To the solid was added 10 mL of dry HMPA, the mixture was frozen and evacuated, and argon was allowed to leak back in. This process was repeated three times and, before the final thawing, the *S* isomer of the sulfonimide from the previous step (0.63 mCi) was added under a strong flow of argon. The mixture was allowed to come to room temperature and then heated at 80 °C for 16 h. After it cooled to 0 °C, 2 mL of H_2O was added, and the pH was adjusted to 7 with HCl. The solution was loaded onto a column of Dowex 50W × 8, 100–200 mesh. The column was washed with 30 mL of H_2O and eluted with 10% NH_4OH. All ninhydrin positive fractions were combined, concentrated under vacuum, and subjected to preparative TLC with *n*-BuOH/HOAc/H_2O (4:1:1) as solvent. The methionine (R_f 0.4) was eluted from the silica with water, concentrated to dryness, and recrystallized from acetic acid (yield 48.7 mg, 0.23 mCi = 36.7%, $^3H/^{14}C$ = 2.3). The *methyl-S* isomer of methionine (31 mg, 0.1 mCi = 44.9%, $^3H/^{14}C$ = 3.2) was prepared in the same manner from the *R* isomer of the sulfonimide (0.21 mCi).

Conversion of Methionines to Ia and III. Fermentations of *S. griseus* strain ATCC 12648 and the isolation of Ia and III were carried out as described earlier.[2] Feeding of 23 μCi of the *methyl-R* isomer of methionine ($^3H/^{14}C$ = 2.3) gave 2.2 μCi of Ia ($^3H/^{14}C$ = 2.5) and 7.89 μCi of III ($^3H/^{14}C$ = 2.4); 9.6 μCi of the *methyl-S* isomer ($^3H/^{14}C$ = 3.2) produced 1.9 μCi of Ia ($^3H/^{14}C$ = 3.2) and 3.3 μCi of III ($^3H/^{14}C$ = 3.2).

Degradation of Ia and III. Indolmycin from the two feeding experiments was hydrolyzed with base[2] to give indolmycenic acid and methylamine, isolated as the hydrochloride. Both the indolmycenic acids from this hydrolysis and the ones isolated directly from the cultures were subjected to Kuhn–Roth oxidation[22] to give acetic acid from the *C*-methyl group.

The two samples of methylamine hydrochloride from the hydrolysis of Ia were converted into the *N*-methyl-*N*,*N*-di-*p*-toluenesulfonimide as described above. The latter, 100 mg of KCN, and 5 mL of dry HMPA were quickly frozen in a 25-mL single-neck recovery flask equipped for magnetic stirring. The flask was connected to a second 25-mL recovery flask containing 1 mL of H_2O and a magnetic stirring bar via a vacuum bridge. Both flasks were cooled to −78 °C and the system was evacuated and closed. The flask containing the reaction mixture was then heated with stirring at 85 °C while the second flask was cooled to −78 C. The heating was continued for 72 h. The reaction flask was then replaced with a clean flask and to the cold flask containing the water and acetonitrile were added 3 mL of 30% H_2O_2 and 0.1 mL of 6 N NaOH. The mixture was heated at 50 °C for 6 h and then evaporated to dryness to yield acetamide (yield 19–23% based on methylamine).

The acetamide was dissolved in 1 mL of H_2O and cooled to 0 °C. Cold 5 N H_2SO_4 (2 mL) was added slowly with stirring, followed by 0.4 g of $NaNO_2$ in 1 mL of H_2O. After 1 h at 0 °C, the mixture was stirred for 5 h at room temperature. The acetate was isolated by steam distillation as described above. The yields in this step were quantitative.

Acknowledgment. We thank Mrs. Kathryn Mascaro, Mrs. Laurel K. Sellers, and Mrs. Janet Weaver for their expert technical assistance in carrying out numerous chirality analyses of acetate samples.

Enthalpy Changes Accompanying Hydrolysis of 3-(2-Furyl)acryloylimidazole by α-Chymotrypsin

J. L. Slightom and D. W. Bolen*

Contribution from the Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, Illinois 62901. *Received January 25, 1980*

Abstract: A calorimetric analysis of the α-chymotrypsin-mediated hydrolysis of 3-(2-furyl)acryloylimidazole at pH 7.85 is presented and compared with the nonenzymic heat of hydrolysis. The enzyme-mediated hydrolysis is characterized by an apparent pK_a for acylation of 6.60 and a deacylation pK_a of 7.55. Activation enthalpy changes of 7.8 ± 1.3 and 15.0 ± 0.4 kcal/mol were determined for acylation (k_2) and deacylation (k_3), respectively. An enthalpy change of 0.04 ± 0.85 kcal/mol for Michaelis complex formation was also determined from the temperature dependence of K_s. Heats of acylation (−10.1 ± 0.2 kcal/mol) and deacylation (−1.2 ± 0.55 kcal/mol) corrected for buffer ionization and product ionization heats were separately determined by making use of the large rate difference between acylation and deacylation ($k_2 = 1000k_3$) which exists at pH 7.85. The sum of these corrected enthalpy changes (−11.3 ± 0.6 kcal/mol) agrees very well with the enthalpy change observed for nonenzymic hydrolysis of furylacryloylimidazole (−11.3 ± 0.5 kcal/mol) and validates the approach used.

Introduction

The monomolecular and bimolecular reactions (elementary events) which characterize an enzyme catalytic sequence are of major interest in enzymology since they contain the factors responsible for catalysis. These elementary events are composed of collections of part processes such as conformational changes, noncovalent binding of reactants and products to enzyme, desolvation of active site and reactants, solvation of products and active site, covalent bond rearrangements, etc.[1] To add to this complexity, perturbations of ionizable groups on the enzyme (and also substrates and products) frequently result in hydrogen ion uptake or release being coupled to the monomolecular or bimolecular event.

Since thermodynamic parameters (e.g., ΔG, ΔH, ΔS) which describe an elementary event are composed of contributions from various part processes, they are not easy to interpret in detail. The evaluation of any part process in thermodynamic terms is one of the single most difficult problems in enzymology and only through the use of a variety of techniques does there appear much hope of quantitating the more prominent terms in an elementary event. We believe that calorimetric measurements hold great promise for defining enthalpy changes of such monomolecular and bimolecular steps and under favorable conditions could lead to dissection of an event into its component part processes. This objective cannot readily be realized since calorimetry has historically been used to measure equilibria rather than kinetic events, and, consequently, only a very limited number of techniques have been developed for application to catenary chain mechanisms. Clearly, the first goals must be to establish procedures for measuring enthalpy changes for elementary events and to place such measurements on a firm foundation.

The work reported here deals with several aspects of measuring the enthalpy changes for hydrolysis of 3-(2-furyl)acryloylimidazole (FAI)[2] as mediated by α-chymotrypsin (EC 3.4.4.5). These aspects include (1) the separation and calorimetric measurements

(1) Jencks, W. P. *Adv. Enzymol.* **1976**, *43*, 219–409.

(2) The abbreviations used: FA-Ct, furylacryloylchymotrypsin; FAH, protonated furylacrylic acid; FA^-, unprotonated furylacrylic acid; FAI, 3-(2-furyl)acryloylimidazole.

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of acylation and deacylation phases of the reaction, (2) the evaluation of enthalpy changes of elementary events in the enzyme-mediated sequence, (3) evaluation of contributions from proton ionization changes which accompany certain elementary events, and (4) verification of experimental procedures by comparison of the sum of ΔH values for elementary events with the nonenzymic FAI hydrolysis enthalpy change.

Experimental Section

3-(2-Furyl)acryloylimidazole (FAI). FAI was prepared by the mixed anhydride method described by Bernhard et al.,[3] except that a third recrystallization from cyclohexane was performed; mp 114–115 °C.

Anal. Calcd for $C_{10}H_8N_2O_2$: C, 63.83; H, 4.30; N, 14.89; O, 17.00. Found: C, 64.01; H, 4.28; N, 14.86; O, 16.85.

α-Chymotrypsin. α-Chymotrypsin was purchased from Worthington Biochemical Corp. as three times recrystallized powder and further purified by the Method of Yapel et al.[4] using a G-75 Sephadex column instead of G-25. Purity of α-chymotrypsin was checked by titration of the active site using *p*-nitrophenyl trimethylacetate as described by Bender et al.[5] Enzyme purities for the various Worthington lots as determined by titration were 92 to 95% pure or active. A molar absorptivity of 5×10^4 L·mol^{-1}·cm^{-1} was used for α-chymotrypsin at 280 nm and pH 5.0.

Furylacryloyl-α-chymotrypsin (FA-Ct). FA-Ct was prepared by dissolving 200 mg of purified α-chymotrypsin in 10 mL of 0.001 N HCl and adjusting the pH to 3. FAI (0.405M, 0.2 mL) in CH_3CN was slowly added dropwise to the stirred chymotrypsin solution and allowed to stand at room temperature for 5 min. The solution was then gently filtered through a coarse sintered glass funnel. The filtrate was placed on a G-25 sephadex column (4 cm × 38 cm) and rapidly eluted at pH 3.0 to separate acyl enzyme from FAI, CH_3CN, and hydrolysis products furylacrylic acid and imidazole. The column was monitored at 320 nm, an optimal wavelength of FA-Ct at pH 3.0. Analysis of elution products proved that effective separation of the small molecules had occurred. Lyophilization was found unsatisfactory for FA-Ct, but solutions could be kept at pH 3 and 4 °C for periods up to 1 week with no noticeable deacylation. After chromatography, the acyl enzyme was concentrated using a Diaflow system.

Acylation Kinetic Measurements. Two different procedures were used to evaluate the FAI acylation kinetics of α-chymotrypsin. The first method has been described by Kezdy and Bender[6] under conditions in which the enzyme concentration is much larger than substrate. Under these conditions a pseudo-first-order experimental rate constant (k_{exp}) can be evaluated from the "presteady-state" phase and an expression derived relating this rate constant to K_s, k_2^{app}, and total enzyme concentration:

$$1/k_{exp} = 1/k_2^{app} + K_s/k_2^{app}[E]_0 \quad (1)$$

K_s and k_2^{app} can then be evaluated from a double reciprocal plot. Six different α-chymotrypsin concentrations ranging from 2.3×10^{-5} to 1.6×10^{-4} M were used for each pH investigated. Buffers were 0.03 M citrate, pH 5–6.5, and 0.04 M phosphate, pH 7–8.

Reaction rates were determined by following either the rate of acyl enzyme production at 311–315 nm[7] (wavelength is pH dependent) or the disappearance of FAI at 370 nm. All rate measurements were performed on a Gilford 2400 spectrophotometer equipped with thermospacers and controlled at the specified temperatures and pH values. The pH values remained essentially constant over the temperature range because of the low heat of ionization of citrate buffer.

Second-order acylation rate constants were determined by the method of Bender et al.[8] In our procedure, 20- to 30-μL aliquots of FAI stock (ca. 10^{-3} M) dissolved in CH_3CN was added to 3.0 mL of buffer followed by a 25- to 30-μL aliquot of α-chymotrypsin stock (ca. 10^{-3} M) (final enzyme concentrations were all less than 7 μM). The temperature was controlled at 25.0 °C and the disappearance of FAI at 370 nm was observed using a Beckman Acta M VI spectrophotometer. Buffers included 0.1 M acetate, 0.042 M pyrophosphate, and 0.05 M phosphate plus sufficient sodium chloride to give 0.1 ionic strength. Second-order rate constants (k_{obsd}) were calculated using either eq 2 or 3. Where $\delta = 2.303/b(n-1)$, a is the initial concentration of the reactant in excess, b is the initial concentration of the other reactant, $a = nb$, c is a constant, and A_0, A_t, and A_∞ are absorbances measured at time zero, time t, and time infinity, respectively.

$$[E_0] \neq [S_0], \quad k_{obsd}t/\delta - C = \log[1 + (n-1)\{(A_0 - A_\infty)/(A_t - A_\infty)\}] \quad (2)$$

$$[E_0] = [S_0], \quad k_{obsd}at = (A_0 - A_t)/(A_t - A_\infty) \quad (3)$$

Deacylation Kinetic Measurements. A small amount (0.03 to 0.10 mL) of concentrated FA-Ct at pH 3.0 was added to a cuvette containing 3.0 mL of buffer at a specified pH, and deacylation was followed by decrease in absorbance at 315 to 319 nm. In all cases the reactions were found to follow first-order kinetics.

Flow Calorimetry Measurements. Acylation, titration, FAH ionization, and FAI hydrolysis enthalpy changes were measured on a LKB flow microcalorimeter adapted to fit in a steel water-tight chamber submerged in a Tronac Model 1005 water bath. The bath was held at 25.0 °C and controlled within 0.0003 °C. Flow rates were compatible with acylation and nonenzymic hydrolysis kinetics such that the entire reaction was completed during the resident time within the reaction mixing cell. In measurements involving heats of acylation, FAI solutions were introduced by means of a Teflon loading coil connected via a four-way valve and inserted between the LKB perpex pump and the calorimeter unit. This assured that the FAI was never in contact with the silicone rubber working part of the LKB pump (which only pumps buffer solution) and avoided the problem of substrate adsorption to the silicone tubing.

Calorimetric acylation measurements were made by flowing buffer against buffered FAI (8×10^{-4} M) solution to establish a steady-state voltage signal and then the buffer solution was switched to buffered chymotrypsin solution (5.0 to 6.6 × 10^{-5} M). The new steady-state voltage signal was proportional to the observed acylation enthalpy change plus enzyme dilution heat. The enzyme dilution enthalpy change was measured in separated experiments. A minimum of three replicate measurements were made for each experiment.

All three solutions (buffer, enzyme, and FAI) contained identical concentrations of buffer plus 1.6% v/v CH_3CN and were adjusted to within ±0.02 pH unit of one another prior to mixing. The pH of the calorimeter effluent was closely monitored after mixing and seldom differed from the original pH by more than 0.02 pH unit.

The enthalpy change for nonenzymic hydrolysis was measured by flowing 0.24 M NaCl, pH 7.0, against 0.24 M NaCl, pH 12.5, to establish a steady-state voltage signal. Then a solution of 1×10^{-2} M FAI in 0.24 M NaCl, pH 7.0, was substituted for the 0.24 M NaCl, pH 7.0, solution and the difference in steady-state voltage signals recorded. FAI hydrolysis was found to be instantaneous above pH 12, and, after correction for the heat of dilution of FAI (measured in a separate experiment at pH 7.0), the hydrolysis enthalpy change (ΔH_{hyd}) was calculated using the expression $\Delta H_{hyd} = \Delta H_{obsd} - \Delta H_{H_2O\ formation} - \Delta H_{ion\ FAH}$. Since FAI slowly hydrolyzes at pH 7.0, the above solutions were freshly prepared and the actual concentration of FAI entering the calorimeter cell was calculated from the first-order hydrolysis rate equation with an observed (pH 7.0) first-order hydrolysis rate constant of 4.45×10^{-5} s^{-1}.

The heat of ionization of furylacrylic acid was measured using standard flow calorimetry techniques.

Batch Calorimetry Measurements. The enthalpy change for deacylation was measured using a LKB batch microcalorimeter. Measurements were made by delivering 0.25 mL of ca. 5×10^{-4} M FA-Ct in pH 3.0 HCl into the 2 mL compartment of the batch microcalorimeter and 4 mL of the appropriate buffer in the adjacent compartment. The same amounts of HCl (pH 3.0) and buffer were placed in their respective compartments in the reference cell to cancel the buffer heat of dilution and titration. FA-Ct dilution heat at pH 3.0 was found to be negligible. Deacylation was initiated by rotating the calorimetric chamber and resulted in a final pH of about 7.85. pH agreement between reference and sample cells was generally within 0.03 pH unit. The time for 99% deacylation was 51 min ($k_3 = 1.6 \times 10^{-3}$ s^{-1}). Calorimetric runs were allowed to proceed for 70 min though return to base line occurred well before that.

Calculations. A computer program was written to calculate the experimental first-order rate constants for acylation and deacylation kinetics using either the endpoint A infinity or Guggenheim methods of analysis.[28,29] For acylation, a weighted least-squares fit of the reciprocal of the experimental rate constants with the reciprocal of total enzyme concentration was made, and k_2 and K_s were determined by slope–intercept analysis.[6]

Least-squares analyses were performed to determine the heats of acylation and deacylation at zero heat of ionization of buffer, as well as the van't Hoff enthalpy changes for FAI binding to α-chymotrypsin and

(3) Bernhard, S. A.; Lau, S. J.; Noller, H. *Biochemistry* **1965**, *4*, 1108–1118.
(4) Yapel, A.; Han, M.; Lumry, R.; Rosenberg, A.; Shiao, D. F. *J. Am. Chem. Soc.* **1966**, *88*, 2573–2584.
(5) Bender, M. L.; Kézdy, F. J.; Wedler, F. C. *J. Chem. Educ.* **1967**, *44*, 84–88.
(6) Kézdy, F. J.; Bender, M. L. *Biochemistry* **1962**, *1*, 1097–1106.
(7) Bernhard, S. A.; Gutfreund, H. *Philos. Trans. R. Soc. London, Ser. B* **1970**, *257*, 105–110.
(8) Bender, M. L.; Schonbaum, G. R.; and Zerner, B. *J. Am. Chem. Soc.* **1962**, *84*, 2562–2570.

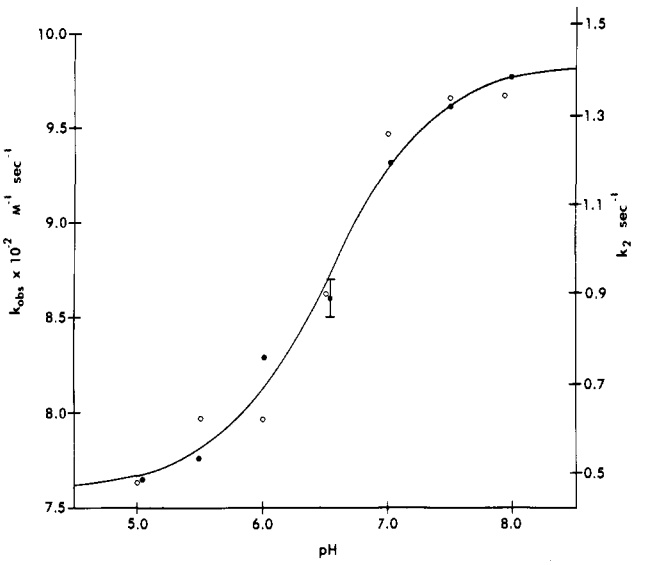

Figure 1. Acylation pH–rate constant dependence. k_{obsd} second-order rate constant data (●) and k_2 values derived from pseudo-first-order rate constants (○). Error bar represents the largest error of the k_{obsd} data set. Solid line represents the nonlinear least-squares best fit to the k_{obsd} data as well as the k_2 data. Buffers and conditions are described in the Experimental Section.

the enthalpies of activation for specific acylation and deacylation.

NLIN 2, a program developed by D. S. Marquart (share distribution no. 3094) for least-squares estimation of nonlinear parameters, was used. Apparent pK_a values were determined from second-order (k_{obsd}) and first-order rate constants (k_3). pH dependencies of rate constants were determined using this program.

Results

α-Chymotrypsin-catalyzed hydrolysis of 3-(2-furyl)acryloylimidazole with (S ≫ E) proceeds in the same fashion as other nonspecific substrates in that a typical first-order "burst" occurs followed by a slower steady-state rate process.[5,7] In fact, FAI is analogous in structure and kinetic behavior to cinnamoylimidazole, perhaps the best known substrate of this type. The kinetic analyses we have performed conform to many of the same procedures used by Bender and co-workers in investigating the action of α-chymotrypsin on cinnamoylimidazole.[6,8] Such data can be interpreted in terms of the following mechanism:[9]

$$E + FAI \underset{}{\overset{K_s}{\rightleftharpoons}} EFAI \xrightarrow{k_2} EFA \xrightarrow{H_2O,\ k_3} E + FAH \quad (4)$$

imidazole

Kézdy and Bender used a number of different conditions ($S_0 \gg E_0$, $E_0 \gg S_0$, $E_0 \simeq S_0$) for evaluating kinetic constants k_2, k_2/K_s, and K_s and found that these different conditions gave identical results.[6] We have used two of these approaches ($E_0 \simeq S_0$ and $E_0 \gg S_0$) to evaluate the dependence of k_2/K_s and k_2, respectively, on pH. Figure 1 gives the pH dependence of the apparent second-order rate constant k_{obsd} under conditions of $E_0 \simeq S_0$ as well as the specific acylation rate constant k_2 evaluated from pseudo-first-order rate data ($E_0 \gg S_0$). The errors in the k_2 values are large (average of ± 15%) in comparison with the second-order data, but both sets of data can be described in terms of a single ionization process with an apparent pK_a of 6.60. Data are not reported below pH 5 because FAI intrinsic hydrolysis becomes significant in the acid region making second-order and pseudo-first-order data analysis difficult.

With this system $k_2 \gg k_3$ and deacylation can be ignored. Since K_s is much greater than the concentration of α-chymotrypsin or FAI, the second-order rate constant k_{obsd} is equal to k_2/K_s.[8]

Deacylation rates were measured by following the decrease in absorbance at 315 to 319 nm (pH dependent λ_{max}) upon addition of purified FA-Ct to a buffer at the specified pH. First-order rate constants were evaluated and a plot of the pH dependence of k_3 is given in Figure 2. These data also appear to fit a single ionization process with an apparent pK_a of 7.55.

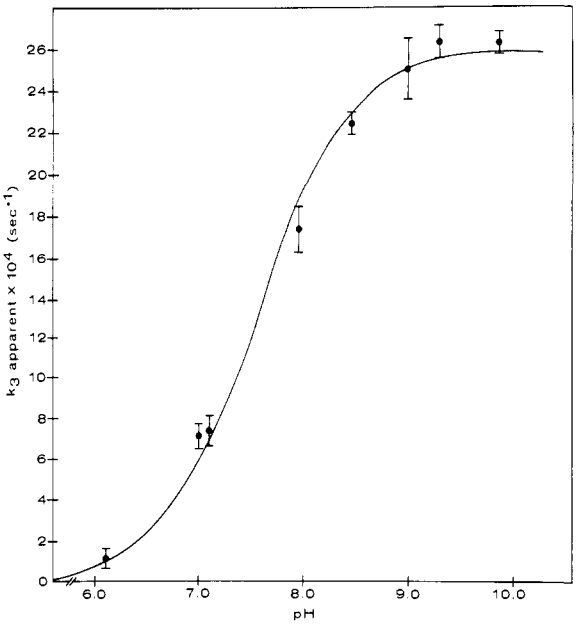

Figure 2. pH dependence of apparent deacylation rate constant (k_3). Buffers include 0.03 M citrate (pH 6–7), 0.09 M phosphate (pH 7–8), and 0.04 M pyrophosphate (pH 8.5–9.9). The temperature was held at 24 °C. The solid line is the nonlinear least-squares best fit titration curve for the data. Other experimental details are given in the Methods section.

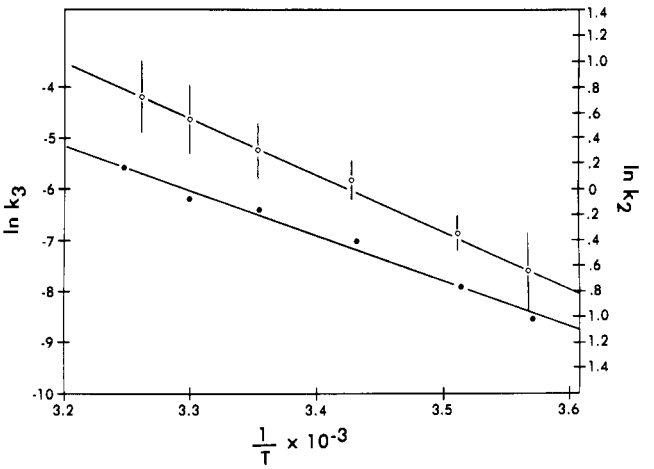

Figure 3. Arrhenius plots of the specific acylation step (k_2) (○) and deacylation step k_3 (●) buffered at pH 7.85 with 0.09 M phosphate buffer.

Analysis of the enthalpy changes accompanying the α-chymotrypsin-mediated hydrolysis of FAI as performed at pH 7.85 and 25 °C. Activation enthalpy changes for specific acylation (k_2) ($\Delta H^{\ddagger}$ = 7.8 ± 1.3 kcal/mol) and deacylation (k_3) ($\Delta H^{\ddagger}$ = 15.0 ± 0.4 kcal/mol) were determined from their respective temperature dependences (Figure 3), and the enthalpy change for FAI binding was determined from the temperature dependence of $1/K_s$. While the errors in $1/K_s$ are actually quite large, it is nonetheless clear from Figure 4 that FAI binding (Michaelis complex formation) is essentially independent of temperature. The best fit straight line of Figure 4 gives a ΔH of zero for FAI binding.

From the rate data in Figures 1 and 2 it is seen that below pH 6, acylation occurs without measurable deacylation. Heats of acylation in the acid region can be determined independently of deacylation by mixing FAI with α-chymotrypsin in either the batch or flow microcalorimeter. Above pH 6, deacylation occurs but at rates which are very low in comparison to acylation (k_2 = $1000k_3$ at pH 7.85). We have used the large rate difference to

(9) Bender, M. L. "Mechanisms of Homogeneous Catalysis from Protons to Proteins"; Wiley-Interscience: New York, 1971; p 494.

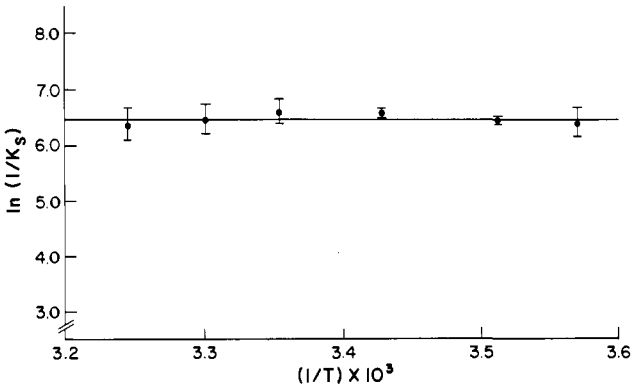

Figure 4. Temperature dependence of the dissociation of FAI from α-chymotrypsin buffered at pH 7.85 with 0.09 M phosphate. K_s values were evaluated from pseudo-first-order rate data (see methods).

measure acylation heats independently of deacylation in the pH region above 6. This was accomplished by flowing reactants FAI and α-chymotrypsin in the flow microcalorimeter at velocities such that the mixed solution only resides in the measuring device 30 to 45 s (resident time). Under conditions in which FAI is in much greater excess than α-chymotrypsin, acylation is complete well within this 30–45 s time frame, but very little deacylation accompanies the process.

Calorimetric determinations of protein ligand interactions are almost invariably complicated by the fact that protons are either taken up or released on forming the protein–ligand complex. These proton changes result in titration of buffer and add an additional contribution to the observed enthalpy change as illustrated by eq 5.[10]

$$\Delta H_{obsd} = \Delta H_{acyl}^{app} - n_{acyl}^{app}\Delta H_{ion\ buffer} \quad (5)$$

A plot of observed reaction enthalpy changes (ΔH_{obsd}) as a function of buffer ionization heats ($\Delta H_{ion\ buffer}$) is predicted with the slope "n_{acyl}^{app}" representing *the number of equivalents of protons released to the buffer as a result of converting enzyme and substrate to acyl enzyme, per equivalent of total enzyme.*[11] The change in apparent acylation enthalpy (ΔH_{acyl}^{app}) at zero buffer ionization heat can be obtained from the intercept. It is tacitly assumed that the presence of buffer does not affect the interaction of substrate with enzyme and that buffer interaction with enzyme is no different from its interaction with acyl enzymes. Use of several buffers having different physical and chemical properties (and presumably different extents of interaction with enzyme) is responsible for the scatter in data with respect to linearity of the plot.

Figure 5 gives the results of experiments from which ΔH_{acyl}^{app} and n_{acyl}^{app} were evaluated from least-squares analyses. The correlation coefficients for such data are 0.99 or better, showing uniformly good agreement between experiment, theory, and values used for buffer ionization heats (ΔH_{ion}).

In addition to the scatter of data due to extent of buffer interaction with enzyme, the accuracy of these plots (Figure 6) is dependent on the range of buffer ionization heats. Some inaccuracies might also arise from differences in ionic strengths used in these experiments in comparison to the literature values for which the ΔH_{ion} buffer data were reported. Most of the literature ΔH_{ion} data (Figure 5 legend) are reported at zero and 0.1 ionic strength. These problems are found to affect the accuracy of the intercept values (ΔH_{acyl}^{app}) to a much smaller extent than the slope (n_{acyl}^{app}) values.[12]

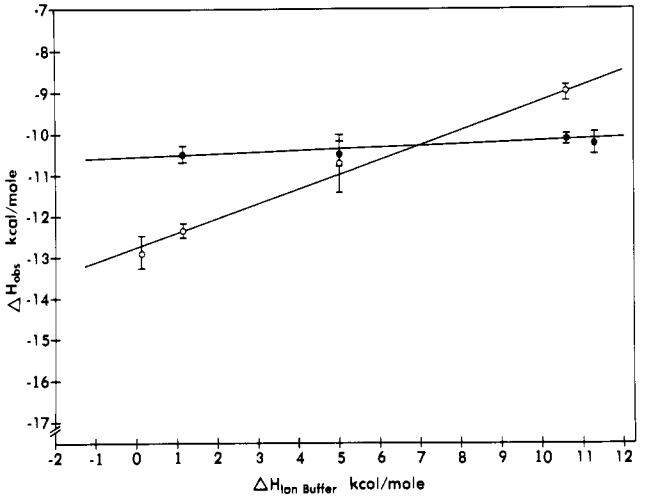

Figure 5. Observed heats of acylation of α-chymotrypsin by FAI at pH 7.5 (○) and pH 8.0 (●). Buffers used include 0.05 M *N*-glycylglycine, ΔH_{ion} = 10.6;[23] 0.05 M HEPES, ΔH_{ion} = 5.01;[10] 0.09 M phosphate, ΔH_{ion} = 1.13;[10,24] 0.033 M pyrophosphate, ΔH_{ion} = 0.11;[24] 0.05 M THAM, ΔH_{ion} = 11.3.[25]

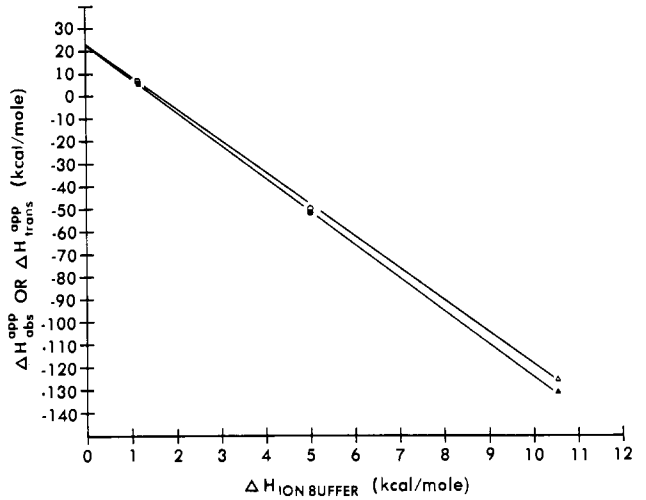

Figure 6. Enthalpy changes measured in the batch (filled symbols) and flow microcalorimeters (open symbols) for the transfer of FA-Ct from pH 3.0 (unbuffered) to pH 7.85 (buffered). Solid lines are the least-squares best fit to the data with the intercepts representing ΔH_{trans} and ΔH_{obsd} independent of buffer ionization (consult Scheme I and eq 6). Buffers include (■, □) 0.09 M phosphate; (●, ○) 0.05 M HEPES; (▲, △) 0.05 M *N*-glycylglycine.

ΔH_{acyl}^{app} and n_{acyl}^{app} are apparent quantities because there are other enthalpy and proton release adjustments which must be made. One such adjustment becomes apparent from consideration of the overall acylation reaction (eq 6) in which the first product,

$$\alpha\text{-chymotrypsin} + \text{FAI} \rightarrow \text{FA-Ct} + \text{imidazole} \quad (6)$$

imidazole, has the potential to protonate. Imidazole leaves the active site of the enzyme as a neutral molecule, and the extent to which it is protonated depends on the pH of the surrounding buffer. The abstraction of a proton by imidazole and its accompanying enthalpy change can be calculated by knowing the pK_a of imidazole (6.99 ± 0.02) and its heat of ionization $\Delta H_{ion\ 1M}$ (8.79 ± 0.03 kcal/mol).[13,14] ΔH_{acyl}^{app} at pH 7.85 can be interpolated from the pH 7.5 and 8.0 data and corrected for imidazole protonation by eq 7. Here ΔH_{acyl} represents the actual enthalpy

$$\Delta H_{acyl} = \Delta H_{acyl}^{app} + n_{1M}\,\Delta H_{ion\ 1M} \quad (7)$$

change for the enzyme associated part of the acylation reaction,

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(11) The numerical value of n_{acyl}^{app} will itself have a sign. A negative sign indicates proton uptake from the buffer by the combined system of enzyme, acyl enzyme, FAI, and imidazole. A positive sign indicates proton release to the buffer by this combined system which occurs on acylation. On the other hand, a negative sign of *n* corresponds to proton uptake from the buffer by the free enzyme and acyl enzyme only, and a positive sign indicates proton release to the buffer by only acyl enzyme and free enzyme.
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Table I. Summary for Calculation of Enthalpy Changes for FA-Ct Deacylation and Overall Enzyme-Mediated Hydrolysis[a]

process	enthalpy change, kcal/mol
ΔH_{obsd} (intercept of batch experiments, Figure 6)	23.31 ± 0.47
ΔH_{trans} (intercept of flow experiments, Figure 6)	23.46 ± 0.28
$\Delta H_{ion\ FAH}$	1.03 ± 0.07[b]
ΔH_{deacyl}(Hess' law calculation)	−1.18 ± 0.55
ΔH_{acyl}	−10.1 ± 0.2[c]
$\Delta H_{overall\ EC}$	−11.28 ± 0.6

[a] The enthalpy change for deacylation (ΔH_{deacyl}) (step III) is determined from Hess' law sum of data in the table applied to steps I, II, IV of Scheme I. All data listed are independent of buffer ionization affects. The overall enzyme catalyzed hydrolysis heat ($\Delta H_{overall\ EC}$) is determined from the sum of reported acylation and deacylation enthalpy changes. [b] Ionization heat of furylacrylic acid ($pK_a = 4.2$) was determined calorimetrically. The anion, initially at pH 7, was abruptly changed (protonation) to pH range 1.3–2.3 in the flow calorimeter at a constant ionic strength (NaCl) of 0.34. [c] Interpolated from data in Figure 6 and corrected for heat of ionization of imidazole as described in text.

$\Delta H_{ion\ 1M}$ is the known heat of dissociation of imidazole,[14] and n_{1M} is the imidazole proton release as calculated from the known pK_a and pH.

Enthalpy changes (ΔH_{acyl}) obtained by the above procedure are the appropriately corrected ones in the pH range of 5 to 6; however, above this pH range the possibility exists that a small amount of deacylation will occur during the resident time of the calorimeter. The experiments are designed to reduce this complication, but up to 6% of the acyl enzyme formed within the calorimeter cell also deacylates within the resident time at pH 8. Since the experiments are carried out under substrate saturating conditions, FAI ≫ E, any deacylation would be followed immediately by reacylation. The amount of excess acylation can be calculated, since the rate of deacylation is known as a function of pH; see Figure 2. This excess concentration of acyl enzyme is used to correct the actual number of moles of enzyme acylated. The correction for deacylation enthalpy change (vide infra) occurring in the flow cell is calculated to be 0.07 kcal/mol at pH 7.85.

At this point, the original observed acylation enthalpy change at a fixed pH has been corrected for buffer ionization heats, product (imidazole) ionization heat, as well as an experimental factor to account for deacylation and reacylation during the resident time of the calorimeter cell. The resultant quantity (ΔH_{acyl}) at pH 7.85 listed in Table I represents the enthalpy change which includes *only* enzyme acylation and accompanying enzyme ionization processes at that pH.

Since FA-Ct is formed stoichiometrically and is stable below pH 6, heats of deacylation can be calorimetrically measured by transfer of acyl enzyme from low pH (pH 3.0) to a pH at which deacylation occurs (e.g., pH 7.85). The observed heat change (ΔH_{obsd}) will contain contributions due to titration of the acyl enzyme from pH 3 to 7.85 (ΔH_{trans}) as well as the deacylation enthalpy change (ΔH_{deacyl}). Scheme I illustrates the experimental approach for evaluating ΔH_{deacyl}. ΔH_{obsd} is measured by use of the batch microcalorimeter since deacylation (step III) is a relatively slow process at pH 7.85 ($t_{1/2}$ = 7 min). Steps II and IV are titration-ionization processes which are very rapid by comparison. Because of the large rate difference between steps II and III, it is possible to titrate acyl enzyme from pH 3 to 7.85 in the flow microcalorimeter without incurring more than 5% deacylation within the time of the flow calorimetric measurement.

In both the batch and flow calorimetric measurements hydrogen ions are absorbed by the buffer in transferring FA-Ct from pH 3.0 (unbuffered) to pH 7.85 (buffered), and an enthalpy change due to the heat of protonation of buffer contributes to the observed heat (eq 8).

$$\Delta H_{trans}^{app} = \Delta H_{trans} - n'\Delta H_{ion\ buffer} \quad (8)$$

Scheme I

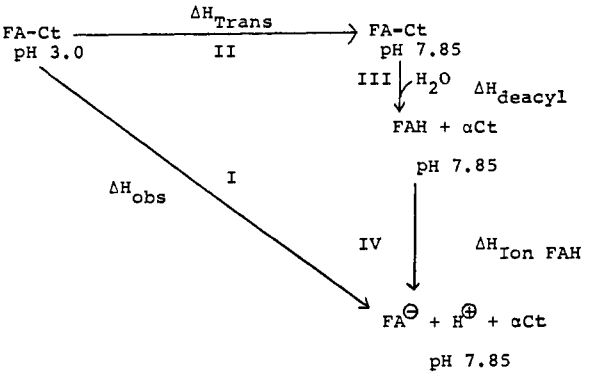

Table II. Enthalpy Change for Base (Nonenzymatic) Hydrolysis of FAI[a]

process	enthalpy change, kcal/mol	no. of obsers
$\Delta H_{hydrolysis}$	−23.59 ± 0.47	8
$\Delta H_{ion\ water}$	13.34	*b*
$\Delta H_{Ion\ FAH}$	1.03 ± 0.07	4[c]
$\Delta H_{overall\ BC}$	−11.28 ± 0.5	

[a] Base-catalyzed hydrolysis enthalpy change ($\Delta H_{overall\ BC}$) is calculated from the data in the table and eq 8–11. [b] See ref 26. [c] See legend to Table I for details.

Here, ΔH_{trans}^{app} is the observed apparent enthalpy change for the transfer process, ΔH_{trans} represents the actual heat of titration of groups on FA-Ct, n' equals the equivalents of hydrogen ion per mole of protein that are absorbed by the buffer, and $\Delta H_{ion\ buffer}$ is the heat of ionization of buffer. The relationship is confirmed by performing both the flow and batch experiments with buffers of differing heats of ionization and the results of such experiments are given in Figure 6. The intercepts in Figure 6 represent either ΔH_{trans} or ΔH_{obsd}, the enthalpy change for step I or II exclusive of buffer influence.

The enthalpy change for step IV is simply the heat of ionization of furylacrylic acid (FAH) determined in separate calorimetric experiments and listed in Tables I and II.

Application of Hess' law to the quantities evaluated using Scheme I permits evaluation of step III (ΔH_{deacyl}), i.e., hydrolysis of FA-Ct to α-chymotrypsin and *protonated* furylacrylic acid. The deacylation enthalpy change and data pertinent to Scheme I are given in Table I.

Nonenzymic hydrolysis of FAI was determined by reacting FAI initially at pH 7 with base at pH 12.5 in the flow microcalorimeter. Hydrolysis is complete within the resident time of the microcalorimeter giving final products, unprotonated imidazole, and unprotonated furylacrylic acid (eq 9). In order to compare base catalyzed with enzyme mediated hydrolysis, it is convenient to convert to a standard state in which reactants and products are uncharged as outlined in eq 9 through 12. Data for this conversion

$$\text{FAI} + \text{OH}^- \rightarrow \text{FA}^- + \text{imidazole} \quad \Delta H_{hyd}^{obsd} \quad (9)$$

$$\text{FA}^- + \text{H}^+ \rightarrow \text{FAH} \quad -\Delta H_{ion\ FAH} \quad (10)$$

$$\text{H}_2\text{O} \rightarrow \text{H}^+ + \text{OH}^- \quad \Delta H_{ion\ water} \quad (11)$$

$$\text{FAI} + \text{H}_2\text{O} \rightarrow \text{FAH} + \text{imidazole} \quad \Delta H_{overall\ BC} \quad (12)$$

are given in Table II along with the calculated standard-state enthalpy change for base-catalyzed hydrolysis ($\Delta H_{overall\ BC}$).

Discussion

pH-dependent rate data for FAI acylation of α-chymotrypsin appear to involve a single ionizable group. Both second-order (k_2/K_s) and first-order (k_2) apparent acylation rate constants (indicated as k_{obsd}) conform to eq 13 and the nonlinear least-squares best fit line results in a pK_a of 6.6 ± 0.1.

$$k_{obsd} = \text{constant} + k_{intrinsic}\{K_a/(K_a + [\text{H}^+])\} \quad (13)$$

It is clear from Figure 2 that deacylation rate data (k_3) can also be explained in terms of a single ionization process which fits the expression $k_3 = k_D K_a/(K_a + [H^+])$. This deacylation pH dependence has been noted for numerous nonspecific substrates[16] (k_D is the pH-independent deacylation rate constant). The nonlinear least-squares best fit line for deacylation in the present study gives a pK_a of 7.55 ± 0.05 which is at variance with the pK_a of 7.25 reported by Bernhard et al.[16]

Though the experimental conditions are very nearly the same in both studies, the techniques for measuring the pH-dependent deacylation rate constants differ. We isolated stable acyl enzyme at low pH by G-25 Sephadex chromatography and measured deacylation at 315 nm on addition of the purified FA-Ct to buffered solution at pH 7.85. Bernhard and co-workers did not attempt to separate FA-Ct from other components but instead followed deacylation by observing the 315-nm absorbance decrease upon mixing FAI and α-chymotrypsin.[7] While we do not as yet know the origin of the pH discrepancy, we believe the procedure we have used is the method of choice since it gives unambiguous first-order rate data and is in accord with the same procedure used for measuring deacylation enthalpy changes.

The acryloylimidazole substrates cinnamoylimidazole, indoleacryloylimidazole, and furylacryloylimidazole appear to have similar pH-dependent kinetics with apparent pK_a's for acylation of 6.8, 6.3, and 6.6 and deacylation values of 7.2, 7.7, and 7.55, respectively.[8,15] The pK_a's for the k_2 and k_3 steps (eq 4) have usually been assigned to histidine 57[17,18] though there has been controversy over assignments in the acidic to neutral range.[19-21] Regardless of the actual assignment, pH-dependent kinetic data and data on proton release on acyl enzyme formation suggest that a single group with a pK_a of <7 is perturbed to a value of >7 on forming acyl enzyme.[8,15]

The pH dependence of rate constants in the α-chymotrypsin mediated hydrolysis of FAI affects the present study in two ways. First, the rates must be known as a function of pH in order to define the proper conditions for flow and batch calorimeter measurements. Secondly, since there are pK_a perturbations on the enzyme during acylation and deacylation, the apparent enthalpy changes obtained at any particular pH will include some contribution from heats of ionization of the groups in addition to the part processes of interest such as binding enthalpy change, covalent bond making and breaking, conformational changes, etc. The enthalpy changes we have obtained for acylation and deacylation at pH 7.85 do contain such ionization heats, but fortunately they have been determined to be minor at this pH.[12] Thus, the acylation and deacylation enthalpy changes reported here reflect the part processes of interest which compose the elementary events.

While acylation and deacylation rate data dictate how the calorimetric studies should be performed, corrections for accompanying ionization of enzyme titratable groups, buffer, reactants, and products are the most troublesome problems to handle. As detailed in the Results, the appropriate corrections have been made for acylation and deacylation and referred to a reference state such that the imidazole and furylacrylic acid products are in their uncharged forms as given by eq 6 and step III of Scheme I. The sum of the corrected heats of acylation (−10.1 ± 0.2 kcal/mol) and deacylation (−1.18 ± 0.55 kcal/mol) gives an overall enthalpy change for furylacryloylimidazole hydrolysis of −11.3 ± 0.6 (Table I). The overall enthalpy change for nonenzymic hydrolysis to the uncharged products was determined to be −11.3 ± 0.5 kcal/mol (Table II). Agreement between these independently determined quantities verifies that the experimental procedures used in evaluating ΔH_{deacyl} and ΔH_{acyl} are fundamentally correct.

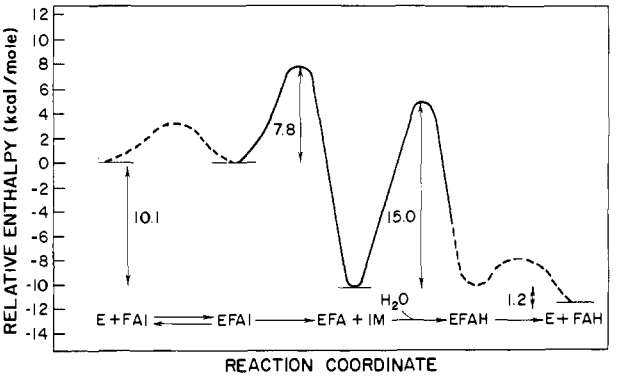

Figure 7. Enthalpy diagram for FAI hydrolysis by α-chymotrypsin. Dashed lines represent barriers not determined in this study while solid lines were calculated from Arrhenius plots of k_2 and k_3 and using $\Delta H^\ddagger = E_a - RT$. Enthalpy levels are relative to the separated reactants α-chymotrypsin, FAI, and water which is set arbitrarily at $H = 0$. Relative enthalpy changes were determined as explained in the Results section.

We have only been able to find one other report in the literature in which enthalpy changes for elementary events were measured independently of the enthalpy change for the overall reaction. The study involved the reversible reaction catalyzed by fumarate hydratase, and the summation of enthalpy changes for the elementary events as determined by van't Hoff procedures agrees quite well with the enthalpy change of the overall reaction.[22] The key to these measurements was the fact that the enzyme reaction is totally reversible and that all equilibrium constants were known and their temperature dependencies evaluated. However, in a large number of enzyme-catalyzed reactions many of the equilibrium constants are not experimentally accessible and this greatly restricts the use of van't Hoff determinations in evaluating enthalpy changes. By contrast, the α-chymotrypsin-mediated hydrolysis of FAI reported here is experimentally an irreversible system and the only possible way of obtaining many of the enthalpy changes is by calorimetric means. We were forced to use van't Hoff procedures for evaluating the ΔH for ES complex formation because we have not yet developed calorimetric methods for separating the substrate binding step from the specific acylation event in the catalytic sequence. It is clear that for some time to come, van't Hoff and calorimetric methods must be used in a complementary fashion in evaluating enthalpy changes for elementary events.

From the data available, it is possible to construct a major part of the enthalpy reaction-coordinate profile for the enzyme-mediated hydrolysis of furylacryloylimidazole. Activation enthalpy changes for acylation and deacylation are incorporated into Figure 7 along with data in Table I and the enthalpy change due to Michaelis complex formation as evaluated from Figure 4. Attempts to measure or estimate enthalpy changes for specific (and nonspecific) binding of imidazole and furylacrylic acid to FA-Ct and α-chymotrypsin, respectively, were unsuccessful, making it impossible to add further detail on product release steps to the diagram.

To fully describe the FAI-α-Ct interaction, Gibbs energy and entropy profiles corresponding to Figure 7 are needed. At present, such diagrams are not available and while this lack of information prevents us from making specific statements about the magnitudes of entropy and Gibbs energy changes, it is possible to make some general statements as to the dominant thermodynamic parameters in the reaction sequence. It is clear, for example, that FAI noncovalent binding to α-chymotrypsin is favorable ($\Delta G^\circ \simeq -4.0$

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kcal/mol as calculated from K_s, Figure 4) and athermal ($\Delta H \simeq 0$ kcal/mol) and that this step must undoubtedly be an entropy-driven process. In contrast, the strongly exothermic acylation step which follows binding suggests that, in terms of enthalpy alone, $\Delta G°$ for conversion of E·FAI to EFA and imidazole would be highly favorable. Though we do not know the actual $\Delta G°$ for acylation it is difficult to imagine how $\Delta S°$ for this step could override (negative $\Delta S°$) or promote (positive $\Delta S°$) acylation to an extent greater than the enthalpy contribution. For this reason we believe the acylation step to be primarily enthalpy driven.

Finally, for the process of EFA deacylation we expect that the Gibbs energy change should not be markedly different from the +7.5 kcal/mol reported for deacylation of *N*-acetyl-L-tryptophanylchymotrypsin.[27] Even if this estimate for EFA is in error by several kcal/mol, the process should at least be endergonic to give protonated furylacrylic acid and α-chymotrypsin. Thus, a positive $\Delta G°$ coupled with the enthalpy change of −1.2 kcal/mol clearly distinguishes this last phase of the reaction sequence as an entropy-controlled process.

The enthalpy profile determined by kinetics and calorimetric methods is the first attempt to calorimetrically evaluate enzyme-mediated enthalpy changes consistent with nonenzymic conversion of substrates to products. The kinetics of transformations between intermediates in this system were well suited for calorimetric analysis and no effort was made to flow reatants in the flow microcalorimeter at rates with a resident time (time in measuring chamber) of much less than 30 s. It is quite possible to flow reactants at much faster flow rates and use the techniques developed here. This can potentially extend the scope of such measurements to more specific substrates provided that the elementary steps are themselves reasonably well separated in time.

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Enzyme Immobilization by Condensation Copolymerization into Cross-Linked Polyacrylamide Gels[1]

Alfred Pollak, Hugh Blumenfeld,[2] Michael Wax,[2] Richard L. Baughn, and George M. Whitesides*

Contribution from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received January 25, 1980*

Abstract: This paper describes a convenient procedure for immobilization of enzymes based on condensation copolymerization of a water-soluble, functionalized prepolymer (PAN, poly(acrylamide-*co*-*N*-acryloxysuccinimide)), a low molecular weight α,ω-diamine (triethylenetetramine (TET), cystamine), and the enzyme in neutral buffered aqueous solution. This procedure is designed specifically for utility with the relatively delicate and expensive enzymes of interest in enzyme-catalyzed organic synthesis. Inclusion of substrates, cofactors, products, or reversible competitive inhibitors during the immobilization protects the enzyme active site against deactivating acylation. Loosely cross-linked gels present little or no resistance to diffusion of macromolecules into the gel interior, and permit the use of gel-immobilized enzymes to catalyze reactions of soluble, high molecular weight substrates. Tightly cross-linked gels *do* inhibit diffusion of macromolecules into the gel, and can be used to protect immobilized enzymes against deactivation by soluble proteases. Either type of gel protects enzymes against shear deactivation. The gels also protect enzymes included in them from deactivation in aqueous-organic solvent mixtures. Enzyme-containing gels can be used directly in suspension. The gels are too soft to be used alone in columns, but by mixing them with filter aides or by forming them on glass beads, columns having excellent flow characteristics can be assembled. The gels can also be supported on filter cloth or the interior surface of glass tubing. Inclusion of a Ferrofluid—a surfactant-stabilized magnetite colloid—in the gel-forming step results in the formation of a ferrimagnetic gel. Particles of this gel can be manipulated in suspension using high-gradient magnetic filtration techniques. Use of a cross-linking agent containing a disulfide group (cystamine) results in gels which can be dissolved by treatment with a reducing thiol under mild conditions. The factors leading to optimum immobilization yields have been examined in detail for hexokinase. Immobilization yields are summarized for approximately 60 other enzymes.

Introduction

Materials and procedures for protein immobilization are an important component of the developing technology which uses enzymes as catalysts for the in vitro synthesis of complex organic substrates.[3-5] The immobilization of enzymes on insoluble supports serves the primary purpose of facilitating their recovery from reaction mixtures, but it can also slow enzyme deactivation (by inhibiting protease attack and minimizing shear, interfacial, temperature or solvent denaturation) and make possible the manipulation of local concentrations of catalytic species.[6] A large number of immobilization methods have been tested: no single method is (or should be expected to be) superior for every application.

The enzymes of interest for chemical synthesis have several general characteristics which distinguish them from enzymes useful in other areas of applied enzymology and which define the features which should be incorporated into procedures for their immobilization. These enzymes are both relatively difficult to obtain and delicate, and thus expensive. They often contain essential cysteine thiol groups and are deactivated rapidly by oxidizing and alkylating

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