These findings suggest that conformational changes in α-CT, which are included by the binding of substrates and inhibitors, are responsible for the observed changes in fluorescence emission. As an aid to understanding the nature of the conformational changes mentioned above and also the various forces involved in the binding reaction, a knowledge of the enthalpic and entropic contributions to the free energies of binding are important. Several workers have estimated the enthalpies of binding of certain inhibitors and virtual substrates to α-CT from the temperature dependence of the binding constants (Doherty and Vaslow, 1952; Yapel, 1967; Hymes *et al.*, 1969). Direct determination of these quantities by calorimetry has been reported by Canady and Laidler (1958) who studied the binding of hydrocinnamate ion to α-CT as a function of pH. In this communication, we report measurements by direct calorimetry of the enthalpy changes associated with the binding of indole, *N*-acetyl-D-tryptophan, and proflavin to α-CT. These compounds were shown to be competitive inhibitors to α-CT by earlier studies (Wallace *et al.*, 1963; Foster and Niemann, 1955; Bernhard *et al.*, 1966).

Experimental Section

Three times recrystallized α-CT (bovine pancreas) was purchased from Worthington Biochemical Corp., Freehold, N. J. (batch CDI-7KD and CDI-8VS). Since purification of the enzyme by Sephadex G-25 (Yapel *et al.*, 1966) was shown to have no significant effect on the results of calorimetric measurements, most of the results reported in this paper were obtained using the enzyme as received. Enzyme concentrations were determined by measuring the optical density at 280 nm, assuming an absorptivity of 5×10^4 M^{-1} cm^{-1}.

The following compounds were used without further purifi-

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[1] The abbreviation used is: α-CT, α-chymotrypsin.

TABLE I: Heats of Dilution of α-Chymotrypsin at pH 7.8 and 25°.

Series No.	Initial Concn (mM) C_i	Final Concn (mM) C_f	Q_{obsd}[a]	Q_c[b]	$-\varphi L_2$[c]
1	0.744	0.744	0.0	0.0	11.0
		0.700	0.2	0.2	10.8
		0.596	0.6	0.6	10.4
		0.372	2.0	2.0	9.0
2	0.612	0.612	0.0	0.4	10.6
		0.576	0.4	0.8	10.2
		0.490	0.8	1.2	9.8
		0.306	2.3	2.7	8.3
3	0.612	0.612	0.0	0.4	10.6
		0.305	2.3	2.7	8.3
4	0.596	0.596	0.0	0.5	10.5
		0.397	1.6	2.1	8.9
		0.298	2.7	3.2	7.8
		0.199	4.2	4.7	6.3
5	0.476	0.476	0.0	1.0	10.0
		0.448	0.5	1.5	9.5
		0.443	0.5	1.5	9.5
		0.317	1.6	2.6	8.4
		0.063	5.0	6.0	5.0
		0.028	9.3	10.3	0.7
6	0.472	0.472	0.0	1.0	10.0
		0.449	0.6	1.6	9.4
		0.382	0.9	1.9	9.1
		0.239	2.5	3.5	7.5
		0.095	3.5	4.5	6.5
		0.028	8.7	9.7	1.3
7	0.464	0.464	0.0	1.0	10.0
		0.436	0.3	1.3	9.7
		0.371	1.0	2.0	9.0
		0.232	2.0	3.0	8.0
		0.093	2.9	3.9	7.1
		0.027	7.6	8.6	2.4
8	0.460	0.460	0.0	1.0	10.0
		0.407	0.9	1.9	9.1
		0.306	1.9	2.9	8.1
		0.153	3.5	4.5	6.5
		0.051	7.3	8.3	2.3
		0.027	9.5	10.5	0.5

[a] Observed heat effect, kcal mole^{-1}. [b] Observed heat effect corrected to C_i = 0.744 mM, kcal mole^{-1}. [c] Apparent relative molar heat enthalpy, kcal mole^{-1}.

cation: indole, Matheson, Coleman and Bell Co., East Rutherford, N.J.; *N*-acetyl-D-tryptophan, Cyclo Chemical Corp., Los Angeles, Calif.; proflavin hemisulfate, British Drug Houses, Ltd., Poole, England. Calorimetric measurements reported here were made with a flow modification of the Beckman Model 190 microcalorimeter.[2] A brief description of this instrument has been presented elsewhere (Sturtevant and Lyons, 1969). All the calorimetric experiments were performed at pH 7.8 and 25°. The buffer solution used was 0.05 M in phosphate and 0.2 M in KCl.

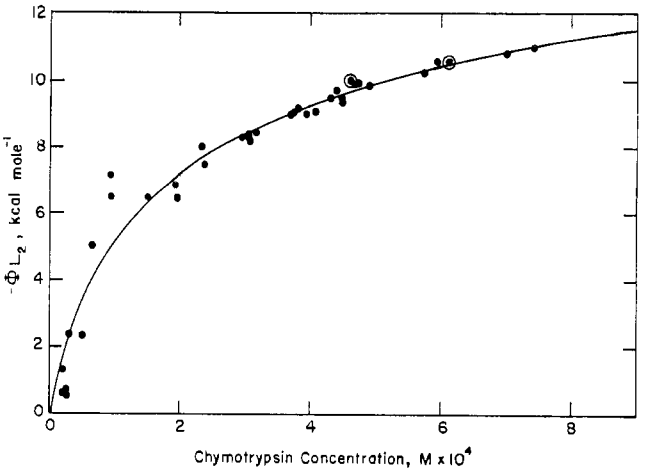

FIGURE 1: The apparent relative molar enthalpy of α-chymotrypsin as a function of protein concentration at pH 7.8 and 25°. The curve is calculated by means of eq 3 using the parameters given in the text.

Results

The Heats of Dilution of α-CT. In determining the heats of binding of small molecules to a protein system by means of flow calorimetry, it is necessary to correct for the heats of dilution of both the protein and the small molecules. α-CT undergoes rapid autolysis at pH 7.8 and 25°, so that precise measurements of its heats of dilution are very difficult. It was found that at a protein concentration of about 1%, the heats of dilution of α-CT became noticeable larger (more positive) after the solution was kept at 25° for about 45 min. At concentrations of 2% or higher, the heats of dilution of α-CT became so strongly time dependent that no useful data could be obtained even if the experiments were performed within 10 min. With these difficulties in mind, we performed our experiments using suitable protein concentrations. The results are summarized in Table I.

The apparent relative molar enthalpy of the solute, φL_2, which is given in the last column of Table I, is defined as follows:

$$\mathrm{CT}(C_f) = \mathrm{CT}(\text{infinite dilution}); \quad \Delta H = -\varphi L_2 \quad (1)$$

The extrapolation involved in obtaining values of φL_2 was performed by running overlapping series of dilutions and correcting each series to the same initial concentration, 7.44×10^{-4} M, by addition of a constant enthalpy estimated by inspection of the dilution curves. φL_2 is plotted as a function of C_f in Figure 1. It is obvious that there is a large uncertainty involved in the extrapolation to infinite dilution.

The form of the plot in Figure 1 suggests that intermolecular interactions occur between molecules of α-CT under

[2] The authors would like to thank Beckman Instruments, Inc., for the gift of the flow modification of the Model 190 microcalorimeter.

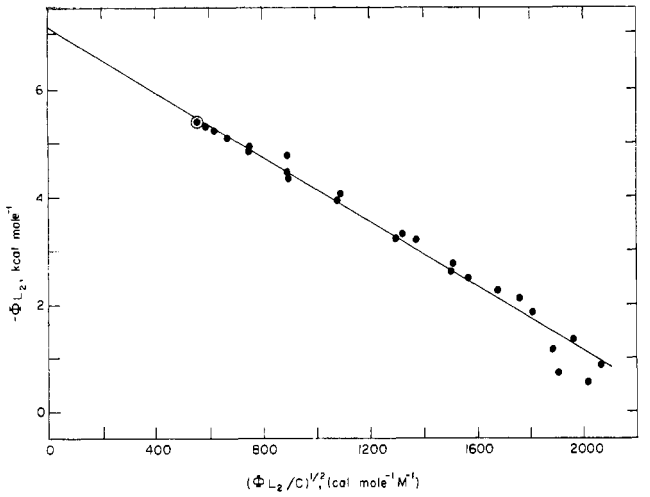

FIGURE 2: The apparent relative molar enthalpy, φL_2, of proflavin at pH 7.8 and 25° as a function of $(\varphi L_2/C)^{1/2}$, where C is the dye concentration. The line is calculated by means of eq 5 using the parameters given in the text.

the present experimental conditions. This suggestion is reasonable, since aggregation of α-CT at various values of pH has been observed by other techniques such as light scattering, depolarization of fluorescence, ultracentrifugation, and gel filtration (Schwert, 1949; Schwert and Kaufman, 1951; Steiner, 1954; Massey *et al.*, 1955; Tinoco, 1957; Rao and Kegeles, 1958; Winzor and Scheraga, 1963, 1964). There are two cases of aggregation for which the mathematical development is particularly simple (Gill *et al.*, 1967; Stoesser and Gill, 1967). For case 1, represented by the equilibrium

$$2P = P_2; \quad K, \Delta H \tag{2}$$

φL_2 is given by the expression

$$\varphi L_2 = {}^1/_2\Delta H - {}^1/_2(\Delta H/K)^{1/2}(\varphi L_2/C_f)^{1/2} \tag{3}$$

For case 2, represented by the equilibria

$$2P = P_2; \quad P + P_2 = P_3; \ldots \quad P + P_{n-1} = P_n; \ldots; \quad K, \Delta H \tag{4}$$

with the same equilibrium constant and enthalpy change for each step,

$$\varphi L_2 = \Delta H - (\Delta H/K)^{1/2}(\varphi L_2/C_f)^{1/2} \tag{5}$$

Equations 3 and 5 are based on the assumptions that the enthalpies of all species involved are independent of concentration, and that volumes are additive. Both of these assumptions are valid within the experimental uncertainties of the present work.

The data for α-CT were shown to be consistent with eq 3 in the following manner. Pairs of values of φL_2 and C_f were read from a visually smoothed plot of all the data, and values for K and ΔH were computed from two such pairs using an iterative procedure.[3] About 25 sets of values of K and ΔH were obtained in this way, and the averages of these values were $K^{-1} = 3.46 \pm 0.4 \times 10^{-4}$ M and $\Delta H = 35.4 \pm 2.3$ kcal (mole of dimer)$^{-1}$. The curve in Figure 1 was calculated using these values. While it is clear that eq 3 gives an adequate representation of the data, it is also evident that other equilibrium schemes, including that of eq 4, could fit the data just as well.

TABLE II: Heats of Dilution of Proflavin at pH 7.8 and 25°.

Series No.	Initial Concn (mM) C_i	Final Concn (mM) C_f	Q_{obsd}[a]	Q_c[b]	φL_2[c]
		17.0	0.0	0.0	5.40
		8.5	0.535	0.535	4.87
		5.67	0.843	0.843	4.56
1	17.0	3.40	1.35	1.35	4.05
		1.89	2.08	2.08	3.32
		1.00	2.92	2.92	2.48
		17.0	0.0	0.0	5.40
		15.1	0.084	0.084	5.32
		13.6	0.160	0.160	5.24
		11.3	0.295	0.295	5.11
2	17.0	8.50	0.526	0.526	4.87
		5.67	0.900	0.900	4.50
		3.40	1.44	1.44	3.96
		1.90	2.20	2.20	3.20
		3.40	0.0	1.34	4.06
		1.70	0.837	2.18	3.22
		1.13	1.30	2.64	2.76
3	3.4	0.680	1.95	3.29	2.11
		0.378	2.72	4.06	1.34
		0.200	3.24	4.58	0.82
		1.70	0.0	2.16	3.24
		0.850	0.958	3.12	2.28
		0.570	1.38	3.54	1.86
4	1.70	0.340	2.09	4.25	1.15
		0.189	2.56	4.72	0.68
		0.100	2.66	4.82	0.58

[a-c] See corresponding footnotes in Table I. Correction factor is C_i = 17.0 mM.

*The Heats of Dilution of Indole and N-Acetyl-*D*-tryptophan.* Under the experimental conditions employed in this work, the heat effects due to the dilution of these compounds were observed to be small but significant. Detailed investigations of these effects were not carried out. However, the necessary experiments were performed so that appropriate corrections could be made to the enthalpies of binding to α-CT discussed below.

The Heats of Dilution of Proflavin. Proflavin is known to undergo aggregation at neutral pH (Li, 1968). Preliminary

[3] The computer program used in these computations was written by Mr. Stephen A. Rudolph.

TABLE III: Enthalpy of Binding of Indole to α-Chymotrypsin at pH 7.8 and 25°.

Concn of Chymotrypsin (mM) (E_0)	Concn of Indole (mM) (I_0)	Observed Heat Effect (kcal mole^{-1}) ΔQ_{app}	Enthalpy of Binding (kcal/mole of Inhibitor Bound) $-\Delta H_b$
0.103	13.6	10.5	16.5
0.115	13.6	9.4	15.8
0.120	11.3	7.6	14.1
0.159	11.3	7.1	14.4
0.172	11.3	8.4	16.0
0.180	8.5	6.2	14.1
0.192	11.3	7.8	15.8
0.238	8.5	6.0	14.9
0.240	5.7	4.8	14.0
0.258	8.5	6.6	15.8
0.288	8.5	6.3	15.8
0.311	5.7	5.4	15.5
0.317	5.7	4.3	14.3
0.384	5.7	4.7	15.5
		Mean	15.2
		Av dev.	±0.8

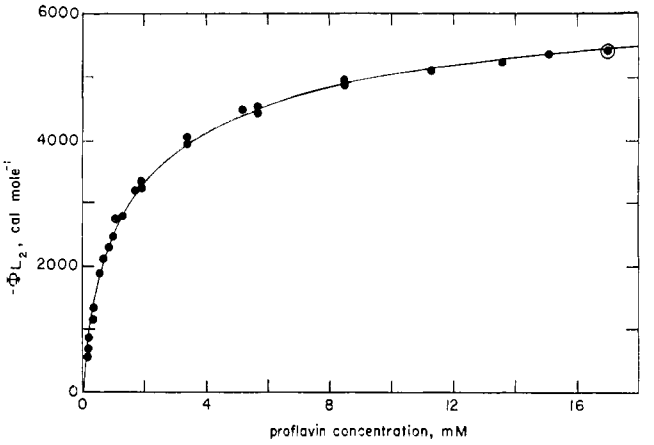

FIGURE 3: The apparent relative molar enthalpy of proflavin as a function of proflavin concentration at pH 7.8 and 25°. The curve is calculated by means of eq 5 using the parameters given in the text.

measurements of the heat of dilution of this compound indicated that the heat effects due to aggregation were quite large. Since the aggregation of proflavin is in itself an interesting phenomenon, we have measured the heat of dilution as a function of dye concentration. The data are presented in Table II. Here it seems most reasonable to assume that case 2 applies. In the process of obtaining values for φL_2 it is necessary, as mentioned above, to extrapolate the observed heats of dilution to infinite dilution. The extrapolation is difficult because of the very rapid change of dilution heat with concentration in the region of low concentrations. We have therefore assumed a series of values for the extrapolated quantity and for each assumed value have fitted the data to eq 5 by the method of least squares. The assumed intercept giving the smallest standard deviation was taken as the correct value. A linear plot of φL_2 *vs.* $(\varphi L_2/C_f)^{1/2}$, according to eq 5, is seen in Figure 2 to fit the experimental data quite satisfactorily. From the slope and intercept of this plot we obtain $K = 803 \pm 56$ M^{-1} and $\Delta H = -7.13 \pm 0.16$ kcal mole^{-1}. The curve in Figure 3 is calculated using these values, and shows that eq 5 gives an entirely adequate description of the results. Again, however, it cannot be concluded that this is a uniquely satisfactory description of the results.

*The Enthalpy of Binding of Indole, N-Acetyl-*D*-tryptophan, and Proflavin to α-CT.* We define the quantity ΔQ_{app} as the change in enthalpy (per mole of protein) associated with the binding of inhibitors to α-CT *after* correction for the heat effects due to dilution. The values of ΔQ_{app} for indole, *N*-acetyl-D-tryptophan, and proflavin as a function of enzyme concentration are listed in Tables III, IV, and V. Since the inhibitor concentrations used in most of our experiments were about ten times larger than the dissociation constants of the enzyme–inhibitor complexes, the fraction of α-CT in the complex forms is expected to be close to unity. Under such circumstances, if the dimerization of α-CT had no effect on the binding of inhibitor or *vice versa*, ΔQ_{app} would be expected to be independent of enzyme concentration. That such is not the case is clearly demonstrated by the results given in Figure 4.

TABLE IV: Enthalpy of Binding of *N*-Acetyl-D-tryptophan to α-Chymotrypsin at pH 7.8 and 25°.

Concn of Chymotrypsin (mM) (E_0)	Concn of Inhibitor (mM) (I_0)	Observed Heat Effect (kcal mole^{-1}) ΔQ_{app}	Enthalpy of Binding (kcal/mole of Inhibitor Bound) $-\Delta H_b$
0.0730	80.0	14.7	20.0
0.0807	67.0	13.5	19.2
0.0944	80.0	14.9	20.8
0.109	80.0	11.8	18.0
0.115	80.0	12.3	18.7
0.121	67.0	11.8	18.5
0.121	50.0	11.2	18.2
0.157	67.0	12.8	20.4
0.161	33.0	9.3	17.8
0.181	67.0	11.1	19.1
0.182	50.0	10.1	18.4
0.196	67.0	10.4	18.6
0.234	50.0	10.5	19.6
0.243	33.0	8.6	18.4
0.272	50.0	9.5	19.1
0.293	50.0	9.2	19.0
0.363	33.0	7.9	18.9
0.391	33.0	7.9	19.2
		Mean	19.0
		Av dev.	±0.6

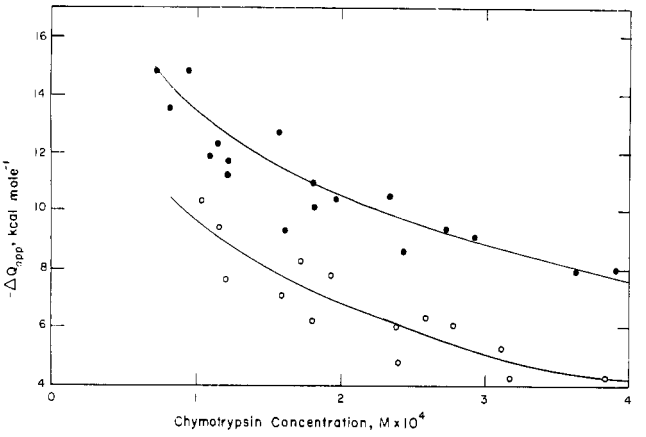

FIGURE 4: The apparent enthalpy change in the binding of *N*-acetyl-D-tryptophan (●) and indole (○) to α-chymotrypsin as a function of enzyme concentration at pH 7.8 and 25°.

The dependence of ΔQ_{app} on enzyme concentration can be understood in terms of the equilibria represented by eq 6 and 7

$$E_2 \rightleftarrows 2E \tag{6}$$

$$E + I \rightleftarrows EI \tag{7}$$

with equilibrium constant K_D and enthalpy change ΔH_D for reaction 6 and equilibrium constant $1/K_I$ and the enthalpy change ΔH_b for reaction 7. The assumption required for writing the above equilibria is that the dimeric form of α-CT does not bind inhibitors. Before equilibration of the mixed solution in the calorimeter, we have

$$(E_{2i})^2 - \left[(E_0) + \frac{K_D}{4}\right](E_{2i}) + {}^1/_4(E_0)^2 = 0 \tag{8}$$

where (E_{2i}) is the initial concentration of the dimer and (E_0) is the total concentration of the enzyme. After equilibration of the mixed solution in the calorimeter, we have

$$(E)^2 + \left(\frac{K_D}{2} + \frac{K_D(I_0)}{2K_I}\right)(E) - \frac{K_D}{2}(E_0) = 0 \tag{9a}$$

provided that $(I_0) \gg (EI)$. If $(I_0) \approx (EI)$, eq 9a becomes

$$(E)^3 + (E)^2\left(K_I + \frac{K_D}{2}\right) + (E)\left[\frac{K_D K_I}{2} + \frac{K_D(I_0)}{2} - \frac{K_D(E_0)}{2}\right] - \frac{K_D K_I(E_0)}{2} = 0 \tag{9b}$$

In addition, we have the equations

$$(E_2) = (E)^2/K_D \tag{10}$$

$$(EI) = (E_0) - (E) - 2(E_2) \tag{11}$$

TABLE V: Enthalpy of Binding of Proflavin to α-Chymotrypsin at pH 7.8 and 25°.

Concn of Chymotrypsin (mM) (E_0)	Concn of Inhibitor (mM) (I_0)	Observed Heat Effect (kcal mole^{-1}) ΔQ_{app}	Enthalpy of Binding (kcal/mole of Inhibitor Bound) $-\Delta H_b$
0.123	1.13	2.9	8.9
0.165	1.13	2.9	9.8
0.165	1.13	3.0	9.9
0.185	1.13	4.1	12.4
0.195	1.13	2.5	10.0
0.195	1.13	2.5	10.0
0.217	0.340	3.5	12.0
0.234	0.850	3.5	11.7
0.248	0.850	2.7	11.1
0.271	0.850	3.7	12.5
0.292	0.850	2.7	11.7
0.312	0.567	2.8	12.5
0.328	0.567	2.2	12.1
0.330	0.567	2.7	12.7
0.389	0.567	2.0	12.7
		Mean	11.3
		Av dev.	±1.0

where (E), (E_2), and (EI) are, respectively, the concentrations of the monomeric enzyme, the dimeric enzyme, and the enzyme–inhibitor complex, and (I_0) is the total concentration of inhibitor. The heat effect produced in killocalories per liter of the mixed solution is then given by

$$\Delta Q = [(E_{2i}) - (E_2)]\Delta H_D - (EI)\Delta H_b \tag{12}$$

The solution of eq 9b was obtained by means of an iterative procedure.[3] Using values of K_I reported by other workers (Wallace *et al.*, 1963; Foster and Niemann, 1955; Bernhard *et al.*, 1966) and the values of K_D and ΔH_D obtained from the experiments on the dilution of CT, the above equations lead to the values of ΔH_b which are summarized in Tables III, IV, and V. The calculated values of ΔH_b for indole and *N*-acetyl-D-tryptophan as a function of enzyme concentration are shown in Figure 5, and those for proflavin as a function of inhibitor concentration in Figure 6. It is clear that ΔH_b as a function of inhibitor concentration in each case is independent of enzyme concentration. The omission of any allowance for the polymerization of proflavin in computing ΔH_b is discussed below.

By combining the available values of K_I and ΔH_b, the thermodynamic functions characterizing the binding of inhibitors to α-CT can be computed, with the results summarized in Table VI. In this table, we have also listed the thermodynamic changes associated with the dimerization of α-CT.

The binding of inhibitors to α-CT may lead to proton liberation or proton uptake (McConn *et al.*, 1968). If so, the values of ΔH_b given in Table VI would have to be corrected

TABLE VI: Thermodynamic Parameters for the Binding of Various Compounds to α-Chymotrypsin at pH 7.8 and 25°.

Compound	Dissocn Const of Enzyme–Compound Complex (mM) K_I	Standard Free Energy of Binding (kcal mole^{-1}) $\Delta G_b'$	Enthalpy of Binding (kcal mole^{-1}) ΔH_b	Standard Entropy of Binding (cal deg^{-1} mole^{-1}) $\Delta S_b'$
Indole	0.70[a]	−4.31	−15.2 ± 0.8	−37 ± 3
N-Acetyl-D-tryptophan	4.0[b]	−3.28	−19.0 ± 0.6	−53 ± 2
Proflavin	0.037[c]	−6.04	−11.3 ± 1.0	−18 ± 3
α-Chymotrypsin	0.35	−4.73	−17.7 ± 1.2	−43 ± 4

[a] Wallace *et al.*, 1963. [b] Foster and Niemann, 1955. [c] Bernhard *et al.*, 1966.

for the contribution resulting from buffer ionization. Since the enthalpy change in the second ionization of phosphoric acid is +1.13 kcal mole^{-1} (G. D. Watt, 1968, personal communications), this correction would be −1.13 kcal mole^{-1} per proton liberated.

Discussion

The results presented above suggest that a monomer–dimer equilibrium of α-CT exists at pH 7.8 and the binding of inhibitors to this enzyme occurs only on the monomers. Our results, however, do not exclude the possible existence of other types of association of α-CT which do not produce a sufficient enthalpy change to allow detection by calorimetry. Rao and Kegeles (1958) have determined the molecular weight of α-CT as a function of protein concentration in phosphate buffer solution at pH 6.2 and ionic strength 0.2 M, by the Archibald ultracentrifuge method. Their results suggest that monomers, dimers, and trimers of α-CT are in equilibrium under their experimental conditions and that this equilibrium is not influenced by the presence of β-phenylpropionate ion, a competitive inhibitor (Sarfare *et al.*, 1966). The apparent disagreement between our observations and those of Rao and Kegeles and Sarfare *et al.* may be due to the difference in experimental conditions used in the two sets of experiments, or it may be that our calorimetric experiments do not see the same reaction as is seen in the ultracentrifuge experiments.

The effect of aggregation on the catalytic activity of α-CT has been studied by several workers (Kézdy and Bender, 1965; Martin and Niemann, 1958; Inagami and Sturtevant, 1965). However, due to uncertainties in assigning parameters in the kinetic equations, no unique conclusion concerning the correlation between catalytic activity and polymerization of α-CT can be deduced from these studies (Inagami and Sturtevant, 1965). In this connection, our experimental evidence suggests that the dimeric forms of α-CT at pH 7.8 do not have catalytic activity since they are incapable of binding competitive inhibitors. On the other hand, kinetic studies with α-CT at pH 6.2 (Inagami and Sturtevant, 1965) indicate that the polymeric species of the enzyme are not completely inactive. This disagreement leads us to suspect that the dimeric form of α-CT at pH 7.8 as detected by calorimetry is not the same as the dimeric species observed at pH 6.2. In view of the fact that autolysis of α-CT occurs at pH 7.8 (Marini and Wunsch, 1963) and that the thermodynamic changes accompanying dimer formation of the enzyme are similar to those for the binding of competitive inhibitors, we further propose that the dimeric form of α-CT as detected by the calorimetric experiments is actually the enzyme–substrate complex preceding the autolysis reaction. It is interesting that under conditions where autolysis is decreased, the calorimetric indication of the presence of dimers is also much decreased. Thus, heats of dilution of α-CT at pH 5.6 and at pH 7.8 in the presence of 0.1 M $CaCl_2$, which is known to

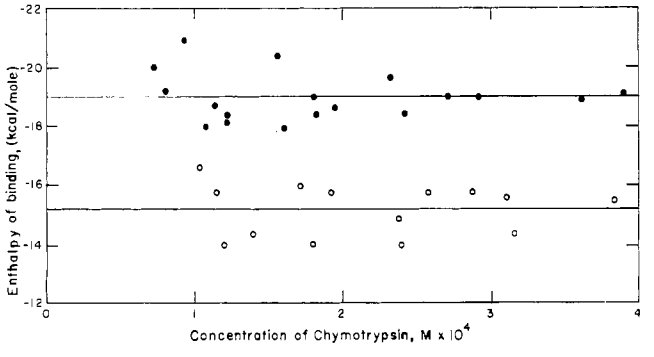

FIGURE 5: The enthalpy of binding of *N*-acetyl-D-tryptophan (●) and indole (○) to α-chymotrypsin corrected for dimerization of the enzyme as a function of enzyme concentration at pH 7.8 and 25°.

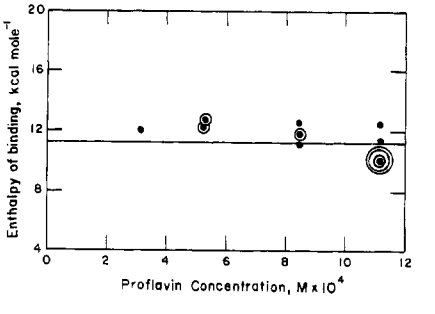

FIGURE 6: The enthalpy of binding of proflavin to α-chymotrypsin at pH 7.8 and 25°, corrected for the dimerization of the enzyme. The constancy of the values indicates that the binding is not affected by the polymerization of proflavin.

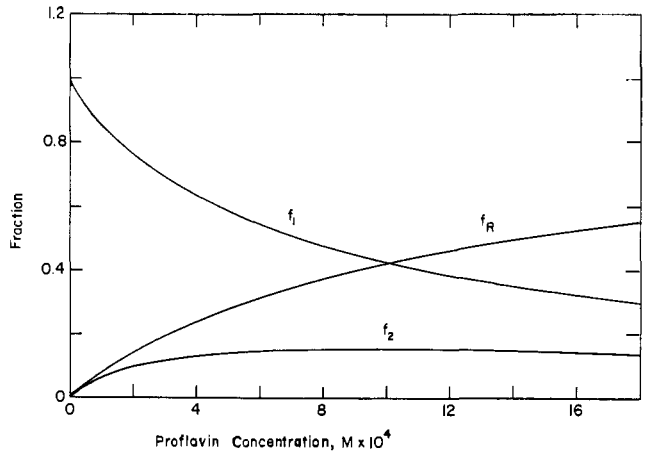

FIGURE 7: The distribution of proflavin among the various polymeric species at pH 7.8 and 25°, according to the scheme indicated by eq 4. The fractions of monomer, dimer and higher polymers are indicated by f_1, f_2 and f_R, respectively.

suppress the rate of autolysis of α-CT (Wu and Laskowski, 1956; Green *et al.*, 1952; Chervenka, 1959), are an order of magnitude smaller than reported above. The correlation between the rate of autolysis and the magnitude of the heats of dilution of α-CT is consistent with our proposal.

Since, as shown by the observations presented in this study, the dimerization of α-CT at pH 7.8 is strongly affected by the binding of inhibitors, it is worthwhile to point out that dimerization should be taken into account in the determination of equilibrium constants for α-CT inhibitor complexes if the experiments are performed at neutral pH and high enzyme concentrations. This point has not been considered by previous workers (Doherty and Vaslow, 1952; Johnson and Knowles, 1966).

The results of the studies of proflavin reveal several interesting features. (1) Despite its positive charge at pH 7.8 (Peacocke and Skerrett, 1956), proflavin polymerizes quite extensively at this pH value. According to the model represented by eq 4, the thermodynamic functions associated with each step in the polymerization of proflavin are calculated to be $\Delta G_p' = -4.0 \pm 0.1$ kcal mole^{-1}, $\Delta H_p = -7.13 \pm 0.16$ kcal mole^{-1}, and $\Delta S_p' = -10.5 \pm 0.6$ cal deg^{-1} mole^{-1}, where $\Delta G_p'$, ΔH_p, and $\Delta S_p'$ are the standard free energy, enthalpy, and standard entropy changes at pH 7.8. (2) These thermodynamic parameters show that the polymerization reaction of proflavin is driven by favorable enthalpic contributions. The entropy change after correction for the cratic contribution (Kauzmann, 1959) is about −2.5 cal deg^{-1} mole^{-1}. Enthalpy terms of the magnitude observed here have been shown to characterize the interaction between the base pairs in the helical form of DNA (Sturtevant and Geiduschek, 1958; Privalov *et al.*, 1965) and also between the self-associated molecules of 6-methylpurine (Stoesser and Gill, 1967). (3) The distribution of polymeric species of proflavin as a function of dye concentration can be calculated according to the model described previously. The results are shown in Figure 7. It is interesting to note that the dimeric forms of the dye do not constitute the major component even at low dye concentrations. The assumption of an equilibrium between monomeric and dimeric forms has been made in interpreting the spectral changes occurring as a function of dye concentration (Li, 1968) and the following thermodynamic parameters were obtained at 25° and neutral pH: $K = 714$ M^{-1} and $\Delta H = -9.0$ kcal mole^{-1} of monomer. These figures, however, cannot be directly compared with our results since the assumption involved in obtaining them is different from the model we employed. It is appropriate to point out that although the similarity of form of eq 3 and 5 makes it clear that our calorimetric data are also consistent with the monomer–dimer model, it is *a priori* much more likely that proflavin undergoes unlimited polymerization than that a simple monomer–dimer equilibrium exists.

In the calculation of values of ΔH_b for proflavin, the self-association of the inhibitor was assumed to have no effect on its binding to α-CT. In view of the facts that the enthalpy change associated with the self-association reaction is quite large, that the population of polymeric species is strongly dependent on dye concentration, and that the calculated values of ΔH_b are independent of proflavin concentration (Figure 6), it appears that the polymeric species of proflavin are capable of binding to α-CT just as well as the monomeric species.

Judging from the results shown in Table VI, the enthalpy of binding of inhibitors to α-CT at pH 7.8 is in general negative and large. According to the van't Hoff relation, the equilibrium constants for the enzyme–inhibitor complexes at this pH must therefore be strongly dependent on temperature. In this connection, it is interesting to note that the binding constant of *N*-acetyl-D-tryptophan to α-CT at 5° and pH 7.9, measured by the method of equilibrium dialysis (Johnson and Knowles, 1966), agrees quite well with that measured by the method of steady-state kinetics (Foster and Niemann, 1955) at 25° and pH 7.9. However, since the extent of dimer formation under the two sets of experimental conditions appears to be different because of the different enzyme concentrations used by these workers, the good agreement between their results is probably fortuitous.

The thermodynamic functions for the binding of indole and *N*-acetyl-D-tryptophan to α-CT have been estimated by Yapel (1967) using a T-jump method in which the effect of inhibitors on the accessibility of the imidazole groups of α-CT to a pH indicator present in the solution is measured. At pH 8.0, the enthalpies of binding of indole and *N*-acetyl-D-tryptophan were estimated to be −22.6 and −9.8 kcal mole^{-1}, respectively. His results are thus in apparent disagreement with ours. The explanation for this discrepancy is not clear at present.

As shown in Table V, the entropy changes accompanying the binding of inhibitors to α-CT are large and negative. These figures are not consistent with those expected for hydrophobic interactions (Kauzmann, 1959). Electrostatic interactions between the charged inhibitors and the negatively charged active center of α-CT probably contribute to changes observed in the binding of *N*-acetyl-D-tryptophan and proflavin to the enzyme, but in the case of indole electrostatic interactions are absent. It thus seems likely that a significant part of the observed values for ΔH_b and $\Delta S_b'$ are due to conformational changes of the enzyme brought about by the binding of inhibitors. Such comformational changes have been detected by independent methods (Sturtevant, 1962; McClure and Edelman, 1967; Bernhard *et al.*, 1966).

The differences in the values of ΔH_b and $\Delta S_b'$ for various inhibitors should reflect some interesting features of the binding forces involved in the formation of complexes of these compounds with α-CT. This point will be discussed in detail in a future communication, in which data on the binding of other inhibitors of α-CT will be reported.

References

Bernhard, S. A., Lee, B. F., and Tashjian, Z. H. (1966), *J. Mol. Biol. 18*, 405.

Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature 221*, 337.

Canady, W. J., and Laidler, K. J. (1958), *Can. J. Chem. 36*, 1289.

Chervenka, C. H. (1959), *Biochim. Biophys. Acta 31*, 85.

Doherty, D. G., and Vaslow, F. (1952), *J. Am. Chem. Soc. 74*, 931.

Foster, R. J., and Niemann, C. (1955), *J. Am. Chem. Soc. 77*, 3365.

Gill, S. J., Downing, M., and Sheats, G. F. (1967), *Biochemistry 6*, 272.

Green, N. M., Gladner, J. A., Cunningham, L. W., and Neurath, H. (1952), *J. Am. Chem. Soc. 74*, 2122.

Hein, G. E., and Niemann, C. (1962), *J. Am. Chem. Soc. 84*, 4495.

Hymes, A. J., Cuppett, C. C., and Canady, N. J. (1969), *J. Biol. Chem. 244*, 637.

Inagami, T., and Sturtevant, J. M. (1965), *Biochemistry 4*, 1330.

Johnson, C. H., and Knowles, J. R. (1966), *Biochem. J. 101*, 56.

Kauzmann, W. (1959), *Advan. Protein Chem. 16*, 1.

Kézdy, F. J., and Bender, M. L. (1965), *Biochemistry 4*, 104.

Li, H. J. (1968), Ph.D. Thesis, Yale University, New Haven, Conn.

Marini, M. A., and Wunsch, C. (1963), *Biochemistry 2*, 1455.

Martin, R. B., and Niemann, C. (1958), *J. Am. Chem. Soc. 80*, 1473.

Massey, V., Harrington, W. F., and Hartley, B. S. (1955), *Discussions Faraday Soc. 20*, 24.

McClure, W. O., and Edelman, G. M. (1967), *Biochemistry 6*, 559.

McConn, J., Czerlinski, G., and Hess, G. P. (1968), *Fed. Proc. 27*, 456.

Peacocke, A. R., and Skerrett, J. N. H. (1956), *Trans. Faraday Soc. 52*, 261.

Privalov, P. L., Monaselidze, D. R., Mrevlishvili, G. M., and Magaldaze, V. A. (1965), *J. Exptl. Theoret. Phys.* (USSR) *47*, 2073.

Rao, N. S., and Kegeles, G. (1958), *J. Am. Chem. Soc. 80*, 5724.

Sarfare, P. S., Kegeles, G., and Kwon-Rhee, S. J. (1966), *Biochemistry 5*, 1389.

Schwert, G. W. (1949), *J. Biol. Chem. 179*, 655.

Schwert, G. W., and Kaufman, S. (1951), *J. Biol. Chem. 190*, 807.

Steiner, R. F. (1954), *Arch. Biochem. Biophys. 53*, 457.

Stoesser, P. R., and Gill, S. J. (1967), *J. Phys. Chem. 71*, 564.

Sturtevant, J. M. (1962), *Biochem. Biophys. Res. Commun. 8*, 321.

Sturtevant, J. M., and Lyons, P. A. (1969), *J. Chem. Thermodynamics 1*, 201.

Sturtevant, J. M., and Geiduschek, E. P. (1958), *J. Am. Chem. Soc. 80*, 879.

Tinoco, I. (1957), *Arch. Biochem. Biophys. 68*, 367.

Wallace, R. A., Kurtz, A. N., and Niemann, C. (1963), *Biochemistry 2*, 824.

Winzor, D. J., and Scheraga, H. A. (1963), *Biochemistry 2*, 1263.

Winzor, D. J., and Scheraga, H. A. (1964), *J. Phys. Chem. 68*, 338.

Wu, F. C., and Laskowski, M. (1956), *Biochim. Biophys. Acta 19*, 110.

Yapel, A. (1967), Ph.D. Thesis, University of Minnesota, Minneapolis, Minn.

Yapel, A., Han, M., Lumry, R., Rosenberg, A., and Shiao, D. F. (1966), *J. Am. Chem. Soc. 88*, 2573.