Calorimetric Investigations of the Binding of Inhibitors to α-Chymotrypsin. II. A Systematic Comparison of the Thermodynamic Functions of Binding of a Variety of Inhibitors to α-Chymotrypsin*

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ABSTRACT: The binding of various inhibitors to α-chymotrypsin has been investigated by direct calorimetry. The enthalpies of binding of benzoate, hydrocinnamate, β-naphthoate, *N*-acetyl-L-tryptophan, and phenol were found to be -18.2 ± 1.0, -15.7 ± 1.0, -17.5 ± 1.0, -21.2 ± 1.2, and -13.5 ± 0.5 kcal mole^{-1}, respectively, at pH 7.8 and 25°. At pH 5.6 and 25°, the enthalpies of binding of hydrocinnamate and *N*-acetyl-D-tryptophan ions and of phenol and indole were found to be -5.8 ± 0.3, -5.5 ± 0.3, -4.1 ± 0.3, and -6.4 ± 0.3 kcal mole^{-1}, respectively. By combining the available dissociation constants for the enzyme–inhibitor complexes, the thermodynamic changes associated with the binding reactions are calculated. On the basis of the data obtained in this study, it is concluded that conformational changes in α-chymotrypsin are induced by the binding of inhibitors at pH 7.8 and that these changes are smaller though not necessarily absent at pH 5.6. Thus, it appears that the conformational changes brought about by the binding of inhibitors to α-chymotrypsin parallel the change in catalytic activity of the enzyme.

The differences in the thermodynamic functions of binding among the inhibitors studied are interpreted in terms of the variation of forces involved in the formation of enzyme–inhibitor complexes. For example, it is concluded that the interaction of two negative charges near the active site of α-chymotrypsin is energetically favorable and the estimated enthalpy and entropy changes accompanying this interaction are -3.8 kcal mole^{-1} and -15 cal deg^{-1} mole^{-1} and that the thermodynamic contributions from the hydrophobic forces are largely entropic in origin. By comparing the thermodynamic functions of binding at pH 7.8 with those at pH 5.6, it can be shown that the number of protons produced by the binding of inhibitors to α-chymotrypsin is strongly temperature dependent and that it changes sign at about 25°.

The enthalpies of binding of indole, *N*-acetyl-D-tryptophan, and proflavin to α-chymotrypsin (α-CT)[1] at pH 7.8 have been reported in a previous communication (Shiao and Sturtevant, 1969). On the basis of these results, we have argued that the binding of inhibitors to α-CT may induce conformational changes of the enzyme. It was also pointed out that since the enthalpies and entropies of binding of the various inhibitors were observed to be different, the comparison of these quantities may reveal useful information concerning the binding forces involved in the formation of α-CT–inhibitor complexes. However, due to the limited structural variation among the inhibitors involved in our previous study, further consideration of this point was postponed to the present communication in which additional data on the enthalpies of binding of *N*-acetyl-L-tryptophan, hydrocinnamate, benzoate, naphthoate, and phenol at pH 7.8 are reported.

The activity of α-CT is known to be strongly pH dependent (Hammond and Gutfreund, 1955). At a pH of about 7.8 the activity is maximal, and it becomes very small at pH values below 5. It is of interest to note that despite the large change in activity of the enzyme between pH 5 and 8, the binding affinities of neutral inhibitors such as *N*-acetyl-D-tryptophan amide in this pH range have been shown to be independent of pH (Johnson and Knowles, 1966). On the other hand the binding constants for negatively charged inhibitors such as *N*-acetyl-L-tryptophan and *N*-acetyl-D-tryptophan have been shown to decrease rapidly above pH 7.8 (Johnson and Knowles, 1966; Foster and Niemann, 1955).

Attempts have been made to estimate the enthalpies of binding of both charged and uncharged inhibitors to α-CT as a function of pH by studying the temperature coefficients of the binding constants (Yapel, 1967; Doherty and Vaslow, 1952). Calorimetric determinations of the enthalpy of binding of hydrocinnamate to α-CT have been reported by Canady and Laidler (1958). In an attempt to investigate further the pH dependence of the binding of inhibitors to α-CT from the calorimetric point of view, we report in this communication measurements of the enthalpies of binding of hydrocinnamate, *N*-acetyl-D-tryptophan, phenol, and indole at pH 5.6 in addition to those listed above at pH 7.8.

Except for *N*-acetyl-L-tryptophan, the compounds used in this study have been shown to be competitive inhibitors of α-CT (Wallace *et al.*, 1963; Canady and Laidler, 1958; Foster and Niemann, 1955). *N*-Acetyl-L-tryptophan has been demonstrated by exchange studies using ^{18}O to form an acyl-enzyme complex with α-CT (Kézdy *et al.*, 1964).

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

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

Chymotrypsin Concn (mM), $(E)_0$	Benzoate Concn (mM), $(I)_0$	Obsd Heat (kcal/mole^{-1}), $-\Delta Q_{app}$	Enthalpy of Binding (kcal/mole of Inhibitor Bound), $-\Delta H_b$
0.073	240	16.3	20.0
0.114	240	13.1	19.6
0.114	150	14.9	19.4
0.121	200	13.0	17.8
0.152	100	9.7	18.0
0.157	200	10.3	17.0
0.182	150	10.7	19.1
0.190	200	9.9	19.6
0.236	150	8.1	17.0
0.242	100	8.1	19.0
0.285	150	8.7	18.4
0.315	150	6.6	17.0
0.380	100	6.0	16.9
		mean	18.2
		av dev	±1.0

Experimental Section

Three times recrystallized α-CT was purchased from Worthington Biochemical Corp., Freehold, N. J., and was used without further purification. Enzyme concentrations were determined by measuring the optical density at 280 nm, assuming an absorptivity of 5×10^4 M^{-1} cm^{-1}.

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

Chymotrypsin Concn (mM), $(E)_0$	Hydrocinnamate Concn (mM), $(I)_0$	Obsd Heat (kcal/mole^{-1}), $-\Delta Q_{app}$	Enthalpy of Binding (kcal/mole of Inhibitor Bound), $-\Delta H_b$
0.041	47.0	10.9	14.4
0.049	40.0	9.2	13.2
0.067	47.0	11.8	16.5
0.073	40.0	10.3	15.3
0.123	33.0	8.3	14.8
0.123	25.0	7.7	14.5
0.180	33.0	7.9	15.7
0.180	33.0	8.6	16.5
0.183	25.0	8.3	16.4
0.243	17.0	6.6	16.0
0.270	25.0	7.0	16.7
0.311	25.0	8.0	17.4
0.360	17.0	8.0	15.2
		mean	15.7
		av dev	±1.0

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

Chymotrypsin Concn (mM), $(E)_0$	β-Naphthoate Concn (mM), $(I)_0$	Obsd Heat (kcal/mole^{-1}), $-\Delta Q_{app}$	Enthalpy of Binding (kcal/mole of Inhibition Bound), $-\Delta H_b$
0.052	16.0	13.0	16.2
0.087	13.0	12.1	17.1
0.092	16.0	13.0	18.0
0.154	13.0	9.8	16.0
0.174	7.0	11.1	17.6
0.217	13.0	9.6	16.0
0.235	10.0	10.5	18.1
0.307	7.0	9.9	17.8
0.325	10.0	9.0	17.7
0.433	7.0	9.1	18.4
		mean	17.5
		av dev	±1.0

Hydrocinnamic acid purchased from Matheson, Coleman & Bell Co., East Rutherford, N. J., was twice recrystallized from glass-distilled water. The following compounds were used without further purification: indole and β-naphthoic acid, Matheson, Coleman & Bell Co.; *N*-acetyl-D-tryptophan and *N*-acetyl-L-tryptophan, Cyclo Chemical Corp., Los Angeles, Calif.; benzoic acid, National Bureau of Standards, Washington, D. C.; phenol, Fisher Scientific Co., Fair Lawn, N. J.

The calorimetric measurements reported here were made with a flow modification of the Beckman Model 190 microcalorimeter. A brief description of this instrument has been presented elsewhere (Sturtevant and Lyons, 1969). The buffer solution used in all the calorimetric experiments was 0.05 M in phosphate and 0.2 M in KCl.

Results

The Enthalpy of Binding of Benzoate, Hydrocinnamate, β-Naphthoate, N-Acetyl-L-tryptophan, and Phenol to α-CT at pH 7.8. 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 of the enzyme and the inhibitors. The information concerning the heat of dilution of α-CT was available from a previous report (Shiao and Sturtevant, 1969) and the heats of dilution of the inhibitors were separately determined in each experimental run. The ΔQ_{app} values for benzoate, hydrocinnamate, β-naphthoate, *N*-acetyl-L-tryptophan, and phenol are listed in Table I, II, III, IV, and V. From the results given in these tables, it is clear that the ΔQ_{app} values are significantly dependent on enzyme concentration. This effect has been explained on the basis of the assumption that the dimeric form of α-CT present at pH 7.8 does not bind inhibitors (Shiao and Sturtevant, 1969). A procedure has been developed, based on this assumption, for computing the

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

Chymotrypsin Concn (mM), $(E)_0$	*N*-Acetyl-L-tryptophan Concn, $(I)_0$	Obsd Heat (kcal/mole^{-1}), $-\Delta Q_{app}$	Enthalpy of Binding (kcal/mole of Inhibitor Bound), $-\Delta H_b$
0.053	87.5	14.1	18.7
0.081	78.0	16.2	22.2
0.109	67.0	15.7	22.8
0.129	8.00	15.7	23.1
0.159	67.0	13.2	21.3
0.164	50.0	12.2	20.9
0.215	67.0	12.4	21.4
0.219	33.0	9.4	19.7
0.238	50.0	10.5	20.3
0.253	67.0	13.7	23.4
0.318	33.0	7.3	18.4
0.322	50.0	9.8	20.4
0.380	50.0	10.5	21.8
		mean	21.2
		av dev	±1.2

enthalpy of binding per mole of inhibitor bound (ΔH_b). The values of ΔH_b so computed are also listed in Tables I, II, III, IV, and V.

In the process of computing ΔH_b values, it is necessary to know the magnitudes of the dissociation constants, K_I, for the enzyme–inhibitor complexes. The values of K_I for *N*-acetyl-L-tryptophan and phenol were available from previous reports (Foster and Niemann, 1955; Wallace *et al.*, 1963). In the case of hydrocinnamate, discrepancies in the reported values of K_I have been noted (Sturtevant, 1962), and we have therefore obtained a value from the calorimetric data by assuming that the computed ΔH_b values should be independent of enzyme concentration. The value so estimated is in good agreement with that determined by the method of steady-state kinetics at pH 7.1 (Canady and Laidler, 1958) but is about an order of magnitude lower than that reported by Wallace *et al.* (1963) at pH 7.9. The K_I values for benzoate and naphthoate were also estimated by this procedure and they too were found to be about an order of magnitude lower than those reported by Wallace *et al.* (1963) at pH 7.9. No explanation for these discrepancies is available.

The Enthalpy of Binding of Hydrocinnamate, N-Acetyl-D-tryptophan, Indole, and Phenol at pH 5.6. Due to the fact that the heat of dilution of α-CT at pH 5.6 was found to be roughly an order of magnitude smaller than that at pH 7.8 (Shiao and Sturtevant, 1969), its determination was not carried out in detail. However, the necessary experiments were performed so that the appropriate small corrections for the heat of dilution of the enzyme as well as the inhibitors could be made to the enthalpies of binding to α-CT reported in this section. In contrast to the situation at pH 7.8, there was no evidence that polymerization of the enzyme influenced the results at pH 5.6.

The ΔQ_{app} values as defined previously are presented in Tables VI, VII, VIII, and IX. It is apparent that at this pH, these quantities are not significantly dependent on enzyme concentration. Under experimental conditions where the total concentration of inhibitor, $(I)_0$, is much greater than the concentration of enzyme–inhibitor complex, the enthalpy of binding per mole of inhibitor, ΔH_b, can be calculated by means of the equation

$$\Delta H_b = \Delta Q_{app}\left(1 + \frac{K_I}{(I)_0}\right) \quad (1)$$

The values of K_I for the neutral molecules indole and phenol were assumed to be the same as those estimated at pH 7.8 and those for hydrocinnamate and *N*-acetyl-D-tryptophan

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

Chymotrypsin Concn (mM), $(E)_0$	Phenol Concn (mM), $(I)_0$	Obsd Heat (kcal mole^{-1}), $-\Delta Q_{app}$	Enthalpy of Binding (kcal/mole of Inhibitor Bound), $-\Delta H_b$
0.161	40.0	4.7	12.7
0.195	40.0	4.0	12.4
0.232	40.0	4.3	13.4
0.241	30.0	4.0	13.6
0.293	30.0	3.4	13.5
0.321	30.0	3.1	14.1
0.349	30.0	3.0	13.6
0.390	20.0	2.5	14.0
0.465	20.0	2.5	14.6
		mean	13.5
		av dev	±0.5

TABLE VI: Enthalpy of Binding of Hydrocinnamate to α-Chymotrypsin at pH 5.6 and 25°.

Chymotrypsin Concn (mM), $(E)_0$	Hydrocinnamate Concn, $(I)_0$	Obsd Heat (kcal/mole^{-1}), $-\Delta Q_{app}$	Enthalpy of Binding (kcal/mole of Inhibitor Bound), $-\Delta H_b$
0.066	44.0	5.8	6.0
0.109	44.0	6.6	6.7
0.118	40.0	6.2	6.2
0.196	40.0	5.8	6.0
0.196	33.0	4.9	5.2
0.295	25.0	5.2	5.6
0.326	33.0	5.2	5.5
0.393	16.7	5.1	5.6
0.489	25.0	5.3	5.8
0.652	16.7	5.1	5.6
		mean	5.8
		av dev	±0.3

TABLE VII: Enthalpy of Binding of *N*-Acetyl-D-tryptophan to α-Chymotrypsin at pH 5.6 and 25°.

Chymotrypsin Concn (mM), $(E)_0$	*N*-Acetyl-D-tryptophan Concn (mM), $(I)_0$	Obsd Heat (kcal/mole^{-1}), $-\Delta Q_{app}$	Enthalpy of Binding (kcal/mole of Inhibitor Bound), $-\Delta H_b$
0.106	41.8	5.8	6.0
0.119	37.6	4.9	5.2
0.191	37.6	5.9	6.2
0.198	31.3	4.8	5.1
0.297	23.5	4.7	5.1
0.319	31.3	5.3	5.6
0.396	15.7	4.5	5.1
0.478	23.5	5.1	5.4
0.637	15.7	4.9	5.6
		mean	5.5
		av dev	±0.3

TABLE IX: Enthalpy of Binding of Phenol to α-Chymotrypsin at pH 5.6 and 25°.

Chymotrypsin Concn (mM), $(E)_0$	Phenol Concn (mM), $(I)_0$	Obsd Heat (kcal/mole^{-1}), $-\Delta Q_{app}$	Enthalpy of Binding (kcal/mole of Inhibitor Bound), $-\Delta H_b$
0.128	48.0	3.2	3.6
0.189	48.0	3.2	3.6
0.210	40.0	4.1	4.8
0.315	40.0	3.7	4.2
0.321	30.0	3.4	4.7
0.328	20.0	2.9	3.7
0.472	30.0	3.3	4.0
0.630	20.0	2.7	3.6
		mean	4.1
		av dev	±0.3

were estimated from data reported by other workers (Canady and Laidler, 1958; Foster and Niemann, 1955). The values of ΔH_b calculated by eq 1 are also listed in Tables VI, VII, VIII, and IX.

The Thermodynamic Functions for the Binding of Inhibitors to α-CT. Combination of the available values for K_I and ΔH_b leads to the thermodynamic functions for the binding of inhibitors to α-CT which are listed in Tables X and XI. The results on the binding of *N*-acetyl-D-tryptophan, indole, and proflavin which were reported earlier (Shiao and Sturtevant, 1969) are also included in Table X. Since the standard free energies of binding of various inhibitors to α-CT reported by other workers (see Table X for references) were determined from inhibition constants obtained by the method of steady-state kinetics, it should be noted that the calculation of entropies of binding using the ΔH_b values obtained in this study assumes that the binding processes as seen by the calorimetric experiments are the same as those seen by the kinetic experiments.

TABLE VIII: Enthalpy of Binding of Indole to α-Chymotrypsin at pH 5.6 and 25°.

Chymotrypsin Concn (mM), $(E)_0$	Indole Concn (mM), $(I)_0$	Obsd Heat (kcal/mole^{-1}), $-\Delta Q_{app}$	Enthalpy of Binding (kcal/mole of Inhibitor Bound), $-\Delta H_b$
0.103	15.1	6.9	7.1
0.122	13.6	5.6	6.0
0.185	13.6	6.2	6.4
0.204	11.3	6.4	6.9
0.306	8.5	5.9	6.4
0.309	11.3	6.3	6.7
0.408	5.7	5.3	6.0
0.463	8.5	5.8	6.2
0.617	5.7	5.2	5.9
		mean	6.4
		av dev	±0.3

Discussion

The binding of hydrocinnamate to α-CT as a function of pH has been studied calorimetrically by Canady and Laidler (1958). According to their report, the enthalpy of binding of this compound at 25° was found to be -5.0 ± 0.4 kcal mole^{-1} at pH 5.6 and -26 ± 4 kcal mole^{-1} at pH 7.8. The present results (Tables X and XI) indicate that ΔH_b for hydrocinnamate at 25° is -5.8 ± 0.3 kcal mole^{-1} at pH 5.6 and -15.7 ± 1.0 kcal mole^{-1} at pH 7.8. Thus the present results are in good agreement with those of Canady and Laidler at low pH value but are in serious disagreement with theirs at pH 7.8. The fact that in the studies of Canady and Laidler, the effect of dimerization of α-CT at pH 7.8 was not considered, whereas our observations show that at the enzyme concentration they used (approximately 0.2 mM), the correction due to dimerization of α-CT is quite large, cannot account for the discrepancy at high pH since the apparent heat of binding, ΔQ_{app}, for hydrocinnamate at an enzyme concentration of 0.2 mM is only −8.5 kcal/mole of protein. Considering the good agreement between their results and the present results at low pH, it seems that the discrepancy observed at pH 7.8 is not due to instrumental inaccuracies. Although the reason for the discrepancy has not been firmly established, it is suspected that it is due to the autolysis of α-CT which is known to occur at pH 7.8; since the batch calorimeter employed by Canady and Laidler usually requires relatively long thermal equilibration periods before the enzyme and inhibitor solutions can be mixed, the occurrence of autolysis of the enzyme before mixing was probably unavoidable in their experiments.

The enthalpies of binding of *N*-acetyl-L-tryptophan and hydrocinnamate to α-CT were estimated by Yapel (1967) from studies of the temperature coefficients of the binding

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

Compd	Dissocn Constant of Enzyme–Compd 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}), $\Delta S_b'$
Benzoate	12.0[b]	−2.64	−18.2 ± 1.0	−52 ± 3
Hydrocinnamate	2.0[b]	−3.71	−15.7 ± 1.0	−40 ± 3
β-Naphthoate	0.14[b]	−5.30	−17.5 ± 1.0	−40 ± 3
N-Acetyl-L-tryptophan	6.0[c]	−2.96	−21.2 ± 1.2	−61 ± 4
N-Acetyl-D-tryptophan[a]	4.0	−3.29	−19.0 ± 0.6	−52 ± 2
Proflavin[a]	0.037	−6.04	−11.3 ± 1.0	−18 ± 3
Phenol	6.0[d]	−3.05	−13.5 ± 0.5	−35 ± 2
Indole[a]	0.70	−4.31	−15.2 ± 0.8	−37 ± 3

[a] Shiao and Sturtevant (1969). [b] See text. [c] Foster and Niemann (1955). [d] Wallace *et al.* (1963).

TABLE XI: Thermodynamic Parameters for the Binding of Various Compounds to α-Chymotrypsin at pH 5.6 and 25°.

Compd	Dissocn Constant of Enzyme–Compd 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}), $\Delta S_b'$
Hydrocinnamate	1.7[a]	−3.80	−5.8 ± 0.3	−7 ± 1
N-Acetyl-D-tryptophan	2.0[b]	−3.71	−5.5 ± 0.3	−6 ± 1
Phenol	6.0[c]	−3.05	−4.1 ± 0.3	−3 ± 1
Indole	0.70[c]	−4.31	−6.4 ± 0.3	−7 ± 1

[a] Canady and Laidler (1958). [b] Foster and Niemann (1955). [c] Wallace *et al.* (1963).

constants from 3 to 13°, the values obtained at pH 8.0 being −28.6 and −29.5 kcal mole^{-1}, respectively. Thus, the present results at pH 7.8 and 25° (see Table X) are not in agreement with those obtained by Yapel. It seems to be quite unlikely that there are such large changes in heat capacity as to be able to account for the observed discrepancies.

As mentioned previously, *N*-acetyl-L-tryptophan has been shown to form an acyl-enzyme complex with α-CT. This being the case, the value of ΔH_b for *N*-acetyl-L-tryptophan calculated according to eq 1 has a different meaning as compared to the values for the other competitive inhibitors. In order to explore this point, let us consider the equilibria represented by eq 2 and 3

$$E + I = EI \qquad K_1; \Delta H_1 \qquad (2)$$

$$EI = EA \qquad K_2; \Delta H_2 \qquad (3)$$

In terms of these equilibria, the apparent dissociation constant for the enzyme–inhibitor complex is operationally defined under conditions where the total inhibitor concentration is much larger than the concentration of the enzyme–inhibitor complex, as

$$K_I = \frac{1}{K_1 + K_2K_1} \qquad (4)$$

Using Hess' law and eq 4 it can be shown that

$$\Delta H_b = \Delta H_1 + \frac{2}{1 + K_2}\Delta H_2 \qquad (5)$$

From studies of the rate of hydrolysis of *N*-acetyl-L-tryptophan ethyl ester by α-CT, the value of K_2 has been estimated to be about 10 (Bender and Kézdy, 1964). If we assume that the value of ΔH_1 is equal to that of ΔH_b for *N*-acetyl-D-tryptophan (−19 kcal mole^{-1}), the value of ΔH_2 is calculated to be −2.4 kcal mole^{-1} by eq 5. On the basis of this number and the value of K_2 mentioned above, the standard entropy change associated with the formation of EA from EI at pH 7.8 is calculated to be 3 cal deg^{-1} mole^{-1}. Such small changes in enthalpy and entropy produced by the reaction indicated by eq 3 appear to be surprisingly small. It should be pointed out, however, that the calculations described above depend heavily on the value assigned to K_2 which may be uncertain due to the compensatory errors in assigning the rate constants derived from kinetic studies (Bender and Kézdy, 1964).

Attempts have been made to determine the enthalpy of binding of *N*-acetyl-L-tryptophan to α-CT at pH 5.6. Preliminary results showed that the apparent enthalpy of binding of this compound was strongly dependent on enzyme concentration while those of the other inhibitors were not. The author is puzzled by these measurements which reflect the

fact that *N*-acetyl-L-tryptophan is very different from its D enantiomer and other inhibitors. However, studies of the inactivation of α-CT by diphenylcarbamyl chloride indicate that the rate of acyl-enzyme formation is very much smaller at low pH values than at pH 7.8 (Erlanger *et al.*, 1966). This conclusion leads the author to suspect that the rate of formation of the acyl-enzyme complex with *N*-acetyl-L-tryptophan at pH 5.6 is so slow as to preclude measurements of the heat of binding by flow calorimetry.

The results given in Table X show that both the enthalpy and the entropy change associated with the binding of inhibitors to α-CT at pH 7.8 are in general negative and large. The changes in unitary entropy are about 8 cal deg^{-1} $mole^{-1}$ less negative than the $\Delta S_b'$ values listed in this table (Kauzmann, 1959). Such large changes in both enthalpy and unitary entropy are difficult to understand in terms of forces such as van der Waal's interactions, hydrophobic bonding and electrostatic interactions. We thus conclude that a significant part of these changes are due to ligand-induced conformational changes of the enzyme. This point has already been discussed in a previous communication (Shiao and Sturtevant, 1969).

The information contained in Table X does not make it clear whether the enthalpy and entropy changes resulting from the conformational changes of the enzyme are dependent on the structure of the inhibitor. However, if we assume that the conformational contributions to the observed enthalpy and entropy changes are relatively independent of the structural differences between the various inhibitors, we can proceed to examine the present data in terms of the differences in enthalpy and entropy changes resulting from other sources.

Since available evidence shows that the active site of α-CT is probably negatively charged at pH 7.8 (Johnson and Knowles, 1966; Blow *et al.*, 1969), the association of *N*-acetyl-D-tryptophan with α-CT should involve an electrostatic interaction between two negative charges. On the other hand, since indole is a neutral molecule, this interaction would be absent. If it is assumed, as seems reasonable, that the hydrophobic and van der Waal's forces involved in the formation of α-CT–inhibitor complexes are similar for indole and *N*-acetyl-D-tryptophan, comparison of the thermodynamic parameters for the binding of these two inhibitors to α-CT should provide information about the magnitude of the electrostatic interaction between two negative charges near the active site of α-CT. Judging from the results given in Table X, this interaction appears to give rise to a free energy change of 1.1 kcal $mole^{-1}$, an enthalpy change of −3.8 kcal $mole^{-1}$, and an entropy change of −15 cal deg^{-1} $mole^{-1}$ at 25°. The positive change in free energy is expected from the repulsive interaction of two negative charges, and simple theoretical calculations based on Coulomb's law also indicate that the association of two charges of the same sign in water is an exothermic process with a negative entropy change (Linderström-Lang and Schellman, 1959). However, one must be cautious in extending such calculations to a protein system since information concerning the effective dielectric constant and its temperature coefficient inside a protein molecule is entirely lacking.

Since proflavin is a positively charged molecule at pH 7.8 (Peacocke and Skerrett, 1956), one would expect that its association with α-CT should produce less negative changes in both enthalpy and entropy than those resulting from the formation of the indole–α-CT complex. The results shown in Table X demonstrate that this expectation is fulfilled. Furthermore using the numbers deduced above, one may estimate for the binding of proflavin the thermodynamic functions excluding the electrostatic effect having the values $\Delta G_b' = -4.9$ kcal $mole^{-1}$, $\Delta H_b = -15.1$ kcal $mole^{-1}$, and $\Delta S_b' = -33$ cal deg^{-1} $mole^{-1}$. Comparison of these numbers with those for the binding of indole indicates that even without the favorable electrostatic interaction, proflavin is somewhat more strongly bound than is indole, perhaps because of a more favorable hydrophobic interaction. A similar argument applied to the differences in binding thermodynamics between benzoate and phenol leads to the conclusion that the hydrophobic forces involved in the association of these two compounds with α-CT are very similar in magnitude, as would be expected.

Since benzoate and β-naphthoate are negatively charged, differences in the thermodynamic functions for their binding to α-CT should result primarily from differences in hydrophobic bonding. The fact that the enthalpies of binding of these compounds are nearly equal shows that the differences in binding affinity are primarily due to differences in entropies of binding. This deduction is consistent with the view that hydrophobic forces are a consequence of favorable entropic contributions (Kauzmann, 1959).

According to the data reported in Table XI, the enthalpies and entropies of binding of inhibitors to α-CT at pH 5.6 are in general negative but much smaller in magnitude than those observed at pH 7.8. It is very interesting that even though the free energies of binding of the inhibitors tested are not sensitive to pH variation between pH 7.8 and 5.6, the enthalpies and entropies of binding are very different at these two pH values. On the basis of our interpretation that the large decreases in enthalpy and entropy observed at pH 7.8 are due in significant measure to conformational changes of α-CT induced by the ligand binding, it is necessary to conclude that the conformational changes are small, although not necessarily absent, at pH 5.6. This argument leads to the suggestion that the conformational changes of α-CT brought about by the binding of inhibitors parallel the changes in the catalytic activity of the enzyme. The question as to whether the conformational changes suggested by the present results are directly involved in the catalytic function of α-CT is still unclear, however, since the rates of these conformational changes may be too slow to participate in the normal catalytic processes of α-CT (Sturtevant, 1962). A more detailed discussion in connection with this question has been recently presented by Lumry and Biltonen (1969).

The observation that the enthalpies of binding of certain inhibitors to α-CT are strongly dependent on pH in the region pH 4–8 has been reported by other workers (Yapel, 1967; Doherty and Vaslow, 1952; Canady and Laidler, 1958). This observation has been interpreted as an indication of conformational changes of α-CT induced by the binding of ligands (Yapel, 1967; Vaslow, 1958; Doherty and Vaslow, 1952). On the other hand, the effect of electrostatic interaction has been offered as an alternative explanation (Canady and Laidler, 1958). Since according to the present observation, and also that of Yapel, indole, which carries no charge, also binds with a pH-dependent enthalpy change, the explanation based solely on the effect of electrostatic interactions

appears to be untenable. Since the free energies of binding of inhibitors to α-CT are relatively insensitive to pH variations in the region pH 4–7.8, as compared to the enthalpies and entropies of binding, it is evident that the changes in the enthalpy of binding must be compensated by corresponding changes in entropy. The implications of compensation effects in enzyme reactions have been recently discussed by Lumry (1968).

Another interesting feature which appears in the results shown in Table XI is that both the enthalpies and entropies of binding determined at pH 5.6 are relatively insensitive to structural differences between the inhibitors as compared to those determined at pH 7.8. It should be mentioned that since the active site of α-CT is probably uncharged at pH 5.6, the electrostatic interactions which exist at pH 7.8 for negatively charged inhibitors are presumably absent at pH 5.6.

The pH dependence of the free energy and enthalpy changes for a chemical reaction is related to the number of protons produced by the reaction, N_H, and the temperature dependence of this quantity, dN_H/dT, by the expressions (Alberty, 1969)

$$\left(\frac{\partial \Delta G^\circ}{\partial \mathrm{pH}}\right)_T = -2.3RTN_H \quad (6)$$

and

$$\left(\frac{\partial \Delta H^\circ}{\partial \mathrm{pH}}\right)_T = -2.3RT^2\left(\frac{\partial N_H}{\partial T}\right)_{\mathrm{pH}} \quad (7)$$

Although the quantities $(\partial \Delta G^\circ/\partial \mathrm{pH})_T$ and $(\partial \Delta H^\circ/\partial \mathrm{pH})_T$ are not accurately established by the data obtained in the present work, rough estimates of their magnitudes can be obtained from the data reported in Tables X and XI, and approximate values for N_H and $(\partial N_H/\partial T)_{\mathrm{pH}}$ at pH 7 and 25° can be calculated. If we assume that $(\partial N_H/\partial T)_{\mathrm{pH}}$ is independent of temperature in the region 0–40°, the values of N_H given in Table XII are obtained. It is interesting that the number of protons produced by the binding of inhibitors to α-CT is in general positive (liberation of protons by the enzyme) at 0°, about 0 at 25°, and negative (uptake of protons by the enzyme) at 40°. This observation is important, since it indicates that the dissociation constants of the enzyme–inhibitor complexes may be strongly pH dependent at temperatures other than 25° even with neutral compounds such as indole and phenol.

TABLE XII: Proton Liberation in the Binding of Various Compounds to α-Chymotrypsin at pH 7.

Compd	$(\partial N_H/\partial T)_{\mathrm{pH}}$ (Protons/Molecule of EI Formed per deg)	N_H (Protons/Molecule of EI Formed) 0°	25°	40°
Hydrocinnamate	-0.011_6	$+0.2_6$	$+0.0_3$	-0.2_0
N-Acetyl-D-tryptophan	-0.015_1	$+0.2_4$	-0.1_4	-0.3_7
Phenol	-0.010_3	$+0.2_6$	0.0_0	-0.1_6
Indole	-0.008_4	$+0.2_1$	0.0_0	-0.1_3

The release of protons from α-CT accompanying the binding of uncharged ligands at approximately neutral pH has been measured by Glick (1968). It was found that the N_H value for anisole is 0.07 and that for formanilide is 0 at pH 7. Our observations are thus consistent with those of Glick.

It is not possible to offer an explanation for the temperature dependence of the values for N_H listed in Table XII. However, it should be mentioned that complexities are found when certain properties of α-CT in the neutral pH region are studied as a function of temperature. For example, it has been reported that two forms of α-CT exist in thermal equilibrium at pH 7 (Kim, 1968). Whether the implications of this report are related in some way to the present observations is a question which remains to be clarified.

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Inhibition of Mitochondrial Respiration by Hydroxylamine and Its Relation to Energy Conservation*

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ABSTRACT: The inhibition of mitochondrial succinate oxidation by hydroxylamine is kinetically characteristic of an inhibitor of adenosine diphosphate phosphorylation, not of electron transport. When ascorbate and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine are used as electron donors, however, the hydroxylamine is an inhibitor of electron transport and is uncompetitive with respect to the dye. The inhibition of succinate oxidation is almost completely released and the inhibition of the *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine oxidation is partially released by Ca^{2+} and uncouplers.

The release of hydroxylamine inhibition of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine oxidation is effected by 1.3 moles of salicylanilide uncoupler per mole of cytochrome *a*, a titer which is independent of the rate of turnover of the cytochrome oxidase. This titer confirms the value obtained by the uncoupler dependent release of azide inhibition and is postulated to represent a titration by the uncoupler of a binding site on a specific protein associated with the respiratory chain.

In recent reports on the inhibition of mitochondrial respiration by hydroxylamine, Wikström and Saris (1969) have concluded that the inhibition of oxidative phosphorylation by hydroxylamine is specific for site 3 (cytochrome oxidase) while Utsumi and Oda (1969) have concluded more generally that the hydroxylamine interacts at an early, as yet unidentified, energy transfer step in mitochondria. These two conclusions are mutually inclusive and indicate that hydroxylamine may be a very useful tool in studying the mitochondrial energy conservation reactions. The properties described for the inhibition are similar but not identical with those of azide inhibition (Wilson and Chance, 1966, 1967; Wilson, 1967; Nicholls and Kimelberg, 1968). The comparisons of the behavior of the two inhibitors are not satisfactory since a quantitative kinetic analysis of the hydroxylamine inhibition has not been reported. Such a comparison is of great interest since azide has been shown to specifically inhibit electron transport between cytochromes *a* and a_3 and has been proposed to inhibit an intermediate common to the electron transfer and energy conservation reactions (Wilson and Chance, 1966, 1967; Wilson, 1967). The present communication presents a kinetic analysis of the hydroxylamine inhibition of respiration under conditions which permit quantitative comparison with azide inhibition. In addition the stoichiometric binding of the salicylanilide uncouplers to the respiratory chain (Wilson and Azzi, 1968; Wilson, 1969) is examined using the uncoupler dependent release of hydroxylamine inhibition.

Methods

Rat liver mitochondria were prepared in a 0.22 M mannitol, 0.07 M sucrose, and 0.2 mM EDTA medium (pH 7.2) essentially according to the method of Schneider (1948).

Mitochondrial respiration was measured polarographically at 23° in either a 0.2 M mannitol, 0.05 M sucrose, 0.02 M Tris, and 0.01 M KH_2PO_4 medium (MST-P_i) or a 0.12 M KCl, 0.02 M Tris, and 0.01 M KH_2PO_4 medium (KClT-P_i). The pH was 7.2 unless otherwise noted in the figure or table legends. The volume of the reaction vessel was 3.4 ml.

The indicated respiration rates are for initial conditions with a minimum time between the addition of the inhibitor and the rate measurement. For succinate inhibition, for example, the order of addition was medium, mitochondria, rotenone, ADP, hydroxylamine, then succinate.

The cytochrome *a* content of the mitochondrial preparation was measured as previously described (Wilson, 1969) assuming an extinction coefficient of 24 for reduced minus oxidized cytochrome *a* at 605 nm minus 630 nm (van Gelder, 1966).

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