the natural bovine B-chain *S*-sulfonate (Figure 3) gave the following amino acid composition expressed in molar ratios after acid hydrolysis: $Lys_{1.0}His_{1.1}Arg_{1.0}Thr_{1.7}Glu_{2.0}Pro_{1.0}Gly_{2.2}Ala_{0.8}Cys_{0.8}$-$Val_{1.8}Leu_{2.8}Tyr_{1.8}Phe_{2.1}$. This material also showed the following amino acid composition after digestion with APM: $Lys_{0.9}His_{0.9}$-$Arg_{0.9}Thr_{1.9}Glu_{1.9}Pro_{0.9}Gly_{2.0}Ala_{1.0}Val_{2.0}Leu_{3.0}Tyr_{1.9}Phe_{2.0}$*S*-sulfocysteine$_{0.9}$. This composition is in excellent agreement with the theoretically expected values for the C-terminal heneicosapeptide derivative of the human B chain. Finally component 3 which is the major component (27 mg) is eluted (Figure 2-II) from the CM-cellulose column at the same position as the natural bovine B-chain *S*-sulfonate (Figure 2-I). The evidence presented below demonstrates that this component is purified human insulin B-chain *S*-sulfonate.

Criteria of Chemical and Stereochemical Homogeneity of Synthetic Human B Chain *S*-Sulfonate and Comparison with Natural Bovine B-Chain *S*-Sulfonate. Amino acid analysis of the purified synthetic human B-chain *S*-sulfonate (component 3) after acid hydrolysis gave the molar ratios of amino acids shown in Table I in excellent agreement with the theoretically expected values. Digestion of the synthetic material with APM and amino acid analysis of the digest with an automatic analyzer gave the amino acid ratios shown in Table I. As can be seen these ratios are almost identical with the theoretically expected values for human B-chain *S*-sulfonate. The APM digestion of the synthetic chain proves that the optical configuration of the constituent amino acids was preserved during the synthetic processes. On CM-cellulose chromatography the synthetic human B-chain *S*-sulfonate is eluted at the same position as the S-sulfonated natural bovine B chain (Figure 2-I, -II). The synthetic human B-chain *S*-sulfonate possessed the specific rotation of $[\alpha]^{24}D$ $-96.1 \pm 2°$ (*c* 0.1, 0.5 *N* acetic acid) compared to $[\alpha]^{25}D$ $-95.8 \pm 4.3°$ (*c* 0.1, 0.5 *N* acetic acid) which we found for natural bovine B-chain *S*-sulfonate. On high-voltage thin-layer electrophoresis in 0.5 *N* acetic acid, pH 2.9 and 3400 V, and in 0.01 *M* NH_4HCO_3, pH 10 and 3100 V, the synthetic human B-chain *S*-sulfonate and the natural bovine B-chain *S*-sulfonate moved as single components (Pauly reaction) and had the same mobility as shown in Figures 4 and 5, respectively. The most decisive proof, however, as to the identity of the synthetic human B chain with its natural counterpart is provided by the fact that when the synthetic human B chain was combined with the synthetic human A chain, the all-synthetic human insulin produced was identical with the naturally occurring protein.[5]

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Studies of the Chymotrypsinogen Family of Proteins. XIII. Inhibitor-Induced Transient Change in Fluorescence of α-Chymotrypsin[1]

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Abstract: Further resolution of the substates of the "best-folded" state, state A, of α-chymotrypsin has been achieved in studies of the effects of inhibitors on the fluorescence behavior of this protein. An interpretation is proposed on the basis of which the substate diagram for the neutral to alkaline pH region is expanded to include effects of substrate-analog inhibitors (see Scheme I). In the presence of inhibitors similar to "good" substrates the single step $A_bH_2 \rightleftharpoons A_g + 2H^+$ of the free protein in which two protons are released cooperatively is resolved into two one-proton steps the first of which has an apparent pK_a of 8.1 at 25° and can take place as well when inhibitors are bound as absent. The second step with an apparent pK_a of 9.1 at 25° eliminates the ability of the protein to bind competitive inhibitors. The group of pK_a 8.1 is linked to the protein process responsible for the fluorescence change on inhibitor binding in such a way that only the protonated species, A_bH_2, undergoes the process which forms the A_eH_2I species. The data are consistent with a simple expansion of the formal mechanism previously found appropriate for fluorescence behavior of the free enzyme. As in the latter, the thermodynamic changes clearly suggest a significant conformational process for the step $A_bH_2 \rightleftharpoons A_fH_2$ only. Enthalpy and entropy changes for rates and equilibria of several steps have been determined. On the basis of the assumption that the step $A_jH \rightleftharpoons A_g$ involves breaking of the Ile-16 to Asp-194 ion pair, estimates of the thermodynamic importance of concomitant changes in protein conformation, charge arrangements, and solvation are estimated.

Studies of the pH dependence of α-chymotrypsin (α-CT)-catalyzed hydrolysis reactions[2-5] and interactions of α-CT with inhibitors[6-9] have provided information about the nature of the substrate and inhibitor binding sites and the substates of the free enzyme. The results of these studies show that an ionizable group with pK_a 8–9 controls the equilibrium between the catalytically active and the alkaline inactive forms of the enzyme in the neutral and alklaine pH ranges and

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thereby controls the rates of the reactions. In a previous paper[10] we have presented evidence for the existence of several substates of the best-folded state of α-CT. Some of these may differ in protein conformation. Of more current interest we reported that the changes taking place as the pH is raised from neutral to alkaline value include the simultaneous loss of two protons, not one.

Parker[11] tentatively concluded on the basis of her studies of the optical rotatory dispersion (ORD) of α-CT and its acyl derivatives that the pH dependence of ORD patterns and the Moffitt a_0 and b_0 parameters for α-CT and its "acyl" derivatives diisopropylphosphyl-α-CT (DIP-CT) and diphenylcarbamyl-α-CT (DPC-CT) might indicate that covalent bonding of substrate moieties at the active site of the enzyme produces a conformational rearrangement. Havsteen and Hess[12] employed the same reasoning in their analysis of the ORD data for α-CT and its derivatives and were more enthusiastic about the hypothesis of conformation change. Moon, Sturtevant, and Hess[13] followed the kinetics of the reaction of α-CT with diisopropyl fluorophosphate (DFP) in the pH region from 5.5 to 10 and postulated a scheme that included the formation of an intermediate characterized by spectral changes at 290 nm. Furthermore, they speculated that an ionizable group with $pK_a \sim 9$ controls the rate of formation of the intermediate in the alkaline pH range. More direct evidence for the existence of a substrate- or inhibitor-induced conformation change in the α-CT molecule is the observation of fluorescence changes in the presence of substrates and inhibitors by Sturtevant.[14] Sturtevant concluded that the transient fluorescence behavior he observed did not represent the formation of an intermediate of the hydrolysis reaction but was rather a result of conformational changes in the vicinity of the active site of α-CT stimulated by the binding of various reagents.

The criteria for using changes in thermodynamic functions as measures of the relative change in the conformational state of a protein molecule have been discussed previously[10] in relation to pH-induced transitions of α-chymotrypsin. In 1952 Vaslow and Doherty,[6,7] using an equilibrium dialysis technique, measured the equilibrium constants for virtual substrate binding to α-CT as a function of pH, temperature, and substrate concentration. They recognized two types of behavior in the variation of $\Delta H_b°$ and $\Delta S_b°$ with pH: (1) that of catalytically active systems (*e.g.*, the interaction of α-CT with L-virtual substrates, etc.), for which $\Delta H°$ and $\Delta S°$ become strongly negative with increasing pH, and (2) that observed with insensitive systems (*e.g.*, interaction of chymotrypsinogen A (CGN) with L-virtual substrates, or α-CT with D enantiomorphs of L-virtual substrates) for which $\Delta H_b°$ and $\Delta S_b°$ are small and insensitive to pH. Using a microcalorimeter, Canady and Laidler[15] measured the heat of binding of hydrocinnamate ions, a substrate-analog inhibitor manifesting type 1 behavior, to α-CT over the range of pH values 5.1–7.8 and found that $\Delta H_b°$ and $\Delta S_b°$ became considerably more negative with increasing pH. They attributed the changes in thermodynamic functions to electrostatic interactions between the charges on the inhibitor and groups at the active center of the enzyme.

The results of temperature-jump experiments by Yapel[16,17] further substantiated the existence of type 1 and type 2 behavior. Yapel was able to classify the virtual substrates and inhibitors into the same two general categories found by Vaslow and Doherty.

Previously, we have reported[10] on the extreme sensitivity of fluorescence as a tool for detecting and measuring changes in the state of the proteins of the CGN family and we applied this tool to α-CT in systems free of substrates or inhibitors. In this report we repeat and extend the transient fluorescence studies of Sturtevant to compare the effects of binding of a variety of small molecules differing in chemical structure or charge state and manifesting both types of behavior. The results of these studies are integrated with those obtained for the free-protein studies to expand the state diagram.

Experimental Section

Chemicals and Reagents. Three times crystallized Worthington α-CT, CGN, and δ-chymotrypsin were used after the purification procedures described elsewhere.[17,18] Protein solutions were prepared by dissolving appropriate amounts of lyophilized samples in buffer solutions of the desired composition.

Hydrocinnamic acid was obtained from K and K Laboratories, Inc. Hydrocinnamoyl alcohol and naphthalene were the products of Eastman Kodak Co. Isopropyl alcohol of analytical reagent grade was purchased from Mallinckrodt Chemical Works. Acetylated amino acids were obtained from Cyclo Chemical Corporation and Mann Research Laboratories. The purity of the acetylated amino acids was assayed with ninhydrin according to the method of Yemm and Cocking[19] to establish that there were no free amino compounds. Unless otherwise indicated, all the reagents were used without further purification. Hydrocinnamic acid had to be recrystallized twice in petroleum ether to remove the minor fluorescing impurities in the sample. The purified hydrocinnamic acid did not yield any detectable fluorescence between 500 and 295 nm.

The reagent stock solutions were prepared by dissolving weighed amounts of the reagents in measured volumes of deionized and distilled water. Concentrations of *N*-acetyl-DL-tryptophan solutions were calculated using the molar extinction coefficient of $\epsilon_{280} = 5.43 \times 10^3\ M^{-1}\ cm^{-1}$.

Apparatus and Method. Transient Fluorescence Experiments with the Stopped-Flow Method. The small fluorescence intensity change produced by an enzyme–inhibitor interaction is not paralleled by detectable changes in spectral shape or peak position of the fluorescence spectrum. The apparatus described for the pH-perturbation experiments[10,18] was used without modification. The procedure used in a typical experiment was the following. (a) The enzyme solutions were prepared in 0.2 *M* NaCl and 0.02 *M* THAM–HCl buffer solutions such that the protein concentration of the sample was twice the concentration desired in the reaction cell. Inhibitor or substrate samples were prepared by diluting stock solutions with water and the buffer solution to give inhibitor concentrations twice the final concentration desired but all solutions contained 0.2 *M* NaCl and 0.02 *M* THAM–HCl buffers. Thus, mixing the two samples in equal volume produced no change in the buffer concentration but did decrease the concentrations of the enzyme and the inhibitor to one-half of the original concentrations. (b) Adjustment of the pH value of a sample was made in the manner previously described.[10] It was essential that the pH values of both the enzyme

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sample and the inhibitor sample be identical within ±0.01 for a given experiment. (c) Samples were introduced into thermostated driving syringes and equilibrated at the desired temperature before being driven into the observation chamber through the mixers.

Control experiments were carried out routinely with the different combinations of proteins, inhibitors, and buffer solutions to show that the experiments were free of instrumental and experimental artifacts.

For most of the experiments saturated inhibitor concentrations were used since the total fluorescence increase during the reaction was very small ($\leq 2.5\%$) and thus quantitatively measurable only at high inhibitor concentrations.

Steady-State Fluorescence Experiments. The fluorimeter used for these experiments has been described.[10] In order to estimate the quantitative increase in fluorescence yield as a result of the binding of hydrocinnamate ion to α-CT three samples were prepared: (a) a protein solution, (b) an inhibitor solution, and (c) a buffer solution. Samples a and b had the same composition as for the transient fluorescence experiments. Sample c was a blank buffer solution of 0.2 *M* NaCl and 0.02 *M* THAM–HCl. All three samples were adjusted to pH 8.0 separately. Using closely matched 5-ml pipets, the following three mixtures could be prepared from the original samples by mixing them two at a time in equal volumes: (1) the protein blank solution, a + c; (2) the inhibitor blank solution, b + c; (3) protein + inhibitor mixture, a + b. The fluorescence spectrum for each sample was measured separately so that the contributions from individual components in the reaction mixture could be identified.

Results

A. Steady-State Fluorescence Change. It was intended to estimate the magnitude of the enhancement of fluorescence and to observe any possible change in shape of the fluorescence spectrum of the free enzyme molecule as a result of the interaction between the enzyme and the hydrocinnamate ion. The experiments carried out at pH 8.0 at 20° following the protocol described in the previous section revealed neither a detectable shift in the peak position nor a change in shape of any part of the fluorescence spectrum of free α-CT solution when purified hydrocinnamate ion was added to the system. The enhancement of fluorescence estimated from the increase in the area under the fluorescence spectrum was 2.3%. To make certain that this enhancement was not due to an artifact the experiment was repeated using CGN-A in place of α-CT. No change in fluorescence was observed.

The calculation of the fluorescence yield of the (α-CT + inhibitor) mixture is complicated due to the fact that the binding of inhibitor changes the absorption spectrum of the enzyme significantly. On the other hand, the system of CGN plus inhibitor does not show any change in absorption spectrum relative to the additive contributions from the components of the mixture.

B. Transient Fluorescence Change. The magnitude of the fluorescence change was found to depend on the pH, temperature, and the inhibitor and the enzyme concentrations. The higher the inhibitor and the enzyme concentrations, the higher the percentage fluorescence change at a constant temperature and pH value. For a given set of values of the variables the fluorescence enhancement became smaller as the pH was adjusted from 8 to 7. The temperature effect was such that the fluorescence enhancement increased when temperature was lowered holding all other conditions fixed. Unless otherwise stated the experimental fluorescence relaxation spectrum consisted of a single relaxation effect. Typically, after mixing, the fluorescence intensity increased as a negative exponential function of time until it reached the final equilibrium state.

A summary of transient fluorescence response results is given in Table I.

Table I. Transient Fluorescence Response

Virtual substrate or inhibitor	Protein	Transient fluorescence change at pH 8.0
Hydrocinnamate ion	α-CT	Yes
	δ-CT	Yes
	CGN A	No
Hydrocinnamoyl alcohol	α-CT	Yes
	CGN A	No
Naphthalene	α-CT	Yes
	CGN A	No
N-Acetyl-D-tryptophan	α-CT	No
N-Acetyl-L-tryptophan	α-CT	Yes
Isopropyl alcohol	α-CT	Yes
	CGN A	No

1. Hydrocinnamate Ion Binding. Only the binding of hydrocinnamate ion, HC, to α-CT was studied in full detail. The apparent first-order rate constants for the virtual substrate and the other inhibitors at varying conditions were compared with those for HC binding at single sets of experimental conditions.

The first-order rate constants determined at varying α-CT and HC concentrations are given in Table II

Table II. Concentration Dependence of the First-Order Rate Constants

pH	Temp, °C	Concn: α-CT[a]	Concn: Hydrocinn (HC), *M*	Rate constant, sec^{-1}
8.00	20.0 ± 0.1	0.05	0.01	0.58 ± 0.02
8.00	20.0	0.10	0.01	0.55 ± 0.03
8.00	28.0 ± 0.1	0.05	0.01	1.22 ± 0.08
8.00	28.0	0.05	0.005	1.22 ± 0.11

[a] The enzyme concentration is expressed in weight per cent. The errors indicated for rate constants are the arithmatic mean deviations.

and demonstrate that the rate constants are independent of enzyme and inhibitor concentrations.

At a given temperature there was a limit to the range of pH values within which the experimental results were consistent. For example, at 20° any experiment carried out at a pH value above 8.8 gave a relaxation spectrum that consisted of two opposing effects: a relatively large increase in fluorescence intensity with time followed by a slow and smaller decrease in fluorescence. In our earlier work[10] it was found that when the pH is perturbed from 8.5 to 9.5, α-CT undergoes a change in state in which two protons are released with an equilibrium constant K_a such that $-\log K_a = 18.82$ at 20°. The second transient in the relaxation spectrum is due to the increasing participation of this process at high pH values. However, the magnitude of the fluorescence decrease for this process was too small to allow any reliable analysis of the results in the more alkaline region. To simplify analysis, experiments were restricted to pH values where this species, A_g or A_c (ref 10), could be ignored.

At pH values lower than 6.7 and 20°, the change in fluorescence intensity became too small to detect. This observation should not be taken to mean that equally significant changes do not occur at lower pH values (see Discussion).

The following two mechanisms are found to be consistent with the present results. (See Appendix for the derivations.) In eq 1 and 2 the step EHI ⇌ (EHI)′ is

$$\begin{array}{ccc} E + I + H^+ & \rightleftharpoons & EI + H^+ \\ \Updownarrow & & \Updownarrow \\ EH + I & \rightleftharpoons & EHI \rightleftharpoons (EHI)' \end{array} \quad (1)$$

$$\begin{array}{ccc} E + I + H^+ & \rightleftharpoons & EI + H^+ \\ \Updownarrow & & \searrow\nearrow \\ EH + I & \rightleftharpoons & (EHI)' \quad EHI \end{array} \quad (2)$$

a slow first-order process of the enzyme–inhibitor complex. We may rewrite eq 1 and 2 as 1_b or 2_b in

$$\begin{array}{ccccc} A_bH + I + H^+ & & \overset{K_{12}}{\rightleftharpoons} & & A_bHI + H^+ \\ {\scriptstyle K_{13}}\Updownarrow & & & & \Updownarrow{\scriptstyle k_{24}}{\scriptstyle k_{42}} \\ A_bH_2 + I & & \underset{K_{34}}{\rightleftharpoons} & & A_bH_2I \underset{k_{54}}{\overset{k_{45}}{\rightleftharpoons}} A_eH_2I \end{array} \quad (1_b)$$

$$\begin{array}{ccccc} A_bH + I + H^+ & & \rightleftharpoons & & A_bHI + H^+ \\ \Updownarrow & & & \overset{k_{24}}{\searrow}\underset{k_{42}}{\nearrow} & \\ A_bH_2 + I & & \underset{k_{54}}{\overset{k_{45}}{\rightleftharpoons}} & & A_bH_2I \end{array} \quad (2_b)$$

view of our previous finding (see ref 10 for details) that α-CT assumes the substate A_bH_2 at neutral pH and loses two protons as the pH is raised above 9. Thus we can identify the enzyme species in eq 1 and 2 as EH = A_bH_2 and E = A_bH and (EHI)′ is replaced by A_eH_2I. Although the mechanisms are not unique, they are consistent with all information available on the interaction of α-CT with HC at neutral and alkaline pH values and they are the simplest such mechanisms we have found. The mechanisms 1_b and 2_b require that both the protonated and the unprotonated enzyme species, A_bH_2 and A_bH, be able to bind inhibitor even though the slow first-order process detectable with fluorescence can occur only with the protonated enzyme–inhibitor complex, A_bH_2I or A_eH_2I.

A solution for the mechanisms 1_b and 2_b based on reasonable assumptions (see Appendix) which can explain the observed slow relaxation process after making appropriate assumptions is eq 3.

$$\lambda_{app} = k_{54} + \frac{k_{45}}{1 + k_{42}/k_{24}a_H} = k_{54} + \frac{k_{45}}{1 + K_a/a_H} \quad (3)$$

In Figure 1 the solid lines drawn through the experimental points are the theoretical curves computed using eq 3. The procedure employed for the computer analysis of the least-squares fit of the data has been described elsewhere.[10] The parameters determined for the "best fit" of the data are included in Table III and the curves calculated with these parameters are plotted in Figure 2. Reasonably good linearity is observed in the van't Hoff plot of pK_a against $1/T$ except at the lowest temperature. The thermodynamic functions for the slowly equilibrating step and the fast ionization step are tabulated in Table IV.

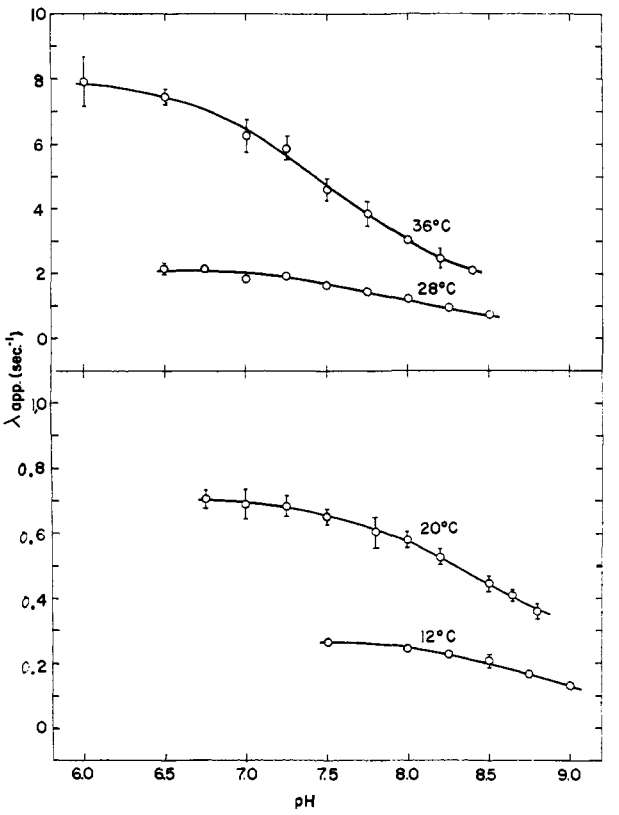


Figure 1. The pH dependence of the apparent first-order rate constant for the binding of hydrocinnamic acid to α-CT at various temperatures: α-CT = 2×10^{-5} *M*, hydrocinnamic acid = 0.01 *M*, THAM–HCl = 0.02 *M*, NaCl = 0.2 *M*. The theoretical curves at different temperatures are shown in solid lines.

Some representative experiments were carried out to investigate the difference in binding of HC to δ-CT and

Table III. Rate Parameters and pK_a Values for Hydrocinnamate Ion Binding to α-CT at Various Temperatures According to Equation 3[a]

Temp, °C	pK_a	k_{54}, sec^{-1}	k_{45}, sec^{-1}
12	8.81 ± 0.1	0.04 ± 0.03	0.24 ± 0.03
20	8.41 ± 0.03	0.23 ± 0.02	0.48 ± 0.02
28	7.9 ± 0.2	0.38 ± 0.17	1.78 ± 0.12
36	7.52 ± 0.01	1.34 ± 0.05	6.67 ± 0.09

[a] The errors in pK_a value are estimated from the result of the least-squares analysis, and the errors included with the parameters, k_{54} and k_{45}, are the standard errors of these quantities.

Table IV. Thermodynamic Functions for the Slowly Equilibrating Step and the Fast Ionization Step of Equation 1b[a]

Type of transition	Properties: $\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, eu/mol	$\Delta F^{\ddagger}$, kcal/mol
$A_bH_2I \overset{k_{54}}{\leftarrow} A_eH_2I$	18.3 ± 2.7	2 ± 9	18.2 ± 0.1
$A_bH_2I \overset{k_{45}}{\rightarrow} A_eH_2I$	28.4 ± 2.3	37 ± 6	17.5 ± 0.1
Type of equilibrium	**Properties: ΔH°, kcal/mol**	**ΔS°, eu/mol**	**ΔF°, kcal/mol**
$A_bH_2I \rightleftharpoons A_eH_2I$	10.1 ± 3.5	35 ± 11	−0.7 ± 0.1
$A_bH_2I \overset{K_a}{\rightleftharpoons} A_bHI + H^+$	22.5 ± 1.8	38 ± 6	11.1 ± 0.1

[a] The errors indicated for ΔS and ΔF are the standard errors.

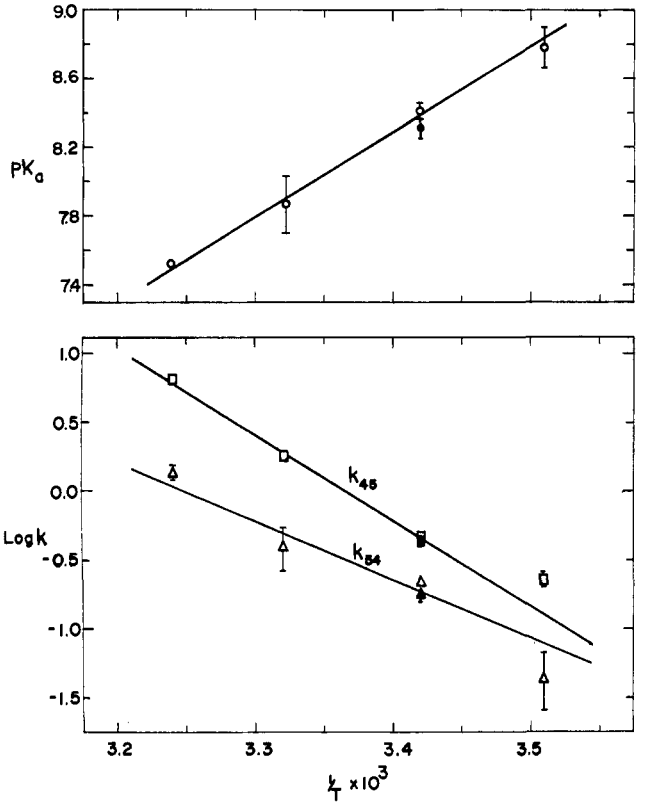


Figure 2. Van't Hoff and Arrhenius plots based on the theoretical analysis of Figure 1 and Table II. The filled symbols at 20° represent the results from Figure 3 for the hydrocinnamoyl alcohol binding to α-CT.

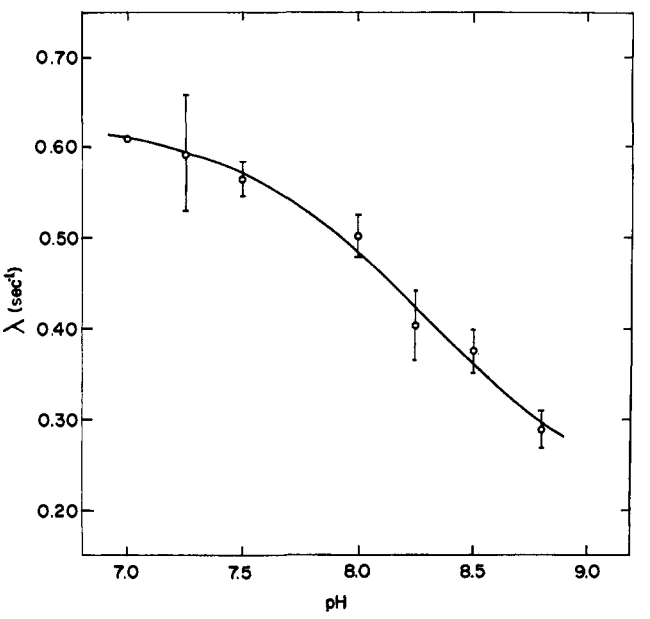


Figure 3. The pH dependence of the apparent first-order rate constant for the binding of hydrocinnamoyl alcohol to α-CT at 20°: α-CT = 2×10^{-5} *M*, hydrocinnamoyl alcohol = 8×10^{-3} *M*, THAM-HCl = 0.02 *M*, NaCl = 0.2 *M*.

to α-CT as reflected in the transient fluorescence change. The results are presented in Table V, in which the apparent first-order rate constants for δ-CT systems under comparable conditions are also included.

Table V. Hydrocinnamate Ion Binding to δ-CT

Temp, °C	pH	Apparent first-order rate constant δ-CT, sec^{-1}	α-CT, sec^{-1}
28.0 ± 0.1	7.50	1.75 ± 0.04	1.6 ± 0.1
	8.00	1.24 ± 0.03	1.22 ± 0.08
	8.50	0.74 ± 0.09	0.74 ± 0.08
20.0 ± 0.1	8.00	0.55 ± 0.04	0.58 ± 0.02
12.0 ± 0.2	8.00	0.26 ± 0.03	0.25 ± 0.01

An experiment was carried out with a system consisting of CGN and HC at pH 8.0, 20°. No detectable transient fluorescence change was seen under conditions at which a significant change in fluorescence intensity had been observed for α-CT.

2. Hydrocinnamoyl Alcohol Binding. Hydrocinnamoyl alcohol has the same "side-chain" structure as hydrocinnamic acid, but carries no charge in our experimental pH range. Hence any effect of the electrical charge of the inhibitor on the binding should appear when the rate processes of the two compounds are compared. The experimental rate constants *vs.* pH at 20° are shown in Figure 3 along with the theoretical curve fitted to the data according to eq 3. The "best-fit" parameters for this system (at 20°) are: pK_a = 8.32 ± 0.07, k_{54} = 0.186 ± 0.035 sec^{-1}, and k_{45} = 8.32 ± 0.02 sec^{-1}. The estimation of the errors was made in the same way as for the hydrocinnamic acid case. These points are given as solid black symbols in Figure 2.

Due to the low solubility of hydrocinnamoyl alcohol it was necessary to check the dependence of the rate constants on the concentrations of inhibitor and enzyme. The results indicate that the apparent rate constants are independent of the inhibitor concentration in the range studied.

3. Naphthalene Binding. Naphthalene is also very insoluble in water. Therefore saturated solutions had to be used for all the experiments. At lower pH (~7.5) and at high temperature (36°) the fluorescence intensity change was so small that only a few reliable rate constants could be evaluated. The results are given in Table VI. An experiment with 0.05% (by weight) CGN solution in place of α-CT, at pH 8.00 and 12°, failed to give any time-dependent fluorescence change.

Table VI. Rate Constants for the Binding of Naphthalene to α-CT[a]

Temp, °C	pH	α-CT, %	Rate constant, sec^{-1}
28.0 ± 0.1	8.00	0.05	1.18 ± 0.03
	8.00	0.075	1.20 ± 0.05
20.0 ± 0.1	8.00	0.05	0.56 ± 0.04
12.0 ± 0.2	7.50	0.05	0.26 ± 0.02
	8.00	0.05	0.26 ± 0.02
	8.50	0.05	0.20 ± 0.01

[a] Naphthalene concentration in the initial reaction mixture was 1.17×10^{-4} *M*. The errors are the arithmetic mean deviations.

4. *N*-Acetyl-D- and *N*-Acetyl-L-tryptophan Binding. The large background fluorescence contributed by the indole groups of the inhibitors did not significantly impair the precision of the experiments. Using saturated solutions of *N*-acetyl-D-tryptophan and *N*-ace-

tyl-L-tryptophan it was possible to compare the D and L steric selectivity of the enzyme for these inhibitors. *N*-Acetyl-D-tryptophan did not produce detectable change in fluorescence but its enantiomer produced a change in fluorescence intensity with a first-order rate constant of 0.243 ± 0.016 sec^{-1} at 12° and pH 8.00. Any change in emission of the indole groups of the inhibitors due to binding is apparently faster than the dead time of our stopped-flow instrument.

5. Effect of Isopropyl Alcohol on the Fluorescence of α-CT. The effect of isopropyl alcohol on α-CT due to solvent perturbation or to direct binding could be detected by a transient fluorescence change but only at isopropyl alcohol concentrations greater than about 0.1 *M*. As in the other experiments discussed so far, adherance to the first-order kinetics was observed over the same pH range as with the other systems and the first-order rate constant within error was independent of alcohol concentration at pH 8.0. However, unlike other inhibitor systems, the dependence of the apparent first-order rate constant on the pH value was anomalous as can be seen in Table VII. The fluorescence intensity

Table VII. Effect of Isopropyl Alcohol on α-CT[a]

Temp, °C	pH	Isopropyl alcohol concn, *M*	Rate constant, sec^{-1}
20.0 ± 0.1	8.00	0.82	0.51 ± 0.02
	8.00	1.64	0.51 ± 0.04
28.0 ± 0.1	7.25	1.64	3.82 ± 0.2[b]
			0.94 ± 0.06
	7.50	1.64	1.78 ± 0.07
	8.00	1.64	1.79 ± 0.05
	8.20	1.64	1.28 ± 0.06
	8.40	1.64	1.16 ± 0.04

[a] α-CT concentration in the final reaction mixture was 0.05% for all the experiments. [b] The two rate constants were calculated by separating the observed relaxation curve into two first-order processes. The errors are the arithmetic mean deviations.

at pH 7.5 relative to that at pH 8.0 was significantly lower than observed with the other inhibitors and a relaxation spectrum which appeared to consist of two first-order processes was observed at pH 7.25. It is noteworthy that with isopropyl alcohol as with the other small inhibitors, CGN demonstrated no transient fluorescence change (at pH 8.0 and 28°).

Discussion

Effect of the Type of Inhibitors on the Transient Increase in Fluorescence Intensity. The possibility that the transient fluorescent change originates from the binding of inhibitors at sites on the enzyme other than those involved in substrate binding is somewhat minimized by the fact that the catalytically active species, α-CT and δ-CT, gave steady-state and transient changes in fluorescence whereas CGN did not. Inhibitor binding either requires a site or sites not available on the zymogen or it does not influence the fluorescence behavior of CGN.

Steitz, Henderson, and Blow[20] have shown that the indole group of *N*-formyl-L-tryptophan in α-CT crystals is held in a small slot which they not unreasonably presume to be the normal binding site for the side chain of substrates. Even more recent studies by Kraut, *et al.*,[21] comparing CGN at 2.5-Å resolution with α-CT suggest that this slot is closed in CGN or at least not so clearly defined. Although such studies may prove to have only secondary relevance to the behavior of these proteins in homogeneous solution, they nevertheless appear to be consistent with the assumption that there is no "normal" inhibitor binding to CGN. However, Doherty and Vaslow[7] reported binding of this type of inhibitor to CGN so that it is not possible to make the distinction between inhibitor binding to an inactive conformation and no inhibitor binding. With the free proteins we found CGN to demonstrate none of the transient effects in fluorescence on pH change which α-CT manifested.[10]

According to Sturtevant[14] and McClure and Edelman[22] the relative increase in fluorescence when an inhibitor is bound to α-CT corresponds to "one-to one" interaction between inhibitor and enzyme for all the inhibitors that they studied except isopropyl alcohol. The interaction of isopropyl alcohol with α-CT did not correspond to a "one-to-one complex" system. In the following discussion, therefore, the results for isopropyl alcohol experiments are treated separately.

(1) The results of our experiments show that there is no direct relation between the size of the aromatic ring of inhibitors, *i.e.*, phenyl, naphthyl, and indolyl, etc., and the rate behavior of the transient fluorescence change. When the fluorescence relaxation process occurs the first-order rate constants are independent of the side-chain structures of the inhibitors within experimental error.

(2) The binding of hydrocinnamate ion and hydrocinnamoyl alcohol to α-CT yields profiles of pH dependence of λ_{app} at a constant temperature which are practically indistinguishable, and analysis of the data with eq 3 also give values for the rate parameters which are strikingly close to each other. Thus the influence of the electrical charge of the inhibitor on α-CT binding reactions is notably absent in the slow relaxation process of mechanism 1_b.

(3) Both optical isomers, *N*-acetyl-D-tryptophan and *N*-acetyl-L-tryptophan, are known to be bound by α-CT at neutral pH, and at 25° the D compound is reported to be bound more strongly than its enantiomer.[23] However, *N*-acetyl-L-tryptophan was bound to α-CT in such a way that the time-dependent fluorescence increase of the system gave a first-order rate constant identical with that for the HC binding under comparable conditions. The D enantiomer gave no transient behavior. It was the only substrate similar compound in this series which was inert with respect to the fluorescence changes. In terms of eq 1_b the specificity of the enzyme is such that only interaction with the category-1 inhibitors (classification according to Yapel[16]) produces the slowly equilibrating step.

(4) *A priori* it is anticipated that isopropyl alcohol can influence α-CT fluorescence through three mechanisms. (i) The presence of a branched aliphatic alcohol in such high concentration (1.6 *M*) produces a

(20) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, **46**, 337 (1969).

(21) J. Freer, J. Kraut, J. Robertus, A. Wright, and Ng Xuong, *Biochemistry*, **9**, 1997 (1970).

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

(23) D. W. Ingles and J. R. Knowles, *Biochem. J.*, 104, 369 (1967).

change in the dielectric environment of the protein which can cause charge rearrangements leading by direct or indirect pathways to differences in local fluorescence quenching. (ii) Isopropyl alcohol at these concentrations has a large influence on the properties of water and these same properties are strongly implicated in both inhibitor binding and enzymic catalysis by α-CT.[24,25] (iii) It has recently been shown[20] that dioxane is bound in the side-chain slit. The slit is detected as being about the size of an indole nucleus and thus should be large enough to accommodate at least one or several isopropyl alcohol molecules in combination with water molecules. At the present time there is no information which excludes any of these alternatives and the absence of an effect of isopropyl alcohol on CGN fluorescence appears irrelevant in this connection.

Conformational Readjustments. Although Parker[11] and Kim[18] find that inhibitors, acylating agents, and substrates produce easily detected changes in α-CT absorption in the indole and phenol absorption regions, X-ray diffraction studies have shown that there are no indole or phenol groups in or very near the surface of the side-chain slot. Hence the range of influence of a bound small molecule through the protein is reasonably large and, as previously mentioned, it is highly variable as shown by the fact that each inhibitor studied by Parker and Kim gave a significantly different difference spectrum. There is thus a considerable part of the protein in which some sort of adjustment to side-chain binding is possible. The studies of fluorescence reported by Sturtevant and in this paper demonstrate the existence of what appears to be a single first-order process of the protein induced in an all-or-none fashion by inhibitor binding but otherwise independent of the inhibitor and its concentration. The independence of the characteristics of the variable fluorescence accompanying the first-order process or inhibitor strongly suggests that this process has no direct relationship to the chromophoric changes responsible for the variety of difference absorption spectra.

Some evidence[11,13,26] has become available to suggest the involvement of an ionizable group with a pK_a value between 8 and 9 in the protein "conformation change." According to our results the ionizable group that is directly connected to the "fluorescence change" has an effective pK_a of 8.1 at 25° but varies with temperature over more than 1 pK unit in the range from 12 to 36°.

δ-CT has only one break in its primary structure and for this reason must be different from α-CT. Studies of the thermal unfolding transitions of these proteins and CGN by thermodynamic and nmr methods[27] indicate that δ-CT is quite different from α-CT in the extent of folding and much more like CGN. The results of several experiments on the binding of hydrocinnamate ion to δ-CT, nevertheless, reveal a remarkable similarity in the binding behavior between δ-CT and α-CT. Evidently, the inhibitor-induced change of both enzyme species must involve the same residues and perhaps the same extent of change.

Viewpoint from Thermodynamics. The thermodynamic functions associated with the apparent ionization step of eq 1_b are too large for the simple ionization process of an amino group even when we take into account the large dielectric constant difference between the protein–water interfacial region and bulk water. Particularly, the entropy change of 38 eu/mol is much too large to be attributed to an ionization reaction of an ammonium group which in normal ionization process has $\Delta S_a^\circ = 0$–2 eu/mol. Hence, we must assume that the rapidly equilibrated ionization step consists of two parts, one of which is the true ionization step and the other a relatively fast isomerization step. To be noted in eq 4 is that a new species, A_jHI, is introduced as

$$A_bH_2I \overset{K_a}{\rightleftharpoons} A_bHI + H^+ \overset{K_c}{\rightleftharpoons} A_jHI + H^+ \qquad (4)$$

an isomeric form of A_bHI. Thus, we separate the part process responsible for the isomerization from the total process and define $K_{app}' = K_aK_c$, $\Delta H^\circ_{app} = \Delta H_a^\circ + \Delta H_c^\circ$, $\Delta S^\circ_{app}{}' = \Delta S_a^\circ + \Delta S_c^\circ$, $\Delta F^\circ_{app}{}' = \Delta F_a^\circ + \Delta F_c^\circ$, where the subscripts a and c refer to the corresponding steps in eq 4.

The results tabulated in Table VIII include two sets of thermodynamic values calculated on the assumption that the intrinsic pK_a of the group is 8.1. An assumption is also made that the ionizable group is an ammonium group (α-amino or highly perturbed ε-amino group) since Yapel's[16] study of the imidazole groups of free CT shows them to be quite normal with pK_a values of about 7. Rajender, Lumry, and Han[28] reached the same conclusion on the basis of steady-state catalysis studies. Particularly noteworthy is the fact that both ΔH° and ΔS° for the $A_bHI \rightleftharpoons A_jHI$ process are positive. A relatively large positive entropy change is accompanied by a moderate positive enthalpy change and a small ΔF° for this step so that the enthalpy and entropy changes tend to compensate each other. The parameters for the net isomerization step also exhibit surprising similarities with the corresponding change for the slow step, $A_bH_2I \rightleftharpoons A_eH_2I$. The precise nature of any relationship existing between the two separate processes is not yet clear but comparisons of thermodynamic changes reveal that the thermodynamic

Table VIII. Analysis of the Thermodynamic Functions for the Apparent Ionization Process of Equation 4[a]

Type of process	Thermodynamic functions: ΔH°, kcal/mol	ΔS°, eu/mol	ΔF°, kcal/mol
$A_bH_2I \rightleftharpoons A_jHI + H^+$ experimental, Table IV	22.5 ± 1.8	38 ± 6	11.1 ± 0.1
$A_bH_2I \rightleftharpoons A_bHI + H^+$ ($-NH_3^+ \rightleftharpoons -NH_2 + H^+$)[b]	12.8[b]	6	11.1[c]
$A_bHI \rightleftharpoons A_jHI$ isomerization	9.7	32	0

[a] Based on the assumption that the acid group of the protein is an ammonium group. [b] ΔH° for this process is taken from ref 27. [c] The intrinsic pK_a of the ammonium group is assumed to be 8.1 at 25°.

(24) S. Rajender, M. Han, and R. Lumry, *J. Amer. Chem. Soc.*, **92**, 1378 (1970).
(25) R. Lumry and S. Rajender, *Biopolymers*, **9**, 1125 (1970).
(26) J. McConn, E. Ku, C. Odell, G. Czerlinski, and G. P. Hess, *Science*, 161, 274 (1968).
(27) R. Lumry in "A Treatise on Electron and Coupled Energy Transfer in Biological Systems," T. King and M. Klingenberg, Ed., Marcel Dekker, New York, N. Y., in press.
(28) S. Rajender, R. Lumry, and M. Han, *J. Phys. Chem.*, in press.

characteristics of the change in structure of the protein during the inhibitor-binding process for the two steps are very similar so that thermodynamically A_jHI bears the same relationship to A_bHI as A_eH_2I bears to A_bH_2I. It appears possible that A_bHI and A_jHI are protonated at different sites so as to complicate the analysis of the pH dependence. Furthermore, this finding suggests that eq 1_b may still be an oversimplified version of more complex sequence of events occurring in the enzymic reaction. However, according to the analysis of this paper A_eH_2I and A_jHI must have different fluorescence properties.

pH Dependence of $\Delta H_b°$ and $\Delta S_b°$. The expression for the overall binding constant for mechanism 1_b is given in eq 5 (consult Appendix for derivation) and the

$$K_b = \frac{K_{12} + K_{13}K_{34}(1 + K_{45})a_H}{1 + K_{13}a_H} \quad (5)$$

expression for the enthalpy change in the overall binding process in eq 6.

$$\Delta H_b° = \frac{K_{12}\Delta H°_{12} + K_{13}K_{34}(1 + K_{45})(\Delta H°_{34} + \Delta H°_{13})a_H + K_{13}K_{34}K_{45}\Delta H°_{45}a_H}{K_{12} + K_{13}K_{34}(1 + K_{45})a_H} - \frac{K_{13}a_H}{1 + K_{13}a_H}\Delta H°_{13} \quad (6)$$

One can visualize the general trend of the pH dependence of $\Delta H_b°$ by applying the limiting conditions to eq 6 and examining the values which $\Delta H_b°$ approaches. If we assume that the limiting value of $\Delta H_b°$ at low pH is -4.0 kcal/mol, *i.e.*, lim $(a_H \rightarrow \infty)$ $\Delta H_b° = -4.0$, as determined by Canady and Laidler using calorimetry,[15] we can estimate $\Delta H_b°$ at very high pH as follows

$$K_{45} = \frac{k_{45}}{k_{54}} = \frac{1.009}{0.336} = 3.0$$

$$\Delta H°_{45} = 10.1 \text{ kcal/mol}$$

The values for k_{45} and k_{54} for 25° are taken from Figure 3. Then

$$\lim_{a_H \rightarrow \infty} \Delta H_b° = \Delta H°_{34} + \frac{K_{45}}{1 + K_{45}}\Delta H°_{45} = -4.0 \text{ kcal/mol}$$

and

$$\Delta H°_{34} = -11.6 \text{ kcal/mol}$$

If we further assume that $\Delta H°_{12} \simeq \Delta H°_{34}$ for the binding of hydrocinnamate ion to the two enzyme species of different ionization states, A_bH_2 and A_jH, then lim $(a_H \rightarrow 0)$ $\Delta H_b° = \Delta H°_{12} \simeq -11.6$ kcal/mol. Thus, according to this model, $\Delta H_b°$ becomes increasingly more negative as the pH value of the system is raised. This result is consistent with the reported results of the other workers for similar systems.[15,16]

Supporting evidence for the present mechanism is also obtained from the observations by Glazer[29] on the binding of some arsenicals which led him to suggest that the initial α-CT–arsenical complex, EI, undergoes a slow rearrangement to give an inactive enzyme species EI′. Two things are clear from his observations: (1) there exists a slowly equilibrating step in the enzyme–inhibitor binding process for some inhibitors which yields a thermodynamically stable species and (2) the slow rearrangement to the stable species is more extensive at pH 6 than at pH 8. These findings are remarkably consistent with the present analysis, since eq 1_b or eq 2_b specifies that the slow conversion of enzyme–inhibitor complex into a thermodynamically stable species is favored at low pH values.

The small free-energy change resulting from compensating changes in $\Delta H°$ and $\Delta S°$ suggests that the process, $A_bH_2I \rightleftharpoons A_eH_2I$, may be of a "subtle-change" type[30] rather than a folding or unfolding of the protein. It is not possible, however, to decide from the magnitude of the thermodynamic parameters whether the change involves a large part of the protein molecule or a small part.

The Nature of the Ionizable Group Responsible for the Inhibitor-Induced Conformation Change of α-CT. The mechanism 1_b indicates that the enzyme in substates A_bH_2 and A_jH binds inhibitor equally well, whereas studies of hydrolysis reactions of ester substrates[24,31-33] reveal that the K_m value but not k_{cat} increases with increasing pH. In other words, substrates cannot be bound by α-CT in the high-pH substates A_g and A_c. Therefore, the ionizable group with "apparent" pK_a 8.1 controlling the process $A_bH_2I \rightleftharpoons A_jHI$ is probably not the group identified in α-CT-catalyzed ester hydrolysis as the essential ionizable group. Apparently, the inhibitors we have studied do not bind (or bind only weakly) to the A_g and A_c species, but they can be bound to both A_bH_2 and A_jH species.

The ionizable group with "apparent" pK_a 8.1 observed in the inhibitor binding of α-CT may be an α-ammonium or an ε-ammonium group. It must be borne in mind, however, that these assignments are based on apparent pK_a values only.

The discrepancies that are found among the values of pK_a reported for the α-CT-catalyzed sul hydrolysis reactions by different authors are not easy to reconcile. One possible source of disagreement revealed by our findings is that the group of pK_a 8.1 which is identified in the inhibitor-binding processes may also influence the substrate–hydrolysis reactions of "poor" substrates, *i.e.*, substrates whose reactions are inefficiently catalyzed by α-CT.

Relation between the pH Transition Process ($A_bH_2 \rightleftharpoons A_c + 2H^+$) and Inhibitor-Induced "Conformation" Change of the Protein. In our earlier report[10] the pH-induced change in the state of the free enzyme at alkaline pH values was discussed. The experimental results were found to be entirely consistent with a scheme in which a rapidly equilibrating ionization step is followed by a slowly equilibrating step. Furthermore, it became evident that in the free enzyme two ionizable groups were involved in the pH transition process though only one of the two groups may participate in the α-CT-catalyzed hydrolysis reactions of "good" ester substrates.

(29) A. N. Glazer, *J. Biol. Chem.*, **243**, 3693 (1968).

(30) R. Lumry and R. Biltonen in "Structure and Stability of Biological Macromolecules," S. Timasheff and G. Fasman, Ed., Marcel Dekker, New York, N. Y., 1969, p 65.

(31) H. Kaplan and K. J. Laidler, *Can. J. Chem.*, **45**, 547 (1967).

(32) H. L. Oppenheimer, B. Labouesse, and G. P. Hess, *J. Biol. Chem.*, **241**, 2720 (1966).

(33) M. L. Bender, M. J. Gibian, and D. J. Whelan, *Proc. Nat. Acad. Sci. U. S.*, **56**, 833 (1966).

The conclusion that the pK_a 8.1 group may not be an essential group controlling the catalytic activity of α-CT in some hydrolysis reactions at alkaline pH values is based on the indications of kinetic studies of the "good" substrate *N*-acetyl-L-tryptophan ethyl ester[24] that the equilibrium constant for formation of the significant secondary-bonded enzyme–substrate complex depends on a single acid group with an apparent pK_a of about 9. However, the pH range above 9 is experimentally difficult and it has not been possible to establish with certainty that only a single acid group participates.[24] The evidence indicates a dependence on hydrogen-ion activity closer to first order than to second order, so that in the remainder of this discussion we shall assume a first-order dependence in which case the reactions at alkaline pH values are described by eq 7 and 8.[34] If we assume that one of the two groups

$$\begin{array}{ccc} A_bH_2 + I & \rightleftharpoons & A_bH_2I \\ {\scriptstyle K_{a,1}}\downarrow\uparrow & & \uparrow\downarrow{\scriptstyle K'_{a,1}} \\ A_jH + H^+ + I & \rightleftharpoons & A_jHI + H^+ \end{array} \qquad (7)$$

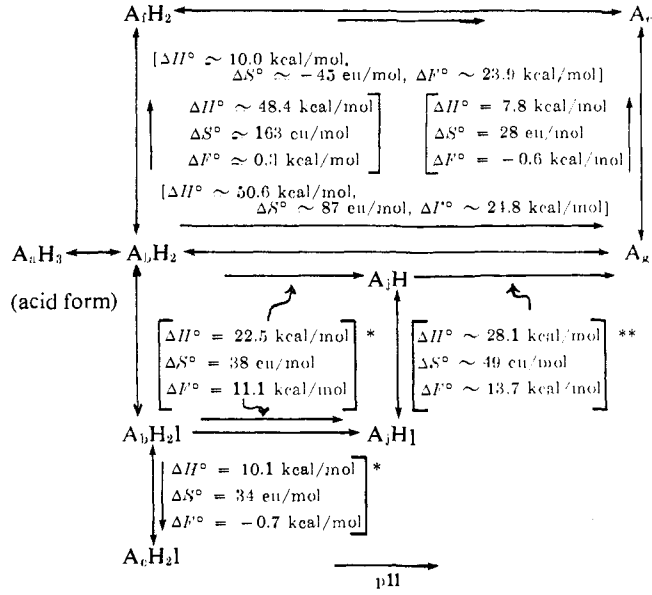
$$\begin{array}{ccc} A_jH + I & \rightleftharpoons & A_jHI \\ {\scriptstyle K_{a,2}}\downarrow\uparrow & & \\ A_g + H^+ + I & \rightleftharpoons & \end{array} \qquad (8)$$

observed in the pH-dependent process of free α-CT is the group of p$K_{a,2}$ in eq 8, *i.e.*, the group of p$K_a \approx 9$ restricting enzyme–substrate binding, then the p$K_{a,1}$ group in eq 7 is the group of p$K_a \approx 8.1$.

Marini and Wunsch[35] have observed in titration experiments that α-CT has two more titratable acid groups than CGN and that these have apparent pK_a values of 7.8. Since in CGN the imidazolium group of His-40 is indicated by X-ray diffraction studies to be buried in an ion pair with the carboxylate of Asp-194[21] and in α-CT this same carboxylate is buried in an ion pair with the ammonium group of Ile-16,[36] the two "new" groups of α-CT are probably the imidazolium group of His-40 and the ammonium group of Ala-149. According to Yapel[16] and Yapel and Lumry[30,37] the imidazolium groups of His-40 and His-57 in α-CT are essentially identical with pK_a values of about 7; the titration results of Marini and Wunsch appear to be explained by the His-40 imidazolium with pK_a of 7 and the Ala α-ammonium group with pK_a somewhat above 8. The latter group is thus the most obvious candidate for the pK_a 8.1 group controlling the fluorescence change through eq 1$_b$. Therefore it is not unreasonable to assume that the two rapid ionization steps, $A_bH_2I \rightleftharpoons A_jHI + H^+$, and $A_bH_2 \rightleftharpoons A_jH + H^+$, are similar in that the same protonic species is involved in each case so that the thermodynamic functions associated with the fast ionization steps are also similar. On the basis of this assumption the interrelations of the pH transition process and the inhibitor-binding reaction are summarized in Scheme I.

The values of the enthalpy and entropy changes estimated for the process $A_bH_2 \rightleftharpoons A_jH$ are approximately

Scheme I. Schematic Diagram of the Thermodynamic States of α-CT and α-CT–Inhibitor Complex[a]



[a] The values indicated by an asterisk are determined in the present study (Table IV) and those marked with a double asterisk are evaluated in Table IX. The other values have been previously reported.[10]

one-half of the corresponding values for $A_bH_2 \rightleftharpoons A_g$. By this criterion the extent of change in protein "conformation" in $A_bH_2 \rightleftharpoons A_jH$ is about half that in $A_bH_2 \rightleftharpoons A_g$.

Scheme I also shows a qualitative agreement with the currently available information of α-CT in neutral and alkaline pH values. In studies of the ORD of α-CT by Parker[11] along the pH scale of Scheme I Parker has noted a contrasting behavior of α-CT at pH 10.4 with that at acid pH in the presence of inhibitors. At acid pH the binding of inhibitors (hydrocinnamic acid or indole) converts α-CT from A_aH_3 (acid form) into A_bH_2 (neutral form). At alkaline pH the protein does not manifest such behavior implying that substrates and inhibitors are not bound to α-CT at alkaline pH with negative free-energy changes large enough to drive the A_c or A_g forms of the protein into the A_jH or A_bH_2 form.[38]

Kezdy[39] has observed that if α-CT solutions at pH 10 are quickly adjusted to pH 8 and the steady-state velocity determined for a good ester substrate, the hydrolytic reaction shows a definite lag period before the enzyme reaches its normal pH 8 catalytic activity. The lag period must be associated with the slow reversal time required for the predominant A_c form at pH 10 to be converted to A_g, and then to A_jH and A_bH_2 or A_fH_2 forms which are catalytically active species and thermodynamically favored at pH 8. This observation is important because it demonstrates that at alkaline

(34) H. Kaplan and K. J. Laidler, *Can. J. Chem.*, **45**, 540 (1967).
(35) M. Marini and C. Wunsch, *Biochemistry*, **2**, 1454 (1963).
(36) B. Matthews, P. Sigler, R. Henderson, and D. Blow, *Nature (London)*, **214**, 652 (1967).
(37) A. Yapel and R. Lumry, submitted for publication.

(38) While this paper was in preparation Garel and Labouesse using optical rotation measurements and potentiometric titrations have examined the "conformational" states of acetylated δ-CT associated with binding of proton and indole (J. R. Garel and B. Labouesse, *J. Mol. Biol.*, **47**, 41 (1970)). Although their optical rotation results at pH $\geq$10 appear to show a qualitative difference from those of Parker,[11] their observations can be interpreted on the basis of the mechanism shown in Scheme I of this paper. However, it should be borne in mind that the conformational states of α-CT and acetylated δ-CT may not be the same,[18] and this possibility must be taken into consideration whenever a comparison of experimental results for the two enzymes is desired.
(39) J. Kezdy, private communication.

pH values the binding of real substrates to the active forms of α-CT (A_bH_2 or A_fH_2 and A_jH) is possible only by way of the process

$$A_c \xrightarrow{\text{slow}} A_g \underset{}{\overset{\text{fast}}{\rightleftharpoons}} A_jH \overset{\text{fast}}{\rightleftharpoons} A_bH_2 \text{ (or } A_fH_2)$$

Since there is no observable curvature in the Arrhenius and van't Hoff plots in Figure 2 over the temperature range in which $A_bH_2 \rightleftharpoons A_fH_2$ transition takes place, it is probable that both A_bH_2 and A_fH_2 participate in the inhibitor binding process quantitatively the same way. Rajender, Han, and Lumry[24] find both substates to be catalytically active with similar values of the same rate parameter. Although the A_bH_2 to A_fH_2 process is not pH dependent, the process is detectable at about pH 7, a maximum at about pH 8, and disappears between pH 8.5 and 9.[24] The analysis used in this report is not immediately consistent with the possibility that the $pK_a = 8.1$ group is responsible for the appearance and disappearance of the A_bH_2 to A_fH_2 process. In view of the complexity of the system this possibility cannot be excluded and must be examined in future work on this important substate transition.

The $A_jH \rightleftharpoons A_g$ Process and Ion-Pair Formation. It is interesting to extend our interpretation one step farther to accommodate the possibility that the process $A_jH \rightleftharpoons A_g + H^+$ (Scheme I) includes the disruption of an ion pair formed between the residues Ile-16 and Asp-194. Let us consider that the process consists of two parts: (1) deprotonation of the ammonium group, $A_jH \rightleftharpoons A_j + H^+$, and (2) breaking of the ion pair, $A_j \rightleftharpoons A_g$. The order is immaterial since we seek only to evaluate contributions to the thermodynamic changes. Therefore, the total process includes loss of a proton from the α-ammonium group, conformational changes, and solvation of the aspartate carboxylate as a result of the migration of this group from the inner position into the solvent.

The thermodynamic changes for the process $A_jH \rightleftharpoons A_g$ can be estimated by combining the results of the studies of the free-enzyme system,[10] $A_bH_2 \rightleftharpoons A_g$, and the inhibitor-binding process of the present results, $A_bH_2 \rightleftharpoons A_jH$. The estimated values of $\Delta H°$ and $\Delta S°$ are presented in Table IX.

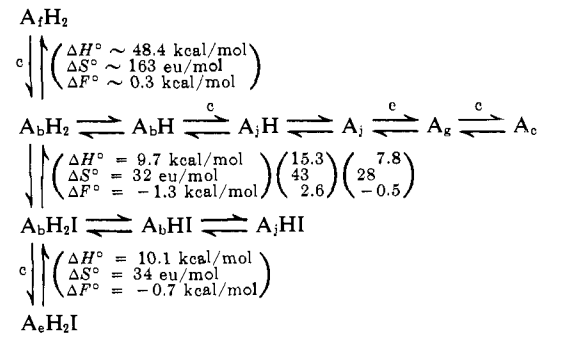
Table IX. Thermodynamic Functions Estimated for the Process $A_jH \rightleftharpoons A_g$ in Scheme I and for Ion-Pair Formation

Type of process	Thermodynamic functions $\Delta H°$, kcal/mol	$\Delta S°$, eu/mol	$\Delta F°$, kcal/mol
$A_bH_2 \rightleftharpoons A_g + 2H^+$ [a]	50.6	87	24.8
$A_bH_2 \rightleftharpoons A_jH + H^+$ [b]	22.5	38	11.1
$A_jH \rightleftharpoons A_g + H^+$	28.1	49	13.7
$Ag + H^+ \rightleftharpoons A_jH$ ($-NH_2 + H^+ + {}^-O_2C- \rightleftharpoons -NH_3^+ \cdots {}^-O_2C-$)	−28.1	−49	−13.7
Protonation of α-amino group $-NH_2 + H^+ \rightleftharpoons -NH_3^+$ [c]	−12.8	−6	−11.1
Ion-pair formation $-NH_3^+ + -CO_2 \rightleftharpoons -NH_3^+ \cdots {}^-O_2C$	−15.3	−43	−2.6

[a] The thermodynamic parameters for this process have been reported previously (ref 10). [b] See Table VIII. [c] Reference 27.

Insofar as our assumptions of mechanism and the type of acid groups involved are correct we can now estimate the net effect of forming an ion pair between the α-ammonium ion and the carboxylate ion from an unprotonated amino group in water solvent. If we assume that the intrinsic pK_a of the α-ammonium group is 8.1 and the intrinsic standard enthalpy change is −12.8 kcal/mol, as has been done for Table VIII, the intrinsic free-energy and entropy changes for the protonation process are −11.1 kcal/mol and −6 eu/mol, respectively. Then the enthalpy and entropy changes when the protonated α-ammonium group and the negatively charged carboxylate group form a buried ion pair are estimated to be −15.3 kcal/mol and −43 eu/mol.

The values of the thermodynamic functions which may be associated with conformational rearrangements in each distinguishable step as based on the present interpretation and as estimated from the present results are summarized in the following diagram in which those steps marked c appear to fall into this category.



Significance of the Results and Interpretation. Several of the substate transitions listed above have ratios $\Delta H°/\Delta S°$ equal to about 300°K. Although this situation may be accidental in any or all of the processes for which it is true, this value of the ratio is also associated with a phenomenological pattern of behavior manifested by a number of small and large solute processes in water and is currently attributed to the two-state behavior of liquid water.[25] The phenomenon and its possible significance are discussed by Lumry and Rajender.[25] The substate transitions of α-CT have been discussed in this connection elsewhere.[27,30] It now appears quite possible that the transition $A_bH_2 \rightleftharpoons A_fH_2$ involves changes in liquid water about the protein as much or more than it involves the protein.

Although the interpretation of the relaxation data given in this and the previous paper satisfies the quantitative requirements of the data very well and is the simplest hypothetical mechanism for this purpose we have found, it is not unique and considerable work remains to be done before the interpretation can be accepted with confidence.

The fluorescence data require that there be several substates of α-CT in the neutral to alkaline pH range. It is possible to rationalize several of these on the basis of the ability of inhibitors to block the removal of the carboxylate group of Asp-194 from its internal position in the protein. Under these circumstances it is improbable that its ion-pair partner from Ile-16 can be removed and deprotonated unless the pH is increased considerably above the effective pK_a of this group in the free enzyme, presumably 9.1 at 25°. If this restriction limits both fluorescence change and changes in circular dichroism and optical rotatory dispersion which occur with pH variation in the free protein, a relatively simple

picture satisfies many of the data of the several types. Parker[11] and Strand, Lumry, and Pool[40] find that acylation of the hydroxyl group of Ser-195 even with small acyl groups, *e.g.*, acetyl and carbamyl, eliminates the pH-dependent ORD and CD changes as might be expected if these changes are dependent on the integrity of the Asp–Ile ion pair. On the other hand the existence of a facile system of substates may be a characteristic of the catalytically active protein. CGN has substates only at extremes of pH and these substates may be due to partial unfolding. CGN has the "charge-relay" system of Blow, Berktoft, and Hartley[41] consisting of the Asp-102 carboxylate, the imidazole group of His-57, and the Ser-195 hydroxy group, and furthermore has these groups arranged in substantially the same geometric way as does α-CT, yet CGN has no catalytic activity.

Appendix

1. Theoretical Analysis of the Mechanism of Inhibitor Binding to the Chymotrypsins. Simple organic molecules with parts resembling the side chains of good substrates form association complexes rather than primary bonded "acyl" products as long as the experimental pH allows only very small fractions of free acid and free amino forms. The binding of hydrocinnamate ion is typical in this respect as has been shown by several studies[14,15,42–44] and by this report. According to these studies a mechanism explaining the fluorescence behavior of α-chymotrypsin when hydrocinnamate ion is bound must be consistent with the following observations. (1) One inhibitor molecule is bound by one enzyme molecule (if more inhibitor molecules are bound, they do not influence the fluorescence behavior). (2) There is a small and relatively slow increase in fluorescence intensity following the initial, rapid, second-order binding step. The first-order rate of this process is independent of the initial concentrations of the enzyme and the inhibitor. (3) The apparent first-order rate constant decreases with increasing pH in a manner which closely resembles a sigmoid titration curve involving only one ionizable group. (4) The results of the steady-state kinetic experiments with hydrocinnamate ion as a competitive inhibitor[42] and of temperature-jump experiments[16,17] indicate that the binding equilibrium constant for the system of hydrocinnamate ion and α-CT decreases at a constant temperature with increasing pH value of the system. (5) Calorimetry experiments[15] and temperature-jump experiments[16] show that the binding enthalpy, $\Delta H_b°$, and the binding entropy, $\Delta S_b°$, become more negative as the pH is increased from 7 to 8.

An enzyme–inhibitor binding reaction can be expressed in the simplest form as

$$E + I \rightleftharpoons EI \qquad \text{(A-1)}$$

The first-order behavior of the fluorescence changes requires an additional step of the form $E \rightleftharpoons E'$ or $EI \rightleftharpoons EI'$. Since the rate constant of the fluorescence change is independent of enzyme and inhibitor concentrations, the change of α-CT must take place according to eq A-2 or A-3 with the steps $EI \rightleftharpoons EI'$ or $E \rightleftharpoons E'$ rate limiting, respectively, in which case the relaxation may be expressed as

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI' \qquad \text{(A-2)}$$

or

$$EI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E + I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E' + I \qquad \text{(A-3)}$$

and the relaxation constants are

$$\lambda_1 = k_{-2} + \frac{k_2}{1 + \dfrac{k_{-1}}{k_1[I]}} \qquad \text{(A-2a)}$$

$$\lambda_2 = k_2 + \frac{k_{-2}}{1 + \dfrac{k_{-1}}{k_1[I]}} \qquad \text{(A-2b)}$$

or

$$\lambda_1 = k_2 + \frac{k_{-2}}{1 + \dfrac{k_1[I]}{k_{-1}}} \qquad \text{(A-3a)}$$

$$\lambda_2 = k_{-2} + k_2\left(1 + \frac{k_1[I]}{k_{-1}}\right) \qquad \text{(A-3b)}$$

When the inhibitor concentration is high, *i.e.*, such that $k_{-1}/k_1[I] \ll 1$, eq A-2a and A-2b reduce to

$$\lambda_1 = \lambda_2 = k_2 + k_{-2} \qquad \text{(A-2c)}$$

while eq A-3a becomes

$$\lambda_1 = k_2 \qquad \text{(A-3c)}$$

Only eq A-3b remains dependent on [I]. Let us now examine the situations in which the condition $k_{-1}/k_1[I] \ll 1$ is valid. We may recognize $k_{-1}/k_1 = K_i$, where K_i is a dissociation constant for the initial enzyme–inhibitor complex formation in eq A-2. However, we cannot compare an apparent dissociation constant, K_i', determined for a competitive inhibitor of α-CT using steady-state kinetics and a good ester substrate with K_i since steady-state data were analyzed as though there were only one inhibitor-bound enzyme species (eq A-2) or one free enzyme species (eq A-3). Hence, the apparent dissociation constant K_i' is related to the true dissociation constant K_i by the relationship, $1/K_i' = 1/K_i(1 + k_2/k_{-2})$. Without knowledge of k_2/k_{-2} one cannot estimate the value of K_i from the observed K_i' value unless there is reason to believe that $k_2/k_{-2} \ll 1$ and therefore $K_i' = K_i$.

It is probable that the value of K_i varies with inhibitor type at a given pH value. Havsteen[45] finds for the binding of proflavin a competitive inhibitor to α-CT, $K_i = 2 \pm 10^{-5}\ M$ at 12° and pH values of 8.4 and 9.2. From the temperature-jump studies of indole binding to α-CT Yapel[16] has estimated $K_i = 5.5 \pm 10^{-4}\ M$ at 25°. If we were to assume $K_i = 6.0 \pm 10^{-4}\ M$ at 12° as an approximation for the inhibitors studied here, the condition $6 \pm 10^{-4}\ M \ll [I]$ would have to be

(40) M. Strand, G. Pool, and R. Lumry, unpublished observations from this laboratory.
(41) D. Blow, J. Berktoft, and B. Hartley, *Nature (London)*, 221, 337 (1969).
(42) H. Neurath and J. A. Gladner, *J. Biol. Chem.*, 188, 407 (1951).
(43) H. T. Huang and C. Niemann, *J. Amer. Chem. Soc.*, 74, 5963 (1952).
(44) R. J. Foster and C. Niemann, *ibid.*, 77, 3370 (1955).
(45) B. H. Havsteen, *J. Biol. Chem.*, 242, 769 (1967).

satisfied in order for eq A-2c and A-3c to be valid. Since no reliable K_i values for the inhibitors studied in this work are available, it was necessary to establish experimentally for each inhibitor whether or not the condition $k_{-1}/k_1[I] \ll 1$ is met in the concentration range used. This was done by observing that there was no dependence of the relaxation constant on [I] over the range of inhibitor concentration. In the subsequent part of this section the same reasoning and procedure has been employed whenever the condition $k_{-1}/k_1[I] \ll 1$ is in question.

Equations A-2c and A-3c represent the relaxation times as independent of [E] and [I]. However, these equations do not describe the pH-dependent change of the observed relaxation constant as indicated in condition 3. A step which is pH dependent must be coupled to the "fluorescence change" in the mechanisms A-2 or A-3.

Several mechanisms can be developed from eq A-3 in which the rate-determining step is the equilibration of the two free-enzyme species, E and E'. Only two representative mechanisms need be examined here. The

$$E' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E + H^+ \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EH + I \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EHI \qquad \text{(A-4)}$$

$$E' + H^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EH + I \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EHI \qquad \text{(A-5)}$$

expressions for the first-order relaxation constants for the mechanisms A-4 and A-5 (with the enzyme isomerization steps, $E' \rightleftharpoons E$ and $EH' \rightleftharpoons EH$, as the rate-limiting steps) can be written, respectively, as

$$\lambda = k_1 + \frac{k_{-1}}{\left(1 + \frac{k_2 a_H}{k_{-2}}\right)\left(1 + \frac{k_3[I]}{k_{-3}}\right)} \qquad \text{(A-4a)}$$

and

$$\lambda = \frac{k_{-2}}{1 + \frac{k_3[I]}{k_{-3}}} + \frac{k_2}{1 + \frac{k_{-1}}{k_1 a_H}} \qquad \text{(A-5a)}$$

These expressions at high inhibitor concentrations such that $k_{-3}/k_3[I] \ll 1$, reduce to the following forms

$$\lambda = k_1 + \frac{k_{-1}}{1 + \frac{k_2 a_H}{k_{-2}}\frac{k_3[I]}{k_{-3}}} \qquad \text{(A-4b)}$$

$$\lambda = \frac{k_2}{1 + \frac{k_{-1}}{k_1 a_H}} \qquad \text{(A-5b)}$$

According to eq A-4b the observed relaxation constant should be dependent on inhibitor concentration, which does not satisfy condition 2. Equation A-5b indicates that the relaxation constant change is first order with respect to hydrogen ion activity in a way similar to the requirement of condition 3. However, eq A-5b also specifies that at low hydrogen ion activity the relaxation constant approaches zero. Since nonzero values have been observed at this condition experimentally, the solution is not compatible with our results. It is thus necessary to build on eq A-2.

It will be shown that the following two mechanisms, eq A-6 and A-7, which are the expanded forms of eq A-2 are the simplest consistent with available observations.

$$E + I + H^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI + H^+ \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EHI \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} [EHI]' \qquad \text{(A-6)}$$

$$EH + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EHI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [EHI]' \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EI + H^+ \qquad \text{(A-7)}$$

One thing that these equations have in common is that the change in the enzyme–inhibitor complex directly responsible for the fluorescence behavior occurs with the protonated enzyme species, EHI, and not with the unprotonated species, EI. This restriction on the mechanisms is required by the experimentally observed pH dependence of the observed rate-limiting step.

One needs to examine only eq A-6 closely, because it can be readily shown that the same reasoning also holds for eq A-7 and, in fact, our kinetic evidence does not allow a distinction between the two mechanisms. The three expressions for the observable relaxation constants for eq A-6 may be written as

$$\lambda_1 = k_1[I] + k_{-1}k_{-2}k_{-3} \qquad \text{(A-6a)}$$

$$\lambda_2 = \frac{k_{-2}}{1 + k_3/k_{-3}} + \frac{k_2 a_H}{1 + k_{-1}/k_1[I]} \qquad \text{(A-6b)}$$

$$\lambda_3 = k_{-3} + \frac{k_3}{(1 + k_{-2}/k_2 a_H)(1 + k_{-1}/k_1[I])} \qquad \text{(A-6c)}$$

(see ref 46 for the calculation of relaxation constants). On the basis of the assumption that $k_{-1}/k_1[I] \ll 1$ at high inhibitor concentrations these expressions reduce to eq A-6a', A-6b', and A-6c'

$$\lambda_1 = k_1[I] + k_{-1}k_{-2}k_{-3} \qquad \text{(A-6a')}$$

$$\lambda_2 = \frac{k_{-2}}{1 + k_3/k_{-3}} + k_2 a_H \qquad \text{(A-6b')}$$

$$\lambda_3 = k_{-3} + \frac{k_3}{1 + k_{-2}/k_2 a_H} = k_{-3} + \frac{k_3}{1 + K_a/a_H} \qquad \text{(A-6c')}$$

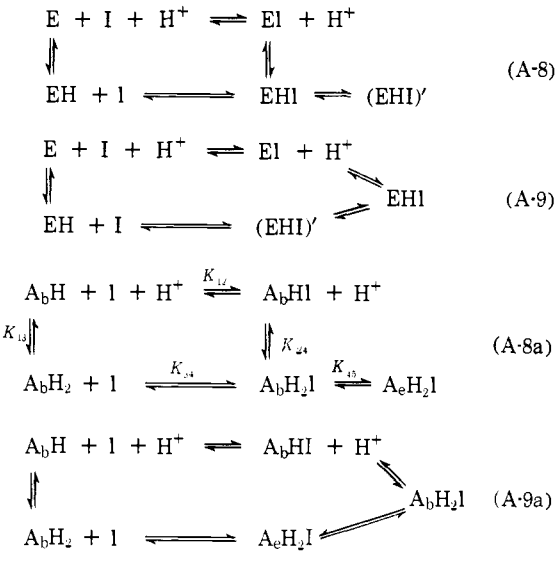
$$\frac{k_{-2}}{k_2} = K_a = \text{ionization constant}$$

λ_1 (eq A-6a') is dominated by $k_1[I]$ and k_{-1} of the initial inhibitor-binding process and λ_2 (eq A-6b') by $k_2 a_H$ and k_{-2} of the ionization of a group in the protein. Hence, λ_1 and λ_2 are expected to be very large compared to λ_3 which representes the slowly equilibrating process of the protein responsible for the fluorescence change. It is apparent also that only eq A-6c' shows pH behavior consistent with condition 3.

Two expanded mechanisms may be postulated on the basis of the present analysis. These are eq A-8 and A-9 and, taking into account the process $A_bH_2 \rightleftharpoons A_c + 2H^+$ previously established, these may be rewritten in conventional substate notation as eq A-8a and A-9a.

II. Binding Constant and Binding Enthalpy as a Function of pH. Referring to the mechanism of eq A-8a, we may write the following relations with respect to the concentration of each species at equilibrium: $K_{12} = [A_bHI]/[A_bH][I]$, $K_{13} = [A_bH_2]/[A_bH]a_H$, $k_{34} =$

(46) G. G. Hammes and P. R. Schimmel, *J. Phys. Chem.*, 70, 2319 (1966).



(A-8)

(A-9)

(A-8a)

(A-9a)

$[A_bH_2I]/[A_bH_2][I]$, $K_{24} = [A_bH_2I]/[A_bHI]a_H$, and $K_{45} = [A_eH_2I]/[A_bH_2I]$. The expression for the overall binding constant for the mechanism A-8a is eq A-8b. Since

$$K_b = \frac{[A_bHI] + [A_bH_2I] + [A_eH_2I]}{([A_bH] + [A_bH_2])[I]} = \frac{\frac{[A_bHI]}{[A_bH][I]} + \frac{[A_bH_2I]}{[A_bH][I]} + \frac{[A_eH_2I]}{[A_bH][I]}}{1 + \frac{[A_bH_2]}{[A_bH]}}$$

$$K_b = \frac{K_{12} + K_{13}K_{34}a_H + K_{13}K_{34}K_{45}a_H}{1 + K_{13}a_H} = \frac{K_{12} + K_{13}K_{34}(1 + K_{45})a_H}{1 + K_{13}a_H} \quad \text{(A-8b)}$$

that equation contains a number of equilibrium parameters whose values are unknown, it is useful to examine some limiting cases

$$\lim_{a_H \to \infty} K_b = K_{34}(1 + K_{45}) \text{ at low pH}$$

$$\lim_{a_H \to 0} K_b = K_{12} \text{ at high pH}$$

As an approximation we assume that $K_{34} \simeq K_{12}$. Since $K_{45} > 0$, we find that K_b (at low pH) $> K_b$ (at high pH).

The expression for the enthalpy change in the overall binding process can be obtained from eq A-8b by differentiating ln K_b with respect to $1/T$.

$$\Delta H_b^\circ = \frac{K_{12}\Delta H^\circ_{12} + K_{13}K_{34}(1 + K_{45}) \times (\Delta H^\circ_{34} + \Delta H^\circ_{13})a_H + K_{13}K_{34}K_{45}\Delta H^\circ_{45}a_H}{K_{12} + K_{13}K_{34}(1 + K_{45})a_H} - \frac{K_{13}a_H}{1 + K_{13}a_H}\Delta H^\circ_{13} \quad \text{(A-8c)}$$

Similar mathematical manipulations may be performed for the mechanism A-9a. The expressions for the binding constant and the binding enthalpy derived according to this scheme look very similar to those obtained for the mechanism A-8a.

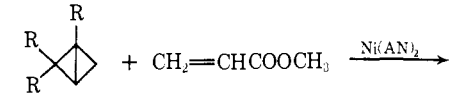
Communications to the Editor

Nickel(0)-Catalyzed Reaction of Bicyclo[1.1.0]butanes with Olefins[1]

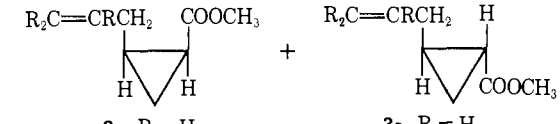
Sir:

As the chemical consequence of the extraordinarily high strain energy of bicyclo[*n*.1.0]alkanes (*n* = 1 and 2), a variety of reactions characteristic of the bicyclic systems are expected.[2] This paper describes a novel reaction of bicyclo[1.1.0]butanes and olefins with the aid of transition-metal catalysis.

When a mixture of bicyclo[1.1.0]butane (**1a**)[3] (5.2 mmol) and methyl acrylate (50 mmol) containing a catalytic amount of bis(acrylonitrile)nickel(0) (Ni(AN)$_2$)[4] (0.15 mmol) was heated at 60° for 36 hr under a nitrogen atmosphere, two isomeric adducts, *cis*- and *trans*-1-allyl-2-carbomethoxycyclopropanes (**2a** and **3a**, 65:35 ratio),[5] were obtained in almost quantitative yield. The spectral data were in accord with the assigned structures: **2a**, ir (CCl_4) 1735 (C=O), 1645,

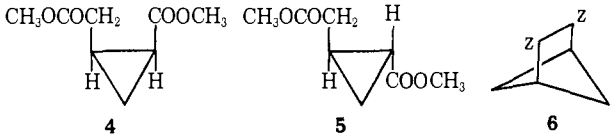


1a, R = H
1b, R = CH_3



2a, R = H
2b, R = CH_3

3a, R = H
3b, R = CH_3



4 **5** **6**

994, and 915 cm^{-1} (CH_2=CH); nmr (CCl_4, TMS) δ 0.7–1.9 (m, 4 H, >CH, >CH_2, and C*H*COOCH_3), 2.1–2.4 (m, 2 H, =CHCH_2), 3.59 (s, 3 H, OCH_3), 4.7–5.2 (m, 2 H, CH_2=CH), and 5.4–6.1 (m, 1 H, CH_2=C*H*);

(1) Nickel-Catalyzed Reactions Involving Strained σ Bonds. II. Part I: R. Noyori, T. Odagi, and H. Takaya, *J. Amer. Chem. Soc.*, **92**, 5780 (1970).
(2) Review: K. B. Wiberg, *Advan. Alicycl. Chem.*, **2**, 185 (1968).
(3) K. B. Wiberg, G. M. Lampman, R. P. Ciula, D. S. Connor, P. Schertler, and J. Lavanish, *Tetrahedron*, **21**, 2749 (1965).
(4) G. N. Schrauzer, *Chem. Ber.*, **94**, 642 (1961).
(5) All new compounds gave correct elemental analyses and/or molecular peaks in exact mass spectra. We thank Professor A. Tatematsu, Meijo University, for measurement of the spectra. Ir and nmr spectra were consistent with the structures assigned.