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Calorimetric determination of linkage effects involving an acyl-enzyme intermediate

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Enthalpy changes of α-chymotrypsin acylation by 3-(2-furyl)acryloylimidazole (FAI) were calorimetrically determined as a function of pH. By observing the functional dependence of acylation enthalpies on buffer ionization heats, a complex pH profile was obtained describing proton release accompanying formation of acyl-enzyme. A pK_a of 4.0 for FAI ionization and apparent pK_a values of 6.8, 7.55 and 8.8 on the enzyme were used to account for the proton release data. A model which accounts for the proton release behavior was used to fit the acylation enthalpy data and values for the apparent dissociation enthalpies of the groups involved were obtained along with a pH-independent intrinsic enthalpy of acylation. This model suggests a group with an apparent pK = 6.8 and ΔH_{ion} = 8.7 kcal/mol which is perturbed to a pK of 7.55 and ΔH_{ion} = 7.6 kcal/mol on attachment of the acyl moiety to the enzyme. The apparent ionization enthalpy change for the active-inactive transition (pK_3 = 8.8; ΔH = 3.0 kcal/mol) corresponds with that calculated from the data of Fersht (J. Mol. Biol. 64 (1972) 497). The pH-independent intrinsic enthalpy of acylation (ΔH = −7.9 kcal/mol) is corrected for group ionizations linked to the acylation process. Consequently, it more closely reflects molecular processes of interest such as substrate binding, covalent bond rearrangement, and product release.

1. Introduction

Interpretation of thermodynamic parameters ($\Delta G°$, $\Delta H°$, $\Delta S°$) for single steps in enzyme-catalyzed reactions has seldom progressed beyond statements relating to the degree of enthalpy or entropy control of the event or the nature (electrostatic, hydrogen bonding, hydrophobic, etc.) of the interactions taking place. What complicates interpretation of thermodynamic parameters is that enzyme-catalyzed reactions generally involve a host of linkage relationships involving, for example, pH and ionic strength which are not easily dissected [2]. Without knowledge of the relationships it is nearly impossible to estimate the values of $\Delta G°$, $\Delta H°$ and $\Delta S°$ which reflect the true character of the event of interest.

Determining linkage relationships and interpreting the linkage data is generally an involved process, requiring information on binding polynomials as a function of ligand concentration. Accurate binding polynomials are sometimes difficult to obtain and it is useful and desirable to develop other means of evaluating thermodynamic linkage. Here, we explore the use of calorimetric methods for evaluating the pH-dependent enthalpy changes associated with formation of fur-

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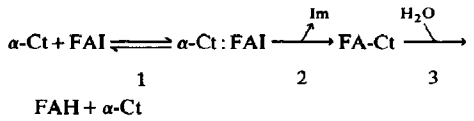
Abbreviations: FAI, 3-(2-furyl)acryloylimidazole; α-Ct, α-chymotrypsin; FA-Ct, furylacryloyl-α-chymotrypsin; FAH, furylacrylic acid.



Scheme 1.

ylacryloyl-α-chymotrypsin (FA-Ct), an intermediate in the α-chymotrypsin catalyzed hydrolysis of 3-(2-furyl)acryloylimidazole (FAI).

α-Chymotrypsin-catalyzed hydrolysis of FAI proceeds through two distinct and separable phases; a relatively fast acylation phase characterized by steps 1 and 2 of scheme 1, followed by a much slower deacylation phase given as step 3.

We have previously shown using flow microcalorimetry that the enthalpy change for acylation can be determined accurately, without substantial interference from deacylation [3]. A full enthalpy profile for α-chymotrypsin-mediated hydrolysis of FAI has been determined at pH 7.8 and shown to be in complete agreement with the nonenzymic heat of FAI hydrolysis at this pH. The agreement demonstrates that the calorimetric methods developed for evaluating acylation and deacylation enthalpy changes are correct. In the present work, we provide a detailed calorimetric study of the enthalpy changes for acylation as a function of pH.

2. Experimental

2.1. 3-(2-Furyl)acryloylimidazole (FAI)

FAI was prepared by the mixed anhydride method described by Bernhard et al. [4], except that a third recrystallization from cyclohexane was performed; m.p. 114–115 °C.

Elemental analysis calculated 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 was purchased from Worthington Biochemical Corp. as three times recrystallized powder and further purified by the method of Yapel et al. [5], 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. [6]. Enzyme purities for the various Worthington lots as determined by titration were 92–95% pure or active using a molar absorptivity of 5×10^4 l mol^{-1} cm^{-1} for α-chymotrypsin at 280 nm and pH 5.0.

2.2. Flow calorimetry measurements

All acylation enthalpies were measured on an 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 to better than 0.0003 °C. Flow rates were compatible with acylation kinetics such that the entire reaction was completed during the residence time of the reaction mixing cell. 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 ensured 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 FAI (8×10^{-4} M) buffer solution to establish a steady-state voltage signal and then switching the buffer solution to buffered chymotrypsin ($5.0–6.6 \times 10^{-5}$ M) solution. The new steady-state voltage signal was proportional to the observed acylation enthalpy plus enzyme dilution heat. The enzyme dilution enthalpy was measured in separate experiments. At least 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 units of one another prior to mixing. The pH of the calorimeter effluent was closely monitored during the course of the experiment and seldom differed from the original pH by more than 0.02 pH units.

Observed acylation heat changes were measured as described above at fixed pH values from 5 to 9. The observed acylation enthalpy changes at each pH were measured in the presence of buffers

Table 1

Data on buffers used for calorimetry

pH	Buffer	pK_a	ΔH_{ion} (kcal/mol)	Ref.
5.00	0.05 M piperazine	5.6	7.12	30
	0.03 M citrate	4.761	0.58	31
	0.05 M acetate	4.756	−0.02	32
	0.05 M propionic acid	4.874	−0.14	33
5.50	0.05 M piperazine	5.6	7.12	30
	0.05 M Mes	6.095	4.50	34
	0.05 M acetate	4.756	−0.02	32
	0.05 M glutarate	5.420	−0.58	33
6.00	0.05 M piperazine	5.6	7.12	30
	0.05 M Mes	6.095	4.50	34
	0.033 M pyrophosphate	6.76	0.11	35
	0.03 M citrate	6.396	−0.80	32
6.50	0.05 M Mes	6.095	4.50	34
	0.09 M phosphate	7.20	1.13	35
	0.033 M pyrophosphate	6.76	0.11	35
	0.03 M citrate	6.396	−0.80	31
7.00	0.05 M Hepes	7.23	5.01	7
	0.09 M phosphate	7.20	1.13	7
	0.033 M pyrophosphate	6.76	0.11	35
	0.03 M citrate	6.396	−0.80	31
7.50	0.05 M *n*-glycylglycine	8.252	10.60	36
	0.05 M Hepes	7.23	5.01	7
	0.09 M phosphate	7.20	1.13	7
	0.033 M pyrophosphate	6.76	0.11	35
8.00	0.05 M Tris	8.07	11.30	37
	0.05 M *N*-glycylglycine	8.252	10.60	36
	0.05 M Hepes	7.23	5.01	7
	0.09 M phosphate	7.20	1.13	7
9.00	0.05 M histamine	9.756	11.00	38
	0.05 M serine	9.208	10.40	39
	0.033 M pyrophosphate	9.41	0.40	35

having different heats of ionization [7]. From these data the true acylation enthalpy at zero buffer ionization enthalpy was determined at each pH as well as the number of protons released by the enzyme-FAI system. The buffers used at each pH and their pertinent thermodynamic quantities are listed in table 1.

Nonlinear least-squares fitting was performed using a program provided by Dr Michael Johnson at the University of Virginia and described elsewhere [8].

3. Results

In order to ensure complete acylation, α-chymotrypsin was reacted with FAI under the condition of excess substrate (FAI ≫ α-Ct) in at least three different buffers at each specific pH. Buffers were selected to give a large range of ΔH_{ion} values and table 1 lists the buffers used, their pK_a values and heats of ionization.

Fig. 1 (a, b) demonstrates the dependence of the observed acylation enthalpy (ΔH_{obs}) on the buffer heats of ionization. The formation of acyl enzyme is generally accompanied by proton uptake or release and this affects ΔH_{obs}, since the buffer must accommodate the proton change. The relationship between ΔH_{obs}, ΔH_{ion}, and the acylation enthalpy change, ΔH^{app}_{Acyl}, is expressed by the simple relationship given in eq. 1 [7,9]:

$$\Delta H_{obs} = \Delta H^{app}_{Acyl} - n^{app}_{Acyl}\Delta H_{ion} \quad (1)$$

The data in fig. 1a and b have been fitted to eq. 1, giving n^{app}_{Acyl} and ΔH^{app}_{Acyl} as slope and intercept quantities, respectively. The correlation coefficients for all such data were 0.99 or better, showing uniformly good agreement between experiment, theory, and values used for ΔH_{ion} buffer. ΔH^{app}_{Acyl} is the apparent acylation enthalpy change in the limit of zero buffer ionization heat while n^{app}_{Acyl} represents the number of equivalents of protons released to the buffer as a result of converting enzyme and substrate to acyl-enzyme per equivalent of enzyme * [3].

The accuracy of the plots in fig. 1a and b is primarily dependent upon the range of buffer dissociation enthalpies and to a lesser degree on differences in ionic strengths used in these experiments in comparison to the literature values for

* The numerical value of n^{app}_{Acyl} will itself have a sign. A negative sign indicates proton uptake from the buffer solution 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. The term '*n*' has been corrected for the proton changes generated by imidazole as it appears as a product on acylation. A negative sign of *n* corresponds to proton uptake from the buffer by the free enzyme, FAI and acyl-enzyme and a positive sign indicates proton release to the buffer by FAI, acyl-enzyme and free enzyme.

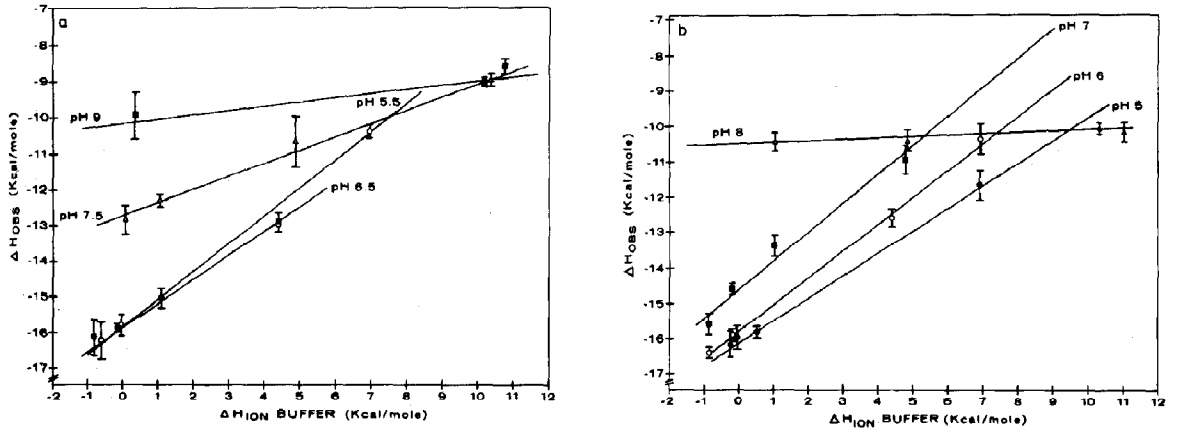


Fig. 1. (a, b) Determination of proton release and buffer-independent apparent acylation enthalpies. The buffers used, along with their corresponding ionization heats, are given in table 1. Slopes and intercepts were evaluated by linear least-squares analysis with points weighted with respect to the number of data points collected with each buffer. Final reaction solutions contained 25–35 μM α-chymotrypsin, 300–400 μM FAI, and 1.4–1.6% CH_3CN. Temperature was held constant at 25.00 ± 0.01°C.

which the ΔH_{ion} buffer data were reported. Most of the literature ΔH_{ion} data of table 1 are reported at zero and 0.1 ionic strength. These problems were found to affect the accuracy of the intercept values (ΔH^{app}_{Acyl}) to a much smaller extent than the slope (n^{app}_{Acyl}) values.

ΔH^{app}_{Acyl} and n^{app}_{Acyl} are apparent quantities since, in addition to buffer ionization heats, there are other enthalpy and proton release adjustments which must be made. One such adjustment becomes apparent from consideration of the overall acylation reaction (see eq. 2).

$$\alpha\text{-Chymotrypsin} + \text{FAI} \rightarrow \text{FA-Ct} + \text{imidazole} \quad (2)$$

It is obvious that both FAI and imidazole have the potential to protonate and deprotonate. On acylation, imidazole leaves the active site of the enzyme as a neutral molecule and the extent to which it becomes protonated depends on the pH of the surrounding buffer. The abstraction of a proton from solution by imidazole and its accompanying enthalpy change can be calculated by knowing the pK_a of imidazole (6.99 ± 0.02) and its ΔH_{ion} (8.79 ± 0.03 kcal/mol) [10,11]. ΔH^{app}_{Acyl} at each pH can be corrected for proton uptake by imidazole by the relationship given in eq. 3.

$$\Delta H_{Acyl} = \Delta H^{app}_{Acyl} + n_{Im}\Delta H_{ion\ Im} \quad (3)$$

where ΔH_{Acyl} represents the actual enthalpy change for the acylation reaction excluding the contribution of imidazole, $\Delta H_{ion\ Im}$ is the known heat of dissociation of imidazole [11], and n_{Im} denotes the proton release as calculated from the known pK_a of imidazole and pH.

The kinetics of acylation and deacylation are known as a function of pH and the flow rates for flow calorimetry experiments were adjusted such that the mixed solutions of α-Ct and FAI reside in the measuring compartment in the range of 30–45 s [3]. The residence time in the calorimeter is sufficient to ensure complete acylation of the enzyme and over much of the pH range, deacylation is insignificant. At the highest pH (pH 9), however, up to 15% of the acyl-enzyme formed within the calorimeter cell becomes deacylated within the residence time and since the experiments are carried out under substrate saturating conditions, any deacylation would be followed immediately by reacylation. The molar enthalpy given as ΔH^{app}_{Acyl} is calculated on the basis of the total amount of acyl-enzyme formed during the residence time of the calorimeter cell. No correction was made for deacylation enthalpy, since it has been determined to be very small (−1.2 ± 0.55 kcal/mol) [3].

Thus, the original observed acylation enthalpy change at a fixed pH has been corrected for buffer ionization heats, product (imidazole) ionization

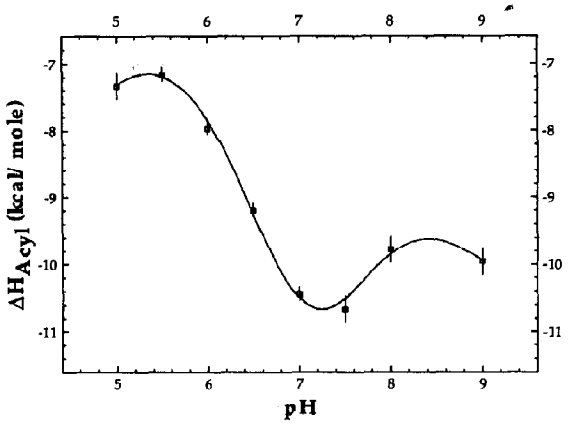


Fig. 2. pH dependence of corrected acylation enthalpy changes. Enthalpy values are free from effects of buffer ionization enthalpy changes and ionization enthalpy change of imidazole generated on acylation. The solid line is the nonlinear least-squares best fit of the data using eq. 6 and the pK_a values 6.8, 7.55, 8.8 and 4.0 for pK values 1–4 given in scheme 2.

heat, as well as a concentration normalization factor to account for deacylation and reacylation during the residence time of the calorimeter cell (maximally 15% reacylation at pH 9.0). The resultant quantity (ΔH_{Acyl}) represents the enthalpy change which deals only with the enzyme acylation and any perturbation of FAI ionization brought about during acylation at the pH of interest. A plot of ΔH_{Acyl} as a function of pH is given in fig. 2.

An additional quantity of interest is the number of protons released by the enzyme system (FAI, FA-α-chymotrypsin and α-chymotrypsin) on forming the acyl-enzyme. These quantities 'n' were determined at each pH of interest by use of eq. 4:

$$n = n^{app}_{Acyl} - n_{Im} \quad (4)$$

This value, n, is important, since it is an indication of the exchange of protons from ionizable residues on the enzyme due to the acylation reaction and any proton uptake or release by FAI on binding to α-Ct (see footnote on p. 305). A plot of n as a function of pH is given in fig. 3.

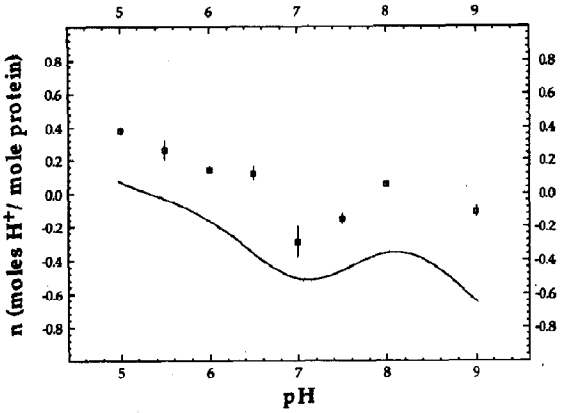


Fig. 3. pH dependence of proton release data generated in fig. 1a, b. Proton release data are corrected for proton uptake by imidazole generated on acylation. The solid line is a simulation using pK_a values 6.6, 7.55, 8.8 and 4.0 for pK values 1–4 given in scheme 2.

4. Discussion

Ionizations which accompany and attenuate enzyme catalysis are a focal point of many discussions of enzyme mechanisms. Chymotrypsin is particularly well studied in this regard but there has been some controversy as to the assignment of observed pK_a values to groups at the active site [12–14]. Regardless of the assignments, it is found with numerous substrates which obey the mechanism of scheme 1 that deacylation (step 3) is dependent on a group with an apparent pK_a of 7.0 ± 0.3, and k_2/K_s is dependent on groups with apparent pK_a values of 6.8 ± 0.3 and 8.8 ± 0.3 [1,15–17]. The pK_a values for steps 2 and 3 of scheme 1, 6.8 and 7.0, respectively, have usually been assigned to His 57 [16,18] though it has been suggested that the pK_a observed in the range of 6.5–7.0 should be assigned to Asp 102 [12–14]. There is general agreement that the apparent pK_a of about 8.8 involves the proton dissociation of the Ile 16 α-amino group, thereby disrupting the Asp 194-Ile 16 salt linkage. This effect also appears to influence the active-inactive transition known to occur with α-chymotrypsin [1,17].

A model which suggests His 57 imidazole as responsible for the pK_a values of 6.8 ± 0.3 and 7.0 ± 0.3 depicts His 57 ionization (pK_a 6.8) coupled with the actual acylation step of scheme 1 [19]. Once the acyl-enzyme is formed, the His 57

imidazole pK_a is perturbed to 7.0 ± 0.3 or higher as evident from the pH dependence of deacylation [19].

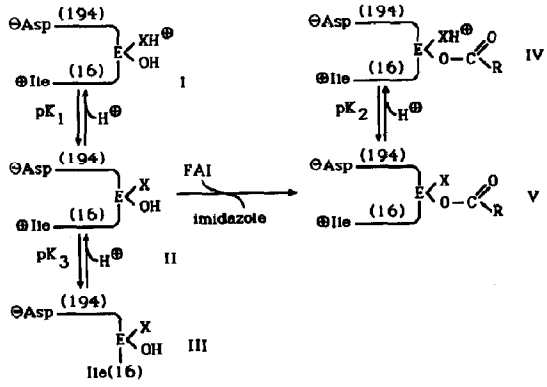
Specific substrates appear to perturb the His 57 imidazole pK_a only marginally while nonspecific substrates appear markedly to accentuate differences between pK_a values associated with steps 2 and 3. For example, the extent to which the pK_a in the free enzyme is altered in the acyl-enzyme has been reported for diphenylcarbamoylation ($6.6 \rightarrow 7.4$) [20], acetylation ($6.4 \rightarrow 7.3$) [21], cinnamoylation ($6.8 \rightarrow 7.2$) [22], and indoleacryloylation ($6.3 \rightarrow 7.7$) [23]. The latter two acylation products are transacryloyl derivatives belonging to the same family as the furylacryloyl acyl-enzyme formed in our experiments. We have found that FAI hydrolysis by α-chymotrypsin is accompanied by an apparent pK_a perturbation of $6.60 \rightarrow 7.55$ associated with acylation and deacylation, respectively [3]. The similarity in pK perturbation behavior among the nonspecific substrates suggests that the transacryloyl derivatives work very much alike.

To investigate further the nature of the apparent pK_a shifts, Keizer and Bernhard [23] directly determined proton release accompanying the stoichiometric acylation of α-chymotrypsin by indoleacryloylimidazole (IAI) over a wide pH range. A variation of the model they used to assess their data is given in scheme 2 and the expression derived for proton release is given by eq. 5.

$$n = n_{\text{intrinsic}} + \frac{[H^+]}{K_1 + [H^+]} - \frac{K_3}{K_3 + [H^+]} - \frac{[H^+]}{K_2 + [H^+]} + \frac{[H^+]}{K_4 + [H^+]} \qquad (5)$$

Here n represents protons released due to interaction of IAI with α-chymotrypsin to form the acyl-enzyme product. All acid dissociation constants given in eq. 5 are defined in scheme 2 except K_4 which represents the proton dissociation from protonated FAI. It is assumed in this model that only unprotonated FAI can bind to α-Ct.

From calorimetric experiments, using buffers with different ionization heats, we have obtained proton release data for α-chymotrypsin acylation by FAI. These data are presented in fig. 3 along



Scheme 2.

with a simulation of proton release expected using the model in scheme 2. The simulation uses our experimentally determined pK values of 6.6 and 7.55 as pK_1 and pK_2 along with the consensus pK_3 of 8.8 [1,16,17] for the Ile 16-Asp 194 salt bond. A pK of 4.0 estimated for FAI ionization was also used in the simulation. It is apparent that the complex shape of the theoretical curve is faithfully described by the experimental data but the data appear to be displaced from the theoretical curve. The values of n obtained from the calorimetric data are uncorrected for the active-inactive transition [17], protein dimerization effects [24], and the contribution from the appearance of furylacrylic acid (FAH) occurring in the basic pH range (0% FAH is formed at pH 7 but up to 15% occurs at pH 9). Such corrections are believed to account, in large part, for the difference between the calculated and observed data.

From the data of Horbett and Teller [24], proton release expected from enzyme dimerization effects over the pH range of 5–7 can be calculated under the conditions of the experiments reported here. From 0.1 to 0.3 mol proton per mol protein are released over this pH range due to the disruption of enzyme dimers when the acyl-enzyme is formed. Similar effects involving the chymotrypsin binding of benzeneboronic acid have been reported by Hanai [25]. It appears that disruption of enzyme dimers accounts for a substantial amount of the discrepancy between calculated and observed values of n.

In the basic pH range (e.g., at pH 9), a small amount of turnover of furylacryloylchymotrypsin occurs within the calorimetric experiment contributing up to 0.15 mol H^+ per mol protein. As with other acryloylimidazole substrates [4], the intrinsic hydrolysis of FAI becomes significant in the pH range of 8 and higher and we have no way of correcting for nonspecific hydrolysis of FAI brought about by groups on the surface of the enzyme. These two effects contribute to the difference between calculated and observed proton release in an increasing manner above pH 7. From the work of Fersht and Requena [17], the transition from the inactive form of the enzyme to the active form upon acylation is also likely to contribute to proton release data in the basic pH range.

How these extraneous effects contribute to the observed enthalpy changes is of major importance to the linkage relationships we are attempting to establish. Jones and Trowbridge [26] found in their calorimetric work a heat of chymotrypsin dimer dissociation of zero over the pH range 5–8 and we have determined the heat of ionization of furylacrylic acid to be 1.03 ± 0.07 kcal/mol [3]. Fersht [1] and Fersht and Requena [17] have evaluated active-inactive equilibrium constants at 25 and 37 °C over a wide pH range allowing determination of van't Hoff enthalpy changes. From these data, corrections can be estimated for the inactive to active conversion forced by acylation. These contributions over the pH range 6–9 are remarkably constant at 0.60 ± 0.20 kcal/mol though there appears to be a slight pH dependency between pH 5 and 6.

The important question for this study is how these proton release corrections affect the results of the enthalpy analysis. For all of the effects believed to account for the discrepancy between the calculated and observed data in fig. 3, the enthalpy change involving the effect is either athermal or very close to athermal. Thus, these effects will contribute essentially nothing to the enthalpy-pH profile presented in fig. 2. We have proceeded to analyze the results entirely within the framework of the events presented in scheme 2.

In accounting for each of the three pK processes evident in scheme 2, their enthalpy contributions should be directly equal to the proton release of each group, at a particular pH, multiplied by its corresponding apparent ionization heat. This transforms eq. 5 into eq. 6.

$$\Delta H_{\mathrm{Acyl}} = \Delta H_{\mathrm{int}} + \frac{\Delta H_1[\mathrm{H}^+]}{K_1 + [\mathrm{H}^+]} - \frac{\Delta H_3 K_3}{K_3 + [\mathrm{H}^+]} - \frac{\Delta H_2[\mathrm{H}^+]}{K_2 + [\mathrm{H}^+]} + \frac{\Delta H_4[\mathrm{H}^+]}{K_4 + [\mathrm{H}^+]} \qquad (6)$$

where $K_1 = 2.54 \times 10^{-7}$ M and ΔH_1 is the apparent ionization enthalpy for the p$K_a = 6.6$ group, $K_2 = 2.83 \times 10^{-8}$ M and ΔH_2 is the apparent ionization enthalpy for the 7.55 pK_a group, $K_3 = 1.74 \times 10^{-9}$ M and ΔH_3 is the apparent ionization enthalpy for the p$K_2 = 8.8$ group. ΔH_{int} represents the pH-independent intrinsic enthalpy

Table 2

Enthalpy changes associated with acylation

Values in parentheses represent the nonlinear least-squares (67%) confidence intervals for the parameters indicated.

Conditions	Enthalpy change (kcal/mol)				
	ΔH_{int}	ΔH_1	ΔH_2	ΔH_3	ΔH_4
p$K_1 = 6.6$, p$K_2 = 7.55$	−8.49	7.12	5.13	2.14	−7.39
p$K_3 = 8.8$, p$K_4 = 4.0$	(−8.94, −8.04)	(6.48, 7.78)	(4.21, 6.06)	(1.38, 2.90)	(−10.44, −4.34)
p$K_1 = 6.8$, p$K_2 = 7.55$	−7.90	8.66	7.61	3.03	−3.82
p$K_3 = 8.8$, p$K_4 = 4.0$	(−8.36, −7.42)	(7.89, 9.43)	(6.52, 8.70)	(2.25, 3.81)	(−6.61, −1.03)
p$K_1 = 6.8$, p$K_2 = 7.55$	−7.89	8.67	7.62	3.04	−1.73
p$K_3 = 8.8$, p$K_4 = 4.4$	(−8.37, −7.41)	(7.88, 9.47)	(6.50, 8.74)	(2.24, 3.83)	(−3.04, −0.40)

change associated with the acylation process. A nonlinear least-squares fit of the ΔH_{Acyl} data at various pH values according to eq. 6 is presented in fig. 2.

Table 2 presents results for nonlinear least-squares fitting of the acylation enthalpy data using different pK_a values. The first row in the table provides the evaluated enthalpy changes using our kinetically derived values for pK_1 and pK_2 [3], along with the consensus value of 8.8 for pK_3 [1,16,17] and an assumed pK_4 of 4.0. The second row presents the fitted values using the consensus pK values of 6.8 and 8.8 for pK_1 and pK_3, our previously determined value for pK_2, and an assumed value of 4.0 for pK_4. The third set of data explores the effect of changing the assumed value of pK_4 to 4.4.

It is seen that whereas the ionization enthalpy changes are affected substantially by slight variations in pK, the intrinsic enthalpy change is altered only modestly. It is the (pH-independent) intrinsic enthalpy change which is of major interest and we believe the best estimates of the fitted quantities are those obtained using the consensus values for pK_1 and pK_3, our pK_2 of 7.55, along with the assumed pK_4 of 4.0.

4.1. Ionization enthalpy changes

The assignment of functional groups at an enzyme active site by their apparent pK_a and ΔH_{ion} values is not necessarily straightforward. The apparent thermodynamic quantities need not be identical to those for a group freely exposed to water, since (1) the protein environment of the ionizing group may perturb the group ionization enthalpy change as well as the pK_a, or (2) a conformational change could be linked to the group ionization resulting in an apparent pK_a and enthalpy change which would be a composite of the group ionization plus the conformational contribution. It is worth pointing out, however, that the quantities in table 2 associated with the pK_1 ionizable group (group X, scheme 2) bear a striking resemblance to those of a free histidine imidazole ($pK \sim 7$, $\Delta H \sim 8.8$ kcal/mol) [10,11]. In fact, the simplest interpretation of the data in table 2 would be the assignment of group X (scheme 2) as a somewhat perturbed histidine imidazole ($pK_1 = 6.6$–6.8, $\Delta H_1 = 7.1$–8.7 kcal/mol) which, after formation of the acyl-enzyme, remains perturbed ($pK_2 = 7.55$, $\Delta H_2 = 5.1$–7.6 kcal/mol).

The pK_a and ΔH_{ion} values we have reported for the three apparent ionizations are comparable with those evaluated from other substrates used with α-chymotrypsin. Ionization enthalpy changes for ΔH_1 of 8.9 and 6.5 kcal/mol were independently evaluated by Hanai [25] and Jones and Trowbridge [26], respectively, from binding studies of benzeneboronic acid to chymotrypsin. Also, a value for ΔH_1 of 7.9 kcal/mol was reported by Tseng [27] from data involving the nonspecific substrate 2,4-dinitrophenyl trimethylacetate. These data, taken with ours, suggest that the ionization regarded by Bender et al. [16] as having an apparent pK of 6.8 ± 0.3 is accompanied by an ionization enthalpy change much like that of free imidazole.

Ionization enthalpy changes associated with the deacylation-dependent pK_a have been reported for a few different substrates. Tseng [27] has obtained ΔH_2 values of 6.59 and 7.01 kcal/mol derived from van't Hoff analyses of deacylation pK_a values for the substrates *p*-nitrophenyl acetate and *N*-acetyl-L-tyrosine ethyl ester, respectively. Rajender et al. [27a] reported a value of 4 kcal/mol for ΔH_2 from studies using *N*-acetyl-L-tryptophan ethyl ester. Our ΔH_2 value of 7.6 kcal/mol compares favorably with Tseng's values though the corresponding acyl-enzymes are distinctly different from FA-Ct. The thermodynamic differences for deacylation of various substrates are probably real, since different acyl-enzymes are likely to impose different constraints on the ionizable group resulting in some variation of apparent pK and ionization enthalpy changes.

The final ionization of importance for α-chymotrypsin involves the pK 8.76 group investigated by Fersht and Requena [17]. This particular ionization is believed to be important for maintaining the electrostatic linkage of Ile 16 and Asp 194. By taking Hess' law sums of the values reported by Fersht [1] and Fersht and Requena [17] one can calculate an apparent enthalpy change (ΔH_3) of 2.1 ± 4.2 kcal/mol. This is comparable

to the ΔH_3 value of 3.0 ± 0.75 kcal/mol derived from our analysis.

The pK designated as pK_4 represents the acid dissociation of FAI and we have assumed in the model given in scheme 2 that only neutral FAI can bind and react with α-Ct. Bender et al. [28] and also Ikeda and Kunugi [29] determined pK_a values of 3.6 and 4.4 for cinnamoylimidazole and indoleacryloylimidazole, two nonspecific substrates closely related to FAI. The pK values suggest that FAI will have a pK in the vicinity of 3.6–4.4. The fitting of enthalpy data using pK values of 4.0 and 4.4 demonstrates little effect of this ionization process on ΔH_{int}. According to the fitting, the FAI dissociation heat is observed to be negative and poorly determined, since this ionization process only partially affects the observed enthalpy at pH 5 and higher.

4.2. Intrinsic enthalpy of acylation

Without doubt, ionization processes as reflected in proton uptake or release are of major importance in the modulation of enzyme action. Regardless of their importance, evaluation of these processes does not evoke the interest that a detailed description of a substrate-enzyme interaction or a conformational change might. In fact, discussion of free energy or enthalpy profiles of catalyzed reactions dwell on substrate-enzyme interactions, conformational changes, product release, etc., often without mention of the attending ionization processes which greatly affect activity. If one is to speak of thermodynamic quantities in terms of elementary events, it is useful to deal with unitary and intrinsic thermodynamic quantities. The pH-independent quantity of -7.9 (-8.36, -7.42) kcal/mol reported here represents the intrinsic reaction in forming the acyl-enzyme intermediate exclusive of contributions from groups with pK_a values in the experimental pH range (5–9). The large and negative value for ΔH_{int} represents a considerable contribution to the driving force for acyl-enzyme formation involving FAI as a substrate.

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References

1 A.R. Fersht, J. Mol. Biol. 64 (1972) 497.
2 J. Wyman, Adv. Protein Chem. 4 (1948) 407.
3 J.L. Slightom and D.W. Bolen, J. Am. Chem. Soc. 102 (1980) 6318.
4 S.A. Bernhard, S.J. Lau and H. Noller, Biochemistry 4 (1965) 1108.
5 A. Yapel, M. Han, R. Lumry, A. Rosenberg and D.F. Shiao, J. Am. Chem. Soc. 88 (1966) 2573.
6 M.L. Bender, F.J. Kedzy and F.C. Wedler, J. Chem. Ed. 44 (1967) 84.
7 L. Beres and J.M. Sturtevant, Biochemistry 10 (1971) 2120.
8 M.J. Johnson and S.G. Frasier, Nonlinear least-squares analysis, eds. S.P. Colowick and N.O. Kaplan, part J (Academic Press, New York, 1985) p. 301.
9 D.H. Atha and G.K. Ackers, Biochemistry 13 (1974) 2376.
10 J.J. Christensen, D.P. Wrathall and R.M. Izatt, Anal. Chem. 40 (1968) 175.
11 I. Wadsö, Acta Chem. Scand. 16 (1962) 479.
12 R.E. Koeppe and R.M. Stroud, Biochemistry 15 (1976) 3450.
13 D.M. Blow, Acc. Chem. Res. 9 (1976) 145.
14 M. Hunkapiller, S.H. Smallcombe, D.R. Whitaker and J.H. Richards, J. Biol. Chem. 248 (1973) 8306.
15 M.L. Bender, G.E. Clement, F.J. Kezdy and H. d'A. Heek, J. Am. Chem. Soc. 86 (1964) 3680.
16 M.L. Bender, M.J. Gibian and D.J. Whelan, Proc. Natl. Acad. Sci. U.S.A. 56 (1966) 833.
17 A.R. Fersht and Y. Requena, J. Mol. Biol. 60 (1971) 279.
18 M.L. Bender and F.J. Kezdy, J. Am. Chem. Soc. 86 (1964) 3704.
19 J.L. Marini and M. Caplow, J. Am. Chem. Soc. 93 (1971) 5560.
20 B.F. Erlanger, H. Castleman and A.G. Cooper, J. Am. Chem. Soc. 85 (1963) 1872.
21 H. Gutfreund and J.M. Sturtevant, Proc. Natl. Acad. Sci. U.S.A. 42 (1956) 719.
22 M.L. Bender, G.R. Schonbaum and B. Zerner, J. Am. Chem. Soc. 84 (1962) 2562.
23 J. Keizer and S.A. Bernhard, Biochemistry 5 (1966) 4127.
24 T.A. Horbett and D.C. Teller, Biochemistry 13 (1974) 5490.
25 K. Hanai, J. Biochem. 79 (1976) 107.

26 J.B. Jones and C.G. Trowbridge, J. Biol. Chem. 258 (1983) 2135.
27 J.K. Tseng, Dissertation, University of North Dakota (1964).
27a S. Rajender, R. Lumry and M. Han, J. Phys. Chem. 75 (1971) 1375.
28 M.L. Bender, G.R. Schonbaum and B. Zerner, J. Am. Chem. Soc. 84 (1962) 2540.
29 K. Ikeda and S. Kunugi, J. Biochem. 88 (1980) 977.
30 P. Paoletti, M. Clampolini and A. Vacca, J. Phys. Chem. 67 (1963) 1065.
31 R.G. Bates and G.D. Pinching, J. Am. Chem. Soc. 71 (1949) 1274.
32 H.S. Harned and R.W. Ehlers, J. Am. Chem. Soc. 55 (1933) 1652.
33 J.J. Christensen, R.M. Izatt and L.D. Hansen, J. Am. Chem. Soc. 89 (1967) 213.
34 S.A. Rudolph and S.J. Gill, Biochemistry 13 (1974) 2451.
35 R.R. Irani and T.A. Taulli, J. Inorg. Nucl. Chem. 28 (1966) 1011.
36 A.P. Brunetti, M.C. Lim and G.N. Nancollas, J. Am. Chem. Soc. 90 (1968) 5120.
37 G. Öjeland and I. Wadsö, Acta Chem. Scand. 22 (1968) 2691.
38 S.N.R. Von Schalien, Suom. Kemistil. B32 (1959) 148.
39 N.E. Good, G.D. Winget, W. Winter, T.N. Connally, S. Izawa and R.M.M. Singh, Biochemistry 5 (1966) 467.