5. For amine general base catalyzed attack of amine the transition state structures of **7**, **8**, and **12** are in accord with out present knowledge. Structures **7** and **12** differ only as to the delocalization of partial negative charge to carbonyl or phenol oxygens. Structure **8** involves a cyclic proton transfer. For the amine conjugate acid general acid catalyzed attack of amine, reasonably concerted or cyclic structures involving proton transfer are not apparent and the lone transition state structure of **13** is suggested.

These considerations apply to water as a solvent. In a poor ion supporting solvent it is to be anticipated that the necessity of internal stabilization of charges will result in cyclic transition state structures. The process associated with k_{ga} has not been seen in such solvents.[10,32,33,35]

Acknowledgments. This work was supported by a grant from the National Institutes of Health.

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Studies of the Chymotrypsinogen Family of Proteins. IX. Steady-State Kinetics of the Chymotryptic Hydrolysis of N-Acetyl-L-tryptophan Ethyl Ester at pH 8.0[1a]

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Abstract: Thermodynamic changes for significant steps in the reaction sequence of the chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester have been determined at pH 8.0 as a function of temperature and ethanol concentration. The reaction follows simple catenary chain kinetics up to 1.5 *M* ethanol. The reaction profile is symmetric about the acyl enzyme at all temperatures. There is no unique rate-limiting step. For ethanol and water standard states of 1 *M* and unit activity, respectively, the phenomenological rate parameters α_0 and β_0 (alcohol independent) and α_p and β_p (alcohol dependent) have the following values at 35° and the following activation enthalpies and entropies in order (α_0, 4.7×10^5 M^{-1} sec^{-1}) (β_0, 78 sec^{-1}) (α_p, 5.0×10^5 M^{-1} sec^{-1}) (β_p, 250 sec^{-1}). There are three "metastable" intermediates which can be distinguished: two Michaelis–Menten complexes ES and EP_2H and an acylenzyme EA with the following standard enthalpies and entropies of formation (ES, −9.9 kcal, −17 eu) (EP_2H, −8.6 kcal, −14 eu) (EA, −12.3 kcal, −26 eu). A cooperative protein transition centered at pH 8 and 25° is detectable in α_0 and α_p and the two substates have different catalytic parameters. EA as well as ES and EP_2H appear to belong to the class of enzyme–inhibitor complexes which manifest a common pattern of linear compensation of enthalpy change by entropy change. Judging by the number of other cases in which the same pattern is found, these results imply that water plays a direct role in the catalytic process.

As a foundation for a study of the transient-phase kinetics of chymotryptic catalysis, a grid of the responses of the steady-state parameters for the variation of a minimum set of independent variables is necessary. No comprehensive study of the steady-state kinetics has been reported thus far. In particular there has been little study of the temperature dependence of the kinetic parameters for chymotryptic catalysis. In addition, the finding by Kim and Lumry[2a,b] that α-chymotrypsin near pH 8.0 and 25° exists in roughly equal proportions of two substates, A_b and A_f, necessitates a reexamination of the catalysis kinetics in this pH and temperature region in order to determine if the transition between these two substates manifests itself in any of the rate parameters. A reexamination of the hydrolysis kinetics of N-acetyl-L-tryptophan ethyl ester has also become necessary to eliminate uncertainties associated with a group of contaminants frequently found in commercial preparations of α-chymotrypsin[3] not treated by the simple purification procedures of Yapel, *et al.*[3] It is important to emphasize the finding of these authors[3] that strong acylating agents[4] used as the "active site" reagents displace the contaminants and thus give spurious assurances of purity. More reliable tests for such purity are provided by Yapel, *et al.*,[3] but the simplest test still appears to be the determination of reaction velocity constants under a fixed set of "standard conditions." We report here a study of the rates of chymotryptic hydrolysis of N-acetyl-L-tryptophan ethyl ester as a function of temperature and ethanol concentration at a fixed pH of 8.0. At this pH, the rate parameters are nearly pH independent thus facilitating their analysis in terms of elementary rate constants or simple combinations of these. The tentative analysis of the rate parameters is based on the work of Bender[5–7] and Wilson[8,9] and

(1) This is publication No. LBC 42. Please request reprint by number. Supported by the National Science Foundation, Grant No. GB-7896, and the National Institutes of Health through Grant No. AM-07853-07.

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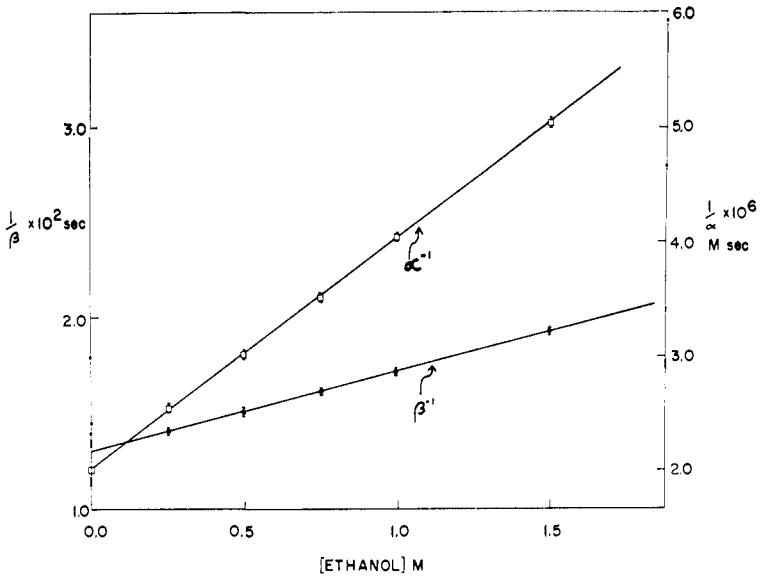

Figure 1. Hydrolysis of N-Ac-L-Tryp EE at 35° and pH 8.0 as a function of ethanol concentr ation: $\frac{1}{\alpha} \times 10^6 M$ sec; $\frac{1}{\beta} \times 10^2$ sec.

their coworkers. The results supplement the finding of these investigators, and in particular, provide additional detail to the studies of the chymotryptic hydrolysis of N-acetyl-L-tryptophan ethyl ester carried out by Bender, *et al.*[5-7]

$$-\frac{dS}{dt} = v = \frac{eS_0}{\frac{1}{\alpha_0 + \alpha_p[P_1]_0} + \frac{S_0}{\beta_0 + \beta_p[P_1]_0}} \quad (1)$$

Figure 4, demonstrate a small but significant curvature.

Table I. Hydrolysis of N-Acetyl-L-tryptophan Ethyl Ester.[a] Rate Parameters and Activation Free Energies

	Temp, °C					
	5	15	25	35	42	50
β_0 (sec^{-1})	12 ± 2	24 ± 2	47 ± 1	78 ± 1	105 ± 1	180 ± 1
$\Delta F^{\pm}(\beta_0 \pm 0.2)$ (kcal/mol)	14.9	15.0	15.0	15.3	15.4	15.6
α_0 (l. mol^{-1} sec^{-1}) × 10^{-5}	5.03 ± 0.3	4.9 ± 0.3	4.7 ± 0.2	4.7 ± 0.2	5.3 ± 0.2	5.9 ± 0.2
$\Delta F^{\pm}(\alpha_0 \pm 0.3)$	9.0	9.3	9.8	10.0	10.2	10.5
β_p (sec^{-1})	60 ± 20	100 ± 15	170 ± 10	250 ± 10	330 ± 10	480 ± 10
$\Delta F^{\pm}\beta_p$ (kcal/mol)	14.0 ± 0.4	14.2 ± 0.4	14.7 ± 0.3	14.9 ± 0.3	14.8 ± 0.3	14.9 ± 0.3
α_p (l. mol^{-1} sec^{-1}) × 10^{-5}	4.9 ± 0.5	4.6 ± 0.5	4.5 ± 0.5	5.0 ± 0.4	5.5 ± 0.4	6.3 ± 0.4
$\Delta F^{\pm}(\alpha_p \pm 0.4)$ (kcal/mol)	9.1	9.4	9.7	10.2	10.1	10.4
$(K_s \pm 0.6) \times 10^4$ (M)	1.3	2.1	3.9	5.2	5.9	7.2
$\Delta F°(K_s \pm 0.5)$ (kcal/mol)	5.0	4.9	4.8	4.7	4.6	4.5

[a] At pH 8.0, 0.2 M KCl, 10^{-4} M Tris.

Results

Dependence of the Hydrolysis on Alcohol and Substrate Concentration. At all temperatures, the velocity of the hydrolysis of N-acetyl-L-tryptophan ethyl ester followed the conventional catenary chain rate law given by eq 1. Figure 1 demonstrates an adherence to this rate law at 35°. The errors shown are two standard deviations estimated by computer analysis of the data. The fitting parameters are thus α_0, α_p, β_0, and β_p. Arrhenius plots for these quantities are shown in Figures 2 and 3 and the values of the parameters obtained at 35° together with apparent enthalpy and entropy of activation corresponding to each parameter are given in Tables I and II. The standard state for water is unit mole-fraction activity and that for ethanol is 1 M. Arrhenius plots for α_0 and α_p as shown in

Table II. Activation Parameters for the Chymotryptic Hydrolysis of N-Acetyl-L-Tryptophan Ethyl Ester at pH 8.0 and 35°

	$\Delta F^{\pm}$ (kcal/mol)	$\Delta H^{\pm}$ (kcal/mol)	$\Delta S^{\pm}$ (eu/mol)
α_0[a]	9.9 ± 0.3	−1.1 ± 1.0	−36 ± 3
α_p	10.2 ± 0.3	−1.3 ± 1.0	−39 ± 3
β_0[a]	15.2 ± 0.2	12.3 + 0.5	−10 ± 2
β_p	15.0 ± 0.2	8.6 ± 0.5	−24 ± 2

[a] These values have been determined independently by two investigators at different times and show very good agreement between the two separate sets of experiments, so that the level of confidence in these parameters is high. Errors reported are computed standard deviations.

Kaplan and Laidler[10] have found abrupt changes in slopes for the Arrhenius plots of β_0 obtained at zero first-product concentration (*i.e.*, with no added alcohol) for the hydrolysis by chymotrypsin of N-benzoyl-L- and D-alanine methyl esters. Martin[11] has found a similar slope change in the Arrhenius plot of β_0 with

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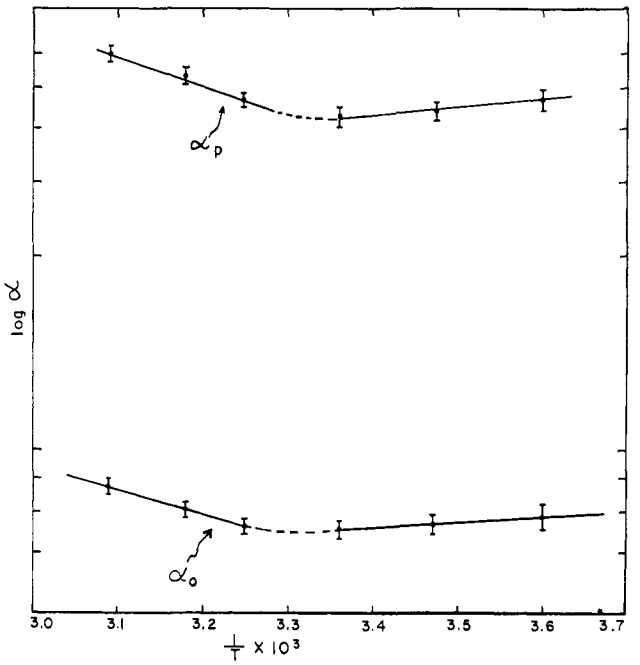

Figure 2. Arrhenius plot for α_0 and α_p for the hydrolysis of N-Ac-L-Tryp EE at pH 8.0.

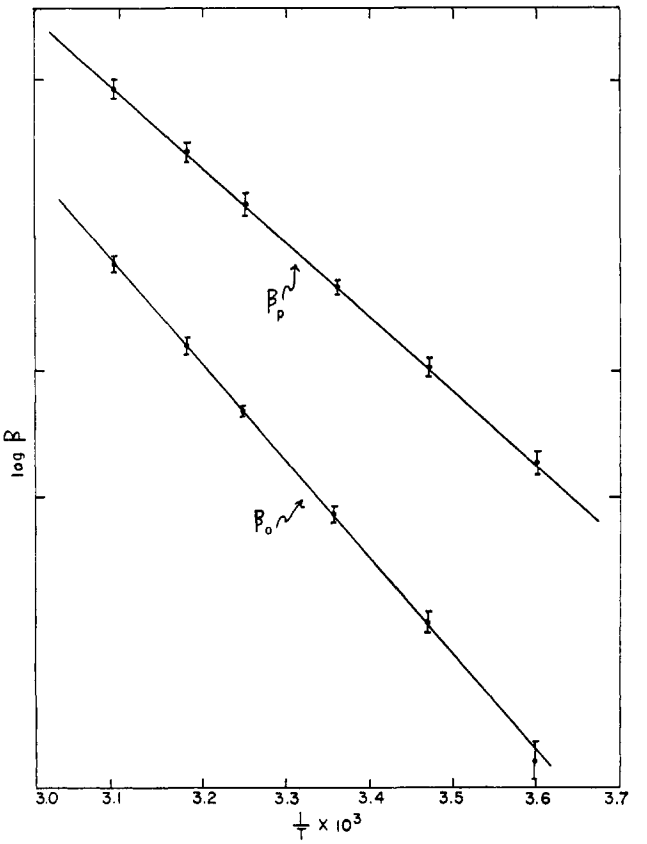

Figure 3. β_0 and β_p *vs.* $1/T$ for the hydrolysis by α-CT of N-Ac-L-Tryp EE at pH 8.0.

N-acetyl-L-tyrosine ethyl ester as substrate, also under conditions of zero first-product concentration. Kim[2a] in his characterization of the substates of the best folded state of α-CT, called state A, found a two-state transition with the half-transition temperature of 25° at pH 8.0. Although the very high experimental precision required to determine accurate values of enthalpy and entropy change in this transition $A_b \rightarrow A_f$ indirectly through catalytic rate studies is not now obtainable, Kim studying the interchange among the substates using fluorescence has roughly estimated the values of the standard enthalpy and entropy of the transition as 48 kcal and 163 eu, respectively.[2a,12] The transition is not unimportant and one object of this study was to determine whether or not the transition would influence the experimental rate parameters with the tryptophan substrate. It is seen in Figure 2 that the transition does influence α_0 and α_p, but that the enthalpies of activation above and below the transition temperature of 25°, as estimated by the average slopes on either side of this temperature, differ by only about 4 kcal. It can also be concluded that both the lower temperature substate, A_b, and the higher temperature substate, A_f, are catalytically active.

Since it is not possible to determine the true values of the slopes on either side of the midpoint of the Arrhenius plot, we have drawn lines through the experimental points in such a way that the curve could be represented by two straight lines. The values of $\Delta H^{\pm}$ and $\Delta S^{\pm}$ for α_0 and α_p are thus estimates of the true values though the error cannot be large. Difficulties involved in the analysis of van't Hoff and Arrhenius plots with abrupt slope changes have been discussed in detail elsewhere.[13,14]

Formal Analysis of Rate Parameters. Studies by Bernhard and Gutfreund[15,16] and by Himoe and Hess[17] suggest that for a chemically adequate description of the hydrolysis of ester substrates by chymotrypsin, a number of metastable intermediates of enzyme–substrate and enzyme–product will be required. In addition, Bender and coworkers[5-7,18] have established the existence of at least one intermediate compound formed by primary bond attachment of the acid moiety of the substrate to the protein. This "acyl–enzyme" intermediate proposed by Wilson[19] will be labeled EA. The validity of the Hartley–Kilby[20] reaction scheme for chymotryptic hydrolysis of simple ester substrates in addition to the adherence of our kinetics measurements to a conventional catenary chain mechanism (of which the Hartley–Kilby scheme is a specified case) provides the basis for the analysis. The limited number of independent variables available in this study makes it impossible to recognize more than three intermediate states and four activated complexes. We are thus restricted to the mechanism shown in eq 2, which is

$$E + S + 2H_2O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES + 2H_2O \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EA + P_1 + H_2O \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_2H + P_1 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P_2H + P_1 \quad (2)$$

(12) Recent calorimetric studies show that the correct values are considerably larger than the estimates of Kim: S. Rajender, unpublished observations from this laboratory.

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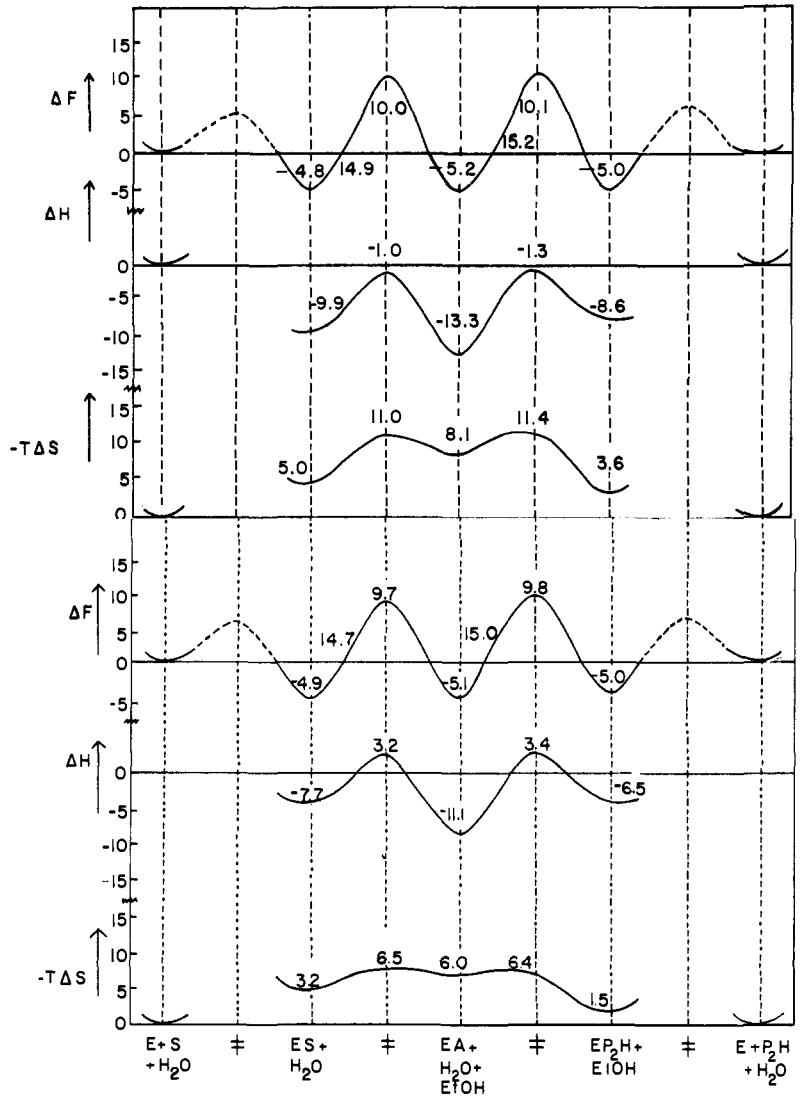

Figure 4. Standard free energy, enthalpy, and entropy *vs.* reaction coordinate profile for the hydrolysis by α-CT of N-Ac-L-Tryp EE at pH 8.0 and 35°: ‡, activated complexes; ES, EA, EP_2H, metastable intermediates.

sufficient to accommodate the data now available. The analysis is essentially a routine application of assumptions based on experimental evidence about rate-limiting steps and follows very closely the procedure of Bender, *et al.*,[5,6] P_1 = ethanol, P_2H = N acetyl-L-

$$\alpha_0 = (1/k_1 + k_{-1}/k_1k_2)^{-1} \quad (3)$$

$$\alpha_p = (k_{-1}k_{-2}/k_1k_2k_3 + k_{-1}k_{-2}k_{-3}/k_1k_2k_3k_4)^{-1} \quad (4)$$

$$\beta_0 = (1/k_2 + 1/k_3 + 1/k_4 + k_{-3}/k_3k_4)^{-1} \quad (5)$$

$$\beta_p = (k_{-2}/k_2k_3 + k_{-2}k_{-3}/k_2k_3k_4)^{-1} \quad (6)$$

tryptophan in free acid form, E, ES, and EP_2H have the usual meaning. Transient phase kinetic results would require additional detail in this analysis, but the data already available indicate that any new detail that might appear for this substrate will require elaboration only in the rate constants k_1, k_{-1}, k_4, and k_{-4}. The data may be analyzed on the basis of the following set of assumptions which are consistent with the data available at the present time: (1) k_1 is no less than 10^6 l. M^{-1} sec^{-1};[21,22] (2) $k_4 \gg k_2$ (ref 7); (3) $k_4 \gg k_3$ (ref 7); (4) $k_{-3} \ll k_4$ (ref 7); and (5) $\Delta F^\circ \approx \Delta H^\circ \approx \Delta S^\circ = 0$ for the overall reaction $S + H_2O \rightarrow P_1 + P_2H$. Considering reported values for standard thermodynamic changes for ester hydrolysis (ester → un-ionized acid).[23-25] These assumptions (5) may lead to an error of ±1 kcal in ΔF°. A recent calorimetric determination of ΔH° for the hydrolysis of N-Ac-L-Tryp EE[26] to the free acid form gives a value of -0.6 ± 0.2 kcal which would give an estimate of 2 ± 4 eu for ΔS°.

On the basis of these considerations, the standard free energy, enthalpy and entropy changes along the formal reaction coordinate have been calculated (see Figure 4). Two sets of enthalpy and entropy values are given corresponding to the participation of the A_b and A_f states. These calculated data can be refined further when the precise ΔH° and ΔS° values for the $A_b \rightarrow A_f$ process become available.

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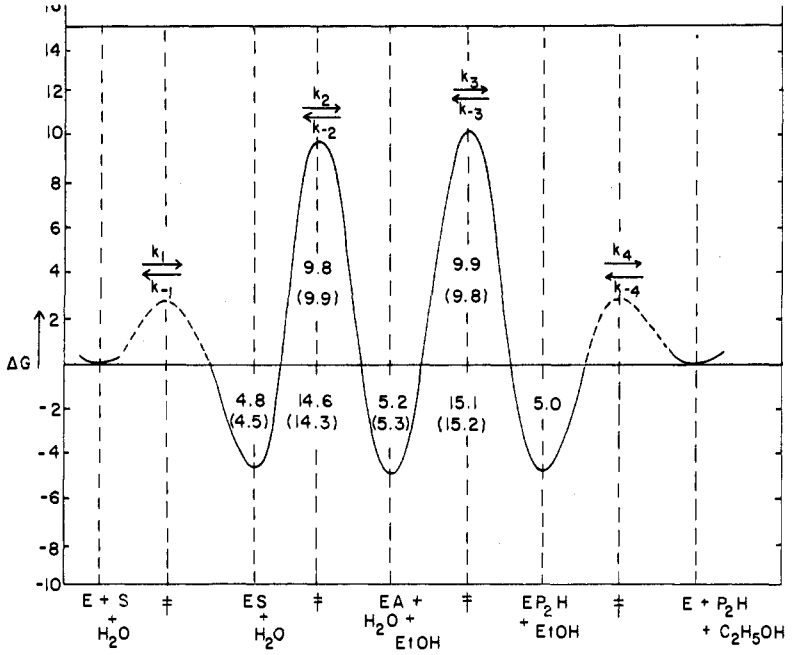

Figure 5. Standard free energy *vs.* reaction coordinate for the hydrolysis of N-Ac-L-Tryp EE by α-CT, at pH 8.0 and 25°. Numbers in parentheses are those recalculated from Bender, *et al.*[7]

The data of Bender, *et al.*,[4,5,27] obtained at pH 7.6 have been recalculated and refitted using statistical methods. The resulting free-energy values are given in parentheses in Figure 5. These authors did not report extensive temperature studies of the kinetics. Our own results agree qualitatively and, to a considerable extent, quantitatively with the recalculated data of Bender, *et al.*[5,6,27] Different solution conditions, presence of acetonitrile in their solution mixture, possible contaminants in the enzyme preparation, concentrations of buffer, etc., may also account for some of the disagreement. However, a factor of two discrepancies in the rate constants is negligible in terms of thermodynamic changes and the free-energy diagrams therefore, should agree within error, as indeed they do (see Figure 5). Differences in the free energy and entropy value are due primarily to different choices of the standard states for water (Bender, *et al.*,[4] used a 1 *M* standard state) and the differences in the assumed overall thermodynamic changes. Our value for $K_s = k_{-1}/k_1$ at 25° and pH 8.0 agrees very well with that reported by Himoe and Hess[28] measured at pH 6.0. K_s is essentially pH independent in this pH region between 5.5 and 8.5.

Discussion

The $A_b \rightarrow A_f$ Transition. It is interesting that this transition which may be due to rather large changes in the protein does not eliminate catalytic activity. In fact, its influence on catalytic function for our substrate does not appear to be large. There is an interesting and potentially very useful inconsistency in the way this transition appears in the rate parameters. As mentioned, it appears only in α_0 and α_p with N-ATEE and as a result can be readily shown to influence our "elementary-step" rate constants k_1, k_{-1}, or k_2. With the L and D forms of benzoylalanine methyl ester[10] and with N-acetyl-L-tyrosine ethyl ester,[11] it appears in β_0. It may also influence β_p for the latter substrates but information about this parameter is not yet available for these substrates. An examination of the formal expressions for the rate parameters given in eq 3–6 shows that a qualitatively different pattern of dominance is required if these observations are to be interpreted on the same formal framework. However, the analysis of kinetics data for N-acetyl-L-tyrosine ethyl ester given by Bender and coworkers[7,18] indicates that the slow steps in the chymotryptic hydrolysis of the two substrates are the same. The implication of this finding is that there is a qualitative difference between the chymotryptic hydrolysis mechanisms of the tryptophan and tyrosine substrates.

It is unfortunate that such a large fraction of the quantitative specificity studies for α-chymotrypsin have been carried out under experimental conditions where the concentrations of the A_b and A_f species are nearly equal, *i.e.*, at pH 8 and 25°.

Characteristics of the Reaction Profile. The symmetry of the reaction profile about the acyl enzyme state as shown in Figure 5 offers considerable support for the formal interpretation given above in the sense that this symmetry is a reasonable expectation on the basis of the assumed chemical mechanism. Although nearly exact symmetry is demonstrated in Figure 5 and this symmetry is not destroyed by changes in temperature, or, as will be shown in a subsequent paper, hydrogen-ion activity, exact symmetry is an accidental consequence of our use of ethanol as the added nucleophile and our choice of standard states. As a nucleophile, 1 *M* ethanol is kinetically equivalent to about 54 *M* water. A consequence of this equivalence is that at 1 *M* ethanol there is no unique rate-limiting step since the free-energy changes corresponding to the dominant terms k_{-1}/k_1k_2 and $k_{-1}k_{-2}(P_1)/k_1k_2k_3$ in $(\alpha_0^{-1} + \alpha_p^{-1})$ are nearly equal as are those corresponding to the

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dominant terms k_2^{-1}, k_3^{-1}, and $k_{-2}(P_1)/k_2k_3$ in $(\beta_0^{-1} + \beta_p^{-1})$. In such a situation, which may be very common, the analysis of the effects of inhibitors, activators, ionic strength, etc. may often be difficult since the system will shift continuously from multiple dominance to single-term dominance with changes in solution composition, addition of inhibitors, etc.

A literal identification of the peaks and valleys of Figure 5 with the chemical processes of eq 2 would allow us to conclude that one or both imidazole groups of the protein participate chemically only as parts of the activated complexes for "on-acylation" and "off-acylation" and that they enter these complexes in the unprotonated form. This conclusion would be consistent with current views that these groups function only as base catalysts as will be discussed in another place. However, additional investigation is required before we can exclude the possibility that the imidazole groups also function as conformation determinants.

Interesting features of the temperature dependence of α_0 and α_p are very low enthalpy of activation and the large negative entropy of activation (see Table II). These features are also characteristic of the kinetics parameters in all other temperature studies of chymotryptic catalysis with small ester and amide substrates which we have been able to find reported in the literature but these experiments were carried out in 20–30 per cent alcohol–water solvents in which inhibition, dielectric constant, and the direct influence of the cosolvent on the protein make interpretation ambiguous. The very small values of the enthalpies of formation of the activated complexes for on-acylation and off-acylation which we find are unlikely to require revision as it becomes possible to add stepwise detail to the catenary chain mechanism. These values are consistent with the idea that some of the negative free-energy change in forming the activated complexes from separated reactants is not released to heat but rather used to increase the free energy of the chemical system by mechanical and electrostatic distortions of geometry.[29] Already lysozyme appears to demonstrate just such an aspect of its total catalytic mechanism.[30,31] Lumry and Biltonen[32] have discussed the next stages in the elaboration of this type of enzymic process in some detail using data obtained from studies of the chymotrypsinogen-A family of proteins. They are forced to conclude that there is a catalytically important first-order process of the protein-plus-water system which occurs following the fast initial binding steps of substrates and of inhibitors resembling substrates. The extent of this process is very sensitive to geometry and chemical characteristics of these small molecules. Doherty and Vaslow[33] appear to have been the first to detect the process and demonstrate its sensitivity and Vaslow's identification[34] as a conformational process still appears to be sound. The process is related to the slow first-order changes in fluorescence described by Sturtevant.[35] Recently Yapel[36] and Kim[2a] have added considerable quantitative detail to the description of the process. For example, Yapel has shown that it varies greatly in rate and magnitude for the enantiomers N-acetyl-L-tryptophan and N-acetyl-D-tryptophan.[37] The "conformational" process is nevertheless qualitatively the same in the two binding reactions as is established by plotting the total standard enthalpy of binding for each *vs.* the respective total standard entropy of binding on what is known as a compensation plot.[32] Figure 6 is a typical compensation plot obtained by Yapel using a technique which measures the interaction between the imidazole group of HIS 57 and a passive pH-indicator molecule. In this example, in which we have given his results for N-acetyl-L-tryptophan and hydrocinnamate ion at a few pH values, it is seen that regardless of pH the $(\Delta H_b^\circ, \Delta S_b^\circ)$ points lie on a single straight line. For a given pH the points for different inhibitors are spread out along this line in a way which is qualitatively correlated with the specificity requirements of chymotrypsin. The half-times for the process vary from microseconds to minutes depending on inhibitor and pH.

It is of some interest to note that the pairs of values of the enthalpy and entropy of formation of ES, EA, and EP_2H from separated reactants fall on or near the compensation line of Figure 6. Unless this situation is coincidental, it must mean that ES, EA, and EP_2H are characteristic members of the family of molecules which can trigger the process in α-chymotrypsin responsible for the compensation behavior. Of the several classes of inhibitors for this protein, thus far all those found to produce the special compensation behavior have the side chain of a good substrate. Even an isolated indole side chain is effective. The observation that the acyl–enzyme species EA also falls into the same class as the other intermediates in catalysis and the inhibitors, none of which can be presumed to have primary bond between protein and small molecule, is especially noteworthy.

Lumry and Biltonen[32] have also implicated changes in the protein–water system to be responsible for compensation behavior in the formation of the on-acylation and off-acylation activated complexes.

It will be noticed in Figure 6 that the points for EP_2H and N-acetyl-L-tryptophan binding at pH 8 are well separated on the compensation line although the two chemical species differ only by the presence of a proton on EP_2H as set forth in the formal mechanism (eq 1). The separation may be attributable to the difference in charge, but studies of the binding of hydrocinnamate ion and hydrocinnamoyl alcohol by Kim[2a] suggest that the presence of the charge is relatively unimportant since although there are easily distinguished differences in the free energy of binding of the two molecules, the changes in ΔH_b^0 and ΔS_b^0 are small relative to the total values of these quantities. Hence, a different explanation for the separation between EP_2H and the protein complex with N-acetyl-L-tryptophan may be necessary. The probable explanation of the difference is that the species EP_2H and also ES are not the major equilibrium species formed be-

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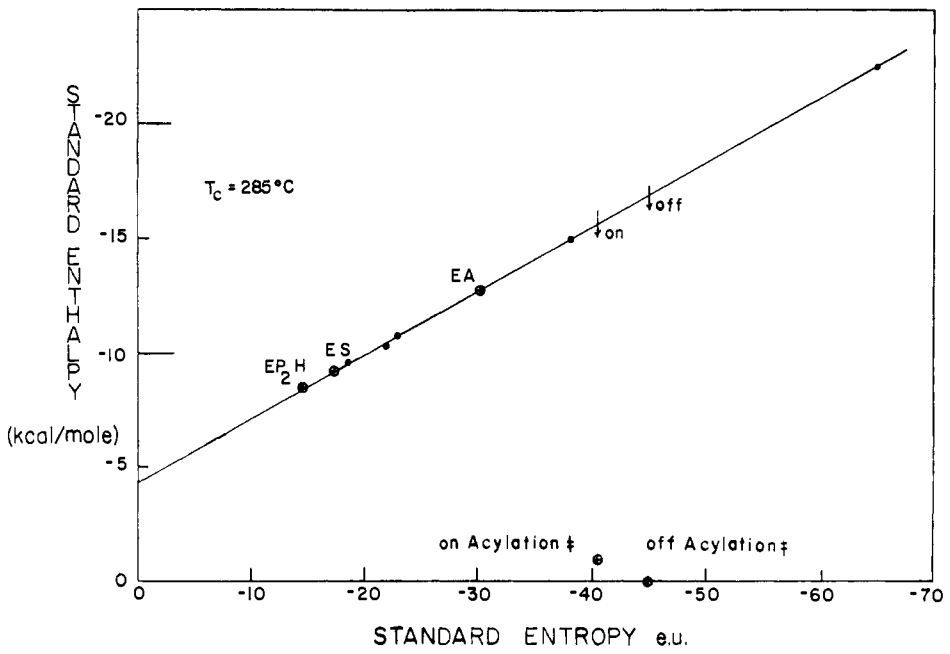

Figure 6. Compensation (ΔH° *vs.* ΔS°) plot for the binding of inhibitors to chymotrypsin: ●, enthalpy and entropy of binding of indole to α-CT; ⊕, standard enthalpies and entropies of formation of the metastable intermediates ES, EA, and EP_2H relative to separated reactants in the hydrolysis of N-Ac-L-Tryp EE, pH 8.0. Points for on- and off-acylation activated complexes are also shown on the plot.

tween chymotrypsin and the protonated form of N-acetyl-L-tryptophan and of N-acetyl-L-tryptophan ethyl ester, respectively. Relative to the true equilibrium species, ES and EP_2H are metastable though only slightly because of the existence of the compensation behavior discussed above. This does not mean that the chemical descriptions of the states are necessarily very similar but only that the free energy is about the same. The chemical differences are measured by their differences in enthalpy or entropy.

The somewhat more detailed description of the proposed mechanism is given in eq 7 (*cf.* eq 2) in which ES′ and EP_2H' are the true equilibrium species. Our data have been analyzed using eq 2 rather than eq 7. If the

$$\begin{array}{ccccccc} & & ES' & & EP_2H' & & \\ & & \downarrow\uparrow k_1 & & \downarrow\uparrow k_2 & & \\ E + S + H_2O & \rightleftharpoons & ES & \rightleftharpoons & EA & \rightleftharpoons & EP_2H \rightleftharpoons \\ & & +H_2O & & +P_1 & & +P_1 \\ & & & & +H_2O & & E + P_2H + P_1 \end{array} \quad (7)$$

steady-state concentration of the "dead-end" complex ES′ is significant relative to the total enzyme concentration, any errors introduced into the rate constants cancel in the α parameters but in β constants $k_3 \rightarrow k_3/(1 + K_1)$ and $k_4 \rightarrow k_4/(1 + K_2)$ so that an error of a factor of $(1 + K_1)$ or $(1 + K_2)$ is introduced in k_3 or k_4. However, our results would not be in error because of a failure to include EP_2H' since the experimental situation was such that reaction velocities could be calculated assuming zero concentration of P_2^- and thus of P_2H.

Lumry and Rajender[38] have examined the quantitative significance of the special compensation behavior in chymotryptic catalysis and conclude that it cannot be responsible for more than one order of magnitude in the on-acylation and off-acylation processes. Although the protein–water process appears to play an important role in determining the magnitude and sign of the enthalpy and entropy along the reaction coordinate, it does so in such a way that the entropy contribution very nearly cancels out the enthalpy contribution. At about 10° the cancellation is exact. Thus the thermodynamic changes associated with the special compensation process appear to indicate a change in the protein–water system which occurs as part of the catalytic process but which plays a major role in the efficiency of the process. The molecular basis for compensation behavior in this system is not yet known. Such behavior is observed in small solute–water solutions as well as in protein solutions and at the present time appears to be linked to changes in the volumes of the solutes during reaction.[38]

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Experimental Section

Materials. Three times crystallized α-chymotrypsin obtained from Worthington Biochemicals Corporation was purified by the method of Yapel, *et al.*[3] Total protein concentration was measured spectrophotometrically using a specific absorbance value at 280 nm of 2.00 g^{-1} cm^{-1} and "active site" concentrations estimated by the cinnamoyl-imidazole titration method.[4] More precise estimates of concentration were made by using the catalytic velocity at saturating substrate concentration under a standard set of conditions (k_{cat} = 47.0 sec^{-1} at pH 8.00, T = 25.00, salt, KCl = 0.2 M, Tris at 1 × 10^{-4} M and no added ethanol). The "active site" titration has an inherent error of −5 to −15% and is not sensitive to the contaminants found by Yapel, *et al.*,[3] which are displaced by cinnamoylimidazole. Concentrations and purity were also checked by the indole-blocking method of Yapel, *et al.*,[3] which serves as a satisfactory secondary standard for such determinations. The latter method and the "maximum-velocity" method are precise to ±1%, so that the two methods should and do agree by ±2%.

N-Acetyl-L-tryptophan ethyl ester was obtained from Mann and Cyclo Chemical Corporation and was used without further purification since identical results were obtained with all preparations from both sources. Tris was a Sigma Chemical Co. product (Trizma Base) and was further recrystallized from water. Sodium and potassium phosphates were Reagent grade from Mallinckrodt Chemical Works. Ethanol was a Fisher product, 95% U.S.P. grade.

Methods. All kinetic measurements were made with a locally built pH-stat assembly. The system was completely thermostated and the deviations in reported temperatures are no more than

±0.05°. An inert atmosphere in the reaction vessel was maintained with helium, which not only eliminates CO_2 interference in the titrations but also removes any complications produced by the direct reaction between CO_2 and chymotrypsin.[39] The total volume of the solution used in each experiment was 10 ml. A minimum of 25 combinations of substrate and alcohol concentrations (5 × 5) was employed in the measurement of initial velocities at each temperature. Ionic strength was maintained at a constant value of 0.2 with 2.0 *M* KCl. Titrations were performed with 0.01 *M* NaOH as the titrating agent. Tris-HCl or phosphate buffers at concentrations of 1.0×10^{-4} *M* were used in all experiments to stabilize the response of the measuring system. Fresh enzyme solutions were prepared each second day in 10^{-4} *M* HCl and stored at 4°. Enzyme concentrations were in the range 10^{-8}–10^{-6} *M* and were adjusted to give roughly identical initial velocities regardless of substrate concentration. Rates were measured from product-formation data taken at less than 10% of the total reactions. No spontaneous hydrolysis of the ester was detected under conditions of the experiments. Alcohol concentrations were varied between 0.2 and 2.0 *M*. Alcohol solutions were made up by volume at 25°. No corrections were made for small electrode errors in pH measurements in alcohol solutions. Initial slopes were obtained using a computer program fitting 30–40 experimental data points read from the product–time record from each titration experiment to a series expansion of the integrated Michaelis-Menten equation written with *P* as the independent variable. The first term of this series is the true velocity at $t = 0$, if the assumption of Michaelis-Menten kinetics is correct. All kinetic and thermodynamic parameters were evaluated using a computer program designed for an iterative, appropriately weighted least-squares analysis of the data according to eq 1, and the van't Hoff and Arrhenius equations.

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Azide Solid Phase Peptide Synthesis[1]

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Abstract: The stepwise synthesis of peptides by the initial attachment of an amino acid *t*-butyloxycarbonylhydrazide through its α-amino group to a polystyrene resin was investigated. The resin–amino acid azide was generated quantitatively and coupled with another amino acid *t*-butyloxycarbonylhydrazide. The peptide chain was elongated by further azide couplings. Finally, the C-terminal amino acid was added as a *t*-butyl ester. The peptide was deprotected and removed from the resin in one step with HBr. The feasibility of the approach was demonstrated by the synthesis of L-leucylglycine and L-leucyl-L-alanylglycyl-L-valine by this stepwise procedure and also by a fragment condensation. Studies on the stability and reactivity of the intermediate azides were carried out.

Modifications in the solid phase peptides synthesis procedure[3] have been directed toward the resin,[4] the coupling reaction,[4a,c,5] the N-protecting group,[4e,5e,6] and the cleavage step.[4d,5a,6a,7] In general the method has involved the attachment of the carboxyl group of the first amino acid to a resin, followed by peptide chain elongation at the amino end.[3] There has been one instance[8,9] in which an amino acid was attached to the resin through its amino group while the carboxyl was protected as an ethyl ester. However, the procedure described in those early experiments was somewhat limited because of the danger of racemization during the subsequent deprotection and coupling steps. In order to develop this general approach further it is necessary to find suitable carboxyl protection and efficient, racemization-free coupling methods. The acid azide route of classical peptide synthesis[10] is known to yield optically pure products[11] not only when amino acids are coupled, but even when the carboxyl groups of peptides are activated. The modified approach of Rudinger and Honzl[12] to the azide reaction utilizing a nonaqueous system, and the use of 1-aminoacyl-2-*t*-butyloxy-

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