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The Effect of Temperature on the Individual Stages of the Hydrolysis of Non-Specific *p*-Nitrophenol Esters by α-Chymotrypsin

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Precise studies were performed on the effect of temperature on the rate and equilibrium parameters characterizing the individual stages of the α-chymotrypsin-catalysed hydrolysis of non-specific *p*-nitrophenol esters at pH7.40 and 8.50. At both pH values the results indicate that a sharp kinetic anomaly is observed in Arrhenius plots of these parameters for the binding and acylation stages of the process, but not for the deacylation stage. Detailed comparison with other kinetic studies was made, and a comparison with thermal transitions observed in α-chymotrypsin by using physical techniques was attempted. A detailed discussion of possible causes of the anomalies is given.

The two-intermediate mechanism (eqn. 1) has been well established for the α-chymotrypsin-catalysed hydrolysis of non-specific (kinetically poor) ester substrates under optimum conditions (Bender & Kezdy, 1965):

$$E+S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES_I \xrightarrow{k_{+2}} \begin{matrix} ES_{II} \\ + \\ P_1 \end{matrix} \xrightarrow{k_{+3}} E+P_2 \quad (1)$$

Despite some evidence to the contrary, a considerable amount of experimental data suggests that eqn. (1) also represents the mechanism by which specific (kinetically good) ester substrates are hydrolysed by the enzyme (Doonan *et al.*, 1970).

Studies of the effect of temperature on the kinetics of the system (eqn. 1) are becoming more common; however, the only stage studied in detail has been the deacylation of the acyl enzyme (characterized by k_{+3}) since this step is the easiest to observe kinetically (Martinek *et al.*, 1972; Marshall & Chen, 1973). Wedler *et al.* (1975) have studied the effect of temperature on the complex parameters $k_{cat.}[k_{+2}k_{+3}/(k_{+2}+k_{+3})]$ and $K_{m(app.)}[k_{+3}k_{-1}/(k_{+1}k_{+2}+k_{+1}k_{+3})]$ for a series of specific substrates at pH7.8 and 9.6, and Baggott & Klapper (1976) have described the effect of temperature on the deacylation of a series of *p*-nitrophenyl esters of 2-(5-*n*-alkyl)-furoates.

The need for comprehensive temperature–kinetic studies of the separate rate constants characterizing individual steps in an enzyme-catalyzed process has been clearly stated by Dixon & Webb (1971). Because of the considerable current interest in the physical properties of α-chymotrypsin–substrate–inhibitor systems it is clearly important to perform such studies. However, we know of no case where the resolution of the kinetic and thermodynamic parameters characterizing the individual stages of eqn. (1), has been extended *in toto* to the study of the effect of temperature on these parameters. To the best of our knowledge, the results reported in this paper are the first examples of such an investigation having been performed.

Although it would be most relevant to study the effect of temperature on α-chymotrypsin-specific substrate systems, the extreme rapidity of the reaction with these substrates makes precise evaluation of rate parameters difficult, even with the most sophisticated equipment. We have therefore studied the effect of temperature on parameters characterizing the individual stages of the mechanism of eqn. (1) for the series of non-specific substrates: *p*-nitrophenyl acetate, *p*-nitrophenyl propionate, *p*-nitrophenyl butyrate and *p*-nitrophenyl trimethylacetate at pH7.40 and 8.50. In the case of the acetate at pH7.40 and the trimethylacetate at pH8.50, the temperature variation of the parameters K_s (k_{-1}/k_{+1}), k_{+2} and k_{+3} have been precisely studied. Although in the case of the acetate, propionate and butyrate at pH8.50 the parameters studied were K_s/k_{+2} and k_{+3}. It should be particularly stressed that a change in rate-determining step in eqn. (1) will not affect the linearity of Arrhenius plots for any of these parameters.

Although methods for the determination of the rate parameters K_s, k_{+2} and k_{+3} have been known in principle for many years (Bender *et al.*, 1967), the attempted evaluation of the parameters (particularly K_s and k_{+2}) from steady-state kinetic data has proven unsatisfactory. The standard deviation in the estimate of k_{+2} for *p*-nitrophenyl trimethylacetate hydrolysis by α-chymotrypsin (Bender & Hamilton, 1962) was of the order of 33%, clearly too great an error margin for quantitative kinetic studies. Since the mechanism (eqn. 1) is of importance in the study of many

enzyme systems, we will consider in some detail the experimental and computational refinements necessary to determine precisely the kinetic rate and equilibrium constants studied.

Theory

Kinetic characterization of eqn. (1)

Under saturation conditions ($[S^0] \gg [E^0]$), and assuming the primary adsorptive step:

$$E + S \overset{K_s}{\rightleftharpoons} ES_1$$

to be a very rapid pre-equilibrium, the sets of dependent differential equations arising from eqn. (1) can be integrated in closed form. Several authors (e.g. Gutfreund & Sturtevant, 1956) have shown that the appearance of *p*-nitrophenoxide anion (abbreviated to P_1 in the equations) with time can be represented by an equation of the form:

$$[P_1] = At + B(1 - e^{-Ct}) \qquad (2)$$

Under the conditions $k_{+2} \gg k_{+3}$, and $k_{+2}[S^0] \gg k_{+3}K_s$ (both of which apply in this study at the two pH values used), the constants A, B and C can be represented by

$$A = k_{+3}[E^0][S^0]/\{[S^0] + K_{m(app.)}\} \qquad (3a)$$

$$B = [E^0][S^0]^2/\{[S^0] + K_{m(app.)}\}^2 \qquad (3b)$$

$$C = k_{+2}[S^0]/([S^0] + K_s) \qquad (3c)$$

Therefore, from the variation of A and C with initial substrate concentration ($[S^0]$) at constant enzyme concentration ($[E^0]$), the values of K_s, k_{+2} and k_{+3} can be calculated by using the appropriate linear transforms of eqn. (3). The precise determination of A, B, and C would appear, however, to present considerable problems. Several methods for the measurement of these parameters have been devised (Gutfreund & Sturtevant, 1956; Bender & Kezdy, 1962), all having the advantage of simplicity, but the disadvantage of being subjective and not allowing any estimate of the true error in the three derived parameters. In particular, owing to solubility limitations, the maximum attainable value of $[S^0]$ for *p*-nitrophenyl trimethylacetate at 25°C and pH 8.50 is less than $0.2K_s$. The determination of K_s and k_{+2} from plots of $[S^0]/C$ against $[S^0]$ will thus be subject to large errors unless very precise values of $[S^0]$ and C are available (Bender & Hamilton, 1962).

In the case of the acetate at pH 7.40, solubility limitations are not of particular importance (solubility $\approx K_s$) and standard methods allow precise evaluation of K_s and k_{+2}. For the trimethylacetate at pH 8.50 we have used non-linear least-squares regression to fit *p*-nitrophenoxide anion concentration–time data directly to an equation of functional form of eqn. (2). The method used is the iterative procedure described by McCalla (1967), and extended to a three-parameter equation of the form of eqn. (2). Initial values of the parameters A, B and C were calculated by various published methods (Gutfreund & Sturtevant, 1956; Bender & Kezdy, 1962). Convergence of the iterative procedure was found to be very rapid, even in cases where the initial estimates were varied by up to ±50% of the converged values. True standard deviations were obtained for the parameters during the regression procedure, and where required were converted into confidence limits by using a Student's *t* factor. Approximately 60 ($[P_1]$ against time) points were used in the determination of A, B and C at each $[S^0]$ value.

In the case of the acetate, propionate and butyrate at pH 8.50, only the deacylation rate constant (k_{+3}) was obtained from saturation kinetic studies. The parameter K_s/k_{+2} was obtained for these substrates at pH 8.50 from a study under single-turnover conditions ($[E^0] = [S^0]$), the method used will be detailed in the Results section of the present paper.

Experimental

Substrates

Preparations of the acetate, propionate, butyrate and trimethylacetate of *p*-nitrophenol were performed by using standard methods (Bender *et al.*, 1962). All substrates were recrystallized at least four times from aq. ethanol. The melting points were in close agreement with literature values (in the case of the butyrate the observed melting point was 10–12°C; no literature value was found). Hydrolysis of stock solutions of the esters by using NaOH (1 M; A.R. grade) after spectrophotometric measurement of the *p*-nitrophenoxide anion gave the purity in all cases as >99.5%.

Enzyme

α-Chymotrypsin was obtained as a three-times-recrystallized freeze-dried powder from Miles Seravac, Cape Town, South Africa, and was used without further purification. Titration with the trimethylacetate (Bender *et al.*, 1966) gave the purity of the enzyme as 83 ± 1%, much of the impurity apparently being water of crystallization (Bender *et al.*, 1967). Stock solutions of the enzyme were made up daily in distilled deionized water to give on dilution an active enzyme concentration of 10 μM. This concentration was used for all kinetic studies at pH 8.50; at pH 7.40 a concentration of 18 μM was used owing to the decreased extinction at this pH. Enzyme solutions were stored at 4°C during use, and were stable at this temperature for at least 48 h.

Buffers and solutions

All reactions at pH8.50±0.01 were performed in Tris/HCl buffer (0.02M), and at pH7.40±0.01 in sodium/potassium phosphate buffer (0.04M). The pH of the buffer was adjusted to exactly 8.50 or 7.40 at each reaction temperature by addition of HCl or NaOH (0.1M) to stock buffer solutions. Ionic strength changes caused by such adjustments were negligible.

Apparatus and experimental techniques

*pH*8.50. The release of *p*-nitrophenoxide anion at pH8.50 was followed by using a Unicam SP.500 series 2 u.v./visible spectrophotometer, the direct readout facility being connected to the 10mV scale of a Phillips PM8100 recorder. Reactions were started by the addition of stock substrate solution (in pure dry acetonitrile) to the temperature-equilibrated solution of the enzyme in buffer. The flattened tip of a glass rod (kept at cuvette temperature ±1°C) was used for addition and mixing. The total time between addition of substrate and recording the release of *p*-nitrophenoxide anion was less than 4s. In cases where relatively high concentrations of *p*-nitrophenyl trimethylacetate were being studied, reaction was initiated by addition of stock enzyme solution to a solution of substrate in the cuvette. In this way precipitation of substrate at the air/solution interface was prevented. In all reactions at pH8.50 the concentration of acetonitrile was maintained constant at 0.33%.

*pH*7.40. The release of *p*-nitrophenoxide anion at this pH was followed by using a Pye–Unicam SP.1700 double-beam spectrophotometer with an AR 25 linear recorder. The spectrophotometer was set up for operation with the sample compartment open as described previously (Adams, 1976). A single-syringe variation of the double-syringe rapid-mixing technique described previously (Adams, 1976) was used to follow the rapid acylation stage of the reaction. Buffer (1.40ml) was incubated in the reaction cuvette, while 1.60ml of stock enzyme solution (34μM) was incubated at cuvette temperature in a thermostatted syringe (2ml). Then 1min before initiation of the reaction 0.0300±0.0002ml of stock substrate *p*-nitrophenyl acetate in acetonitrile solution was added to the reaction cuvette. The reaction was then initiated by rapid injection of the enzyme solution into the reaction cuvette, A_{400} was continuously monitored. The syringe needle was replaced by a plastic tube (2mm internal diameter) to facilitate the very rapid injection of enzyme. The plastic tube was maintained at reaction temperature by withdrawing the enzyme solution 2cm from the mouth of the tube (to avoid mixing at the interface), and holding the jacketted syringe vertically above the controlling water bath with the injection tube immersed.

A typical reaction trace obtained by using this mixing technique is shown in Fig. 1; analysis of this trace to obtain the first-order acylation rate parameter C is shown in Fig. 2. This trace is typical, and illustrates the adequacy of this mixing method in the present study. The fastest 'burst' reaction rate measured for the enzyme–substrate system had a half-life of approx. 430ms, and it was shown that the spectrophotometer–recorder responded to a step change in extinction, equal to one-half the 'burst' extinction, in approx. 120ms. The response of the experimental system was therefore considerably faster than the fastest reaction rate measured. Experiments on the hydrolysis of the acetate in strong base have indicated that first-order rate constants of $3s^{-1}$ could be accurately measured with the present apparatus and technique.

For data obtained at both pH7.40 and 8.50, the observed value of the *p*-nitrophenoxide anion concentration was corrected for spontaneous hydrolysis of substrate by buffer during the enzymic hydrolysis.

Temperature control. Temperature control to better than ±0.05°C was achieved by using a thermostatting heater–cooler unit coupled to Unicam constant-temperature cuvette holders. Temperature measurement was made by using copper–constantan thermocouples immersed in the reaction solution. The e.m.f.

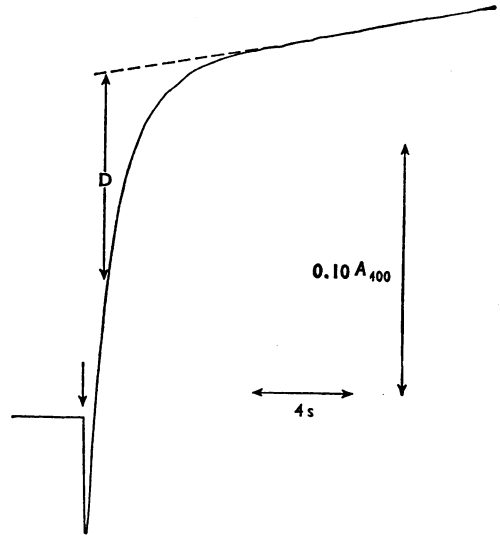

Fig. 1. *Hydrolysis of p-nitrophenyl acetate by α-chymotrypsin at pH* 7.40 *and* 25.06°C
This shows the resolution of the rapid acylation of the enzyme from the steady-state deacylation stage. The α-chymotrypsin and *p*-nitrophenyl acetate concentrations were 18μM and 0.6541mM respectively. The arrow shows where the enzyme was injected. D is equal to $(At+B)-P_1$, which is Be^{-Ct}.

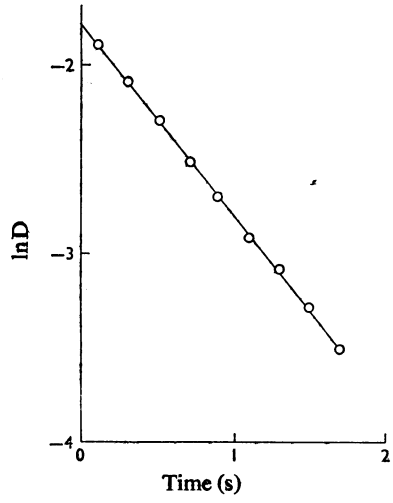

Fig. 2. *Analysis of the trace shown in Fig.* 1 *illustrating the precise first-order kinetic dependence of the acylation kinetics and the evaluation of the rate parameter C*
This plot gives the value of C as $0.994 \pm 0.013\,s^{-1}$.

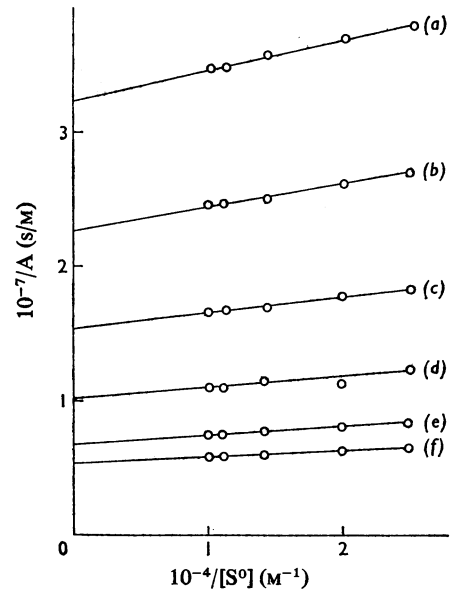

Fig. 3. *Reciprocal plots for the evaluation of the deacylation rate constant* (k_{+3}) *for the hydrolysis of p-nitrophenyl propionate by α-chymotrypsin at pH* 8.50

The temperatures for hydrolysis are (°C): (*a*) 11.94; (*b*) 16.15; (*c*) 20.29; (*d*) 25.46; (*e*) 29.70; (*f*) 32.72.

differential (and hence the temperature differential) between the reaction solution and the water bath was measured by immersing the reference junction in the circulating bath. The temperature of the circulating bath was measured by National Physical Laboratory using calibrated thermometers (calibrated in 0.05°C) and read to 0.01°C.

Determination of substrate concentration, [S^0]. The substrate concentration was determined on completion of each experimental run by addition of 0.50ml of 1M-NaOH to the reaction cuvette After complete hydrolysis of the substrate, A_{400} was measured, and [S^0] was calculated (with correction for dilution by the added NaOH) directly by using an extinction coefficient of 17990 litre·mol^{-1}·cm^{-1} for the *p*-nitrophenoxide anion.

Results and Discussion

Before presentation of the detailed results several points regarding the analysis of the data need to be clarified. In the calculation of K_s, k_{+2} and k_{+3} from the variation of A and C with [S^0] at constant [E^0], the ideal method of constant evaluation would be the non-linear regression method of Wilkinson (1961). In the present study this method cannot be applied for the following reasons. (i) In the case of the parameter determinations for *p*-nitrophenyl trimethylacetate at pH8.50, the limited solubility of the substrate confines the data to the origin region of the C against [S^0]curve (i.e. $[S^0] \gg K_s$). Attempted use of an iterative non-linear regression technique fails therefore, owing to non-convergence of the iterative process. (ii) In the determination of k_{+3} by using eqn. (3*a*), for all substrates, $[S^0] \gg K_{m(app.)}$ (limited by sensitivity considerations for the spectrophotometric monitoring of the reaction), and the experimental data are therefore confined to the region where A approaches its limiting maximal value. Again, non-linear regression techniques fail to converge, since data cannot be obtained in the optimum concentration region $[S^0] = 0.3\text{–}2K_{m(app.)}$.

In the determination of K_s and k_{+2} for the acetate at pH7.40, data could be obtained in the concentration region $[S^0] = 0\text{–}K_s$, and non-linear regression techniques converged. The parameter values obtained by using non-linear regression techniques were found to be virtually identical with those obtained by linear transformation of eqn. (3*c*).

Determination of the deacylation rate constant, k_{+3}

This rate constant was determined in the same way for all substrates. A reciprocal plot of 1/A against 1/[S^0] gives (see eqn. 3*a*) a straight line of slope $k_{+3}[E^0]/K_{m(app.)}$ and intercept $1/k_{+3}[E^0]$. Since [E^0] is known, k_{+3} can be evaluated. Fig. 3 shows data

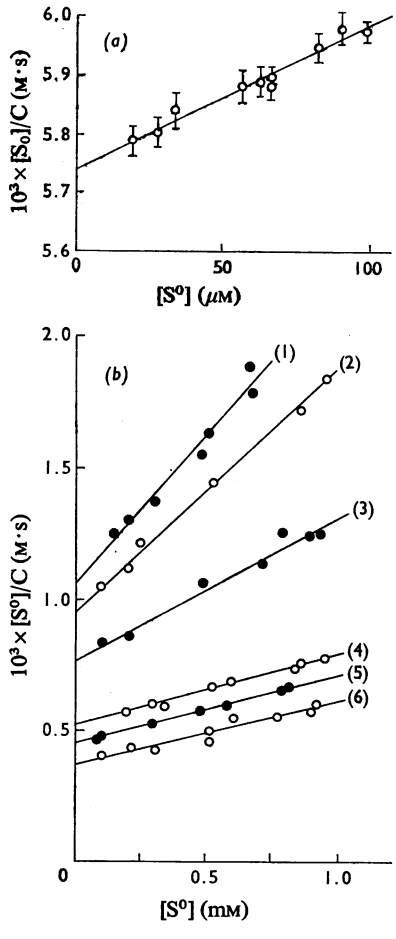

Fig. 4. *Evaluation of K_s/k_{+2} and k_{+2} for the hydrolysis of p-nitrophenyl trimethylacetate (a) and p-nitrophenyl acetate (b) by α-chymotrypsin*

(*a*) pH8.50 at a temperature of 24.46°C. Error limits shown correspond to 95% confidence limits in the rate parameter C. (*b*) pH7.40 at various temperatures. Six representative plots of data on the enzymic hydrolysis of *p*-nitrophenyl acetate at the following temperatures (°C) are shown: (1) 9.6; (2) 12.26; (3) 15.15; (4) 23.11; (5) 26.88; (6) 35.20. Other results at different temperatures have been omitted to avoid overcrowding of the Figure.

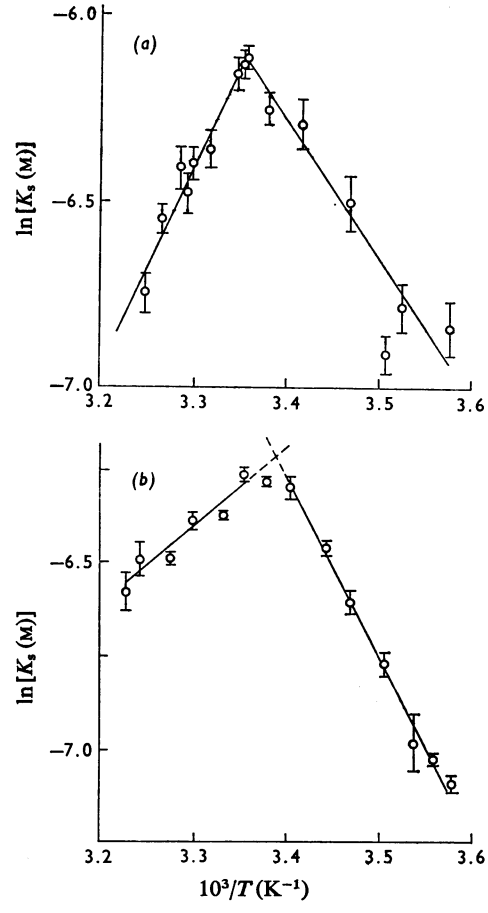

Fig. 5. *Van't Hoff plots for the pre-acylation equilibrium constant (K_s) obtained for the hydrolysis of p-nitrophenyl trimethylacetate (a) and p-nitrophenyl acetate (b) by α-chymotrypsin*

(*a*) pH8.50. The intersection of the lines occurs at 25.8°C. Error limits are 1 S.D. in K_s. (*b*) pH7.40. Error limits are 90% confidence limits in K_s. Intersection of the lines occurs at 21.8°C.

obtained from the enzymic hydrolysis of the propionate at pH8.50 plotted in this fashion. Plotting the results in either alternative form ([S°]/A against [S°] or A/[S°] against A) does not significantly alter the values of k_{+3} obtained. Use of a weighted least-squares procedure, as suggested by Wilkinson (1961), does not significantly alter the results.

Determination of k_{+2} and K_s for p-nitrophenyl acetate at pH7.40, and p-nitrophenyl trimethylacetate at pH8.50

From eqn. 3(*c*) a plot of [S°]/C against C should give a straight line of slope $1/k_{+2}$ and intercept K_s/k_{+2}. Fig. 4(*a*) shows the plot obtained for the enzymic hydrolysis of the trimethyl acetate at pH8.50 and 24.46°C; Fig. 4(*b*) shows a representative series of plots obtained from the enzymic hydrolysis of the acetate at pH7.40. All straight lines were fitted by using a linear least-squares criterion. The S.D. in k_{+2} for the hydrolysis of the trimethylacetate at pH8.50 lay between 2 and 8% of the value of k_{+2}, although for

the hydrolysis of the acetate at pH 7.40 the S.D. ranged between 2 and 11% of k_{+2}.

Determination of K_s/k_{+2} at pH 8.50

In the case of the acetate, propionate and butyrate at pH 8.50, the parameter K_s/k_{+2} was obtained by following the formation of the *p*-nitrophenoxide anion under conditions where $[E^0] = [S^0]$. Under this condition (single-turnover) the system *p*-nitrophenyl acetate–α-chymotrypsin has been reported to follow second-order kinetics with respect to *p*-nitrophenoxy anion formation, the second-order rate constant being k_{+2}/K_s [the exact conditions reported for second-order kinetics are $K_{m(app.)} < [E^0] \approx [S^0] \ll K_s$ (Bender & Kezdy, 1962; Bender *et al.*, 1966)].

We investigated the kinetics of the hydrolysis reaction under conditions where $[S^0]$ lay within ±3% of the active enzyme concentration, the ratios

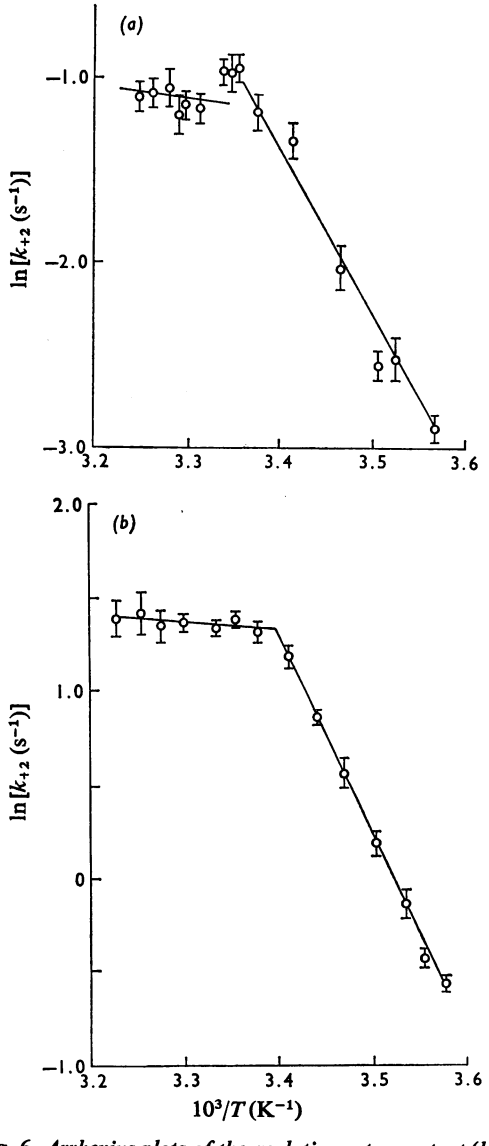

Fig. 6. *Arrhenius plots of the acylation rate constant (k_{+2}) for the hydrolysis of p-nitrophenyl trimethylacetate (a) and p-nitrophenyl acetate (b) by α-chymotrypsin*

(*a*) pH 8.50. (*b*) pH 7.40. Error limits are 90% confidence limits in k_{+2}. The intersection of the straight lines occurs at 20.9°C.

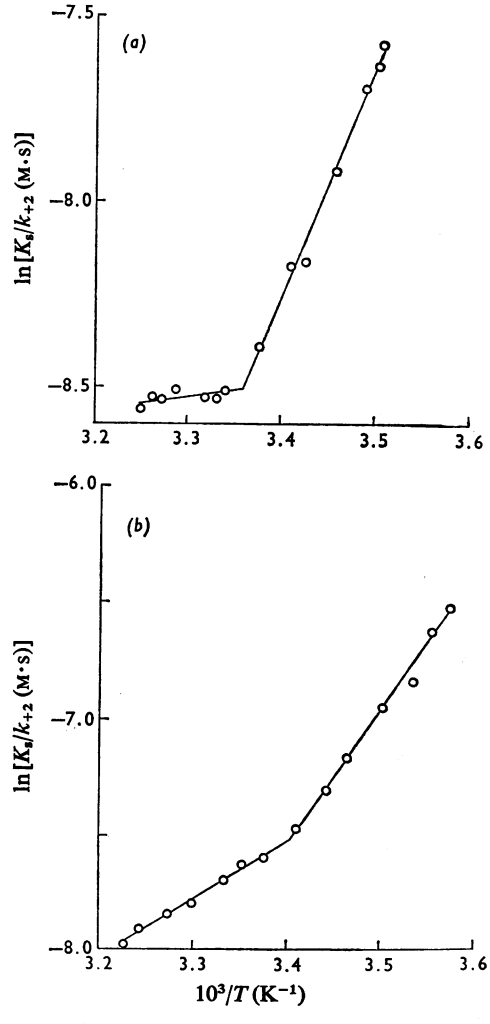

Fig. 7. *Arrhenius-type plots of the parameter K_s/k_{+2} for the hydrolysis of p-nitrophenyl propionate (a) and p-nitrophenyl acetate (b) by α-chymotrypsin*

(*a*) pH 8.50. Intersection of the two straight lines occurs at 24.5°C. (*b*) pH 7.40. Intersection of the two straight lines occurs at 20.1°C.

$K_s/[E^0]$ and $[E^0]/K_{m(app.)}$ being 10^2 and 10 (approx.) respectively. It was found that second-order kinetics were only observed in the initial 50% of the reaction range. However, the values of K_s/k_{+2} obtained at 25°C were in good agreement with other determinations of the parameter (Bender & Kezdy, 1962), and this method was therefore considered satisfactory.

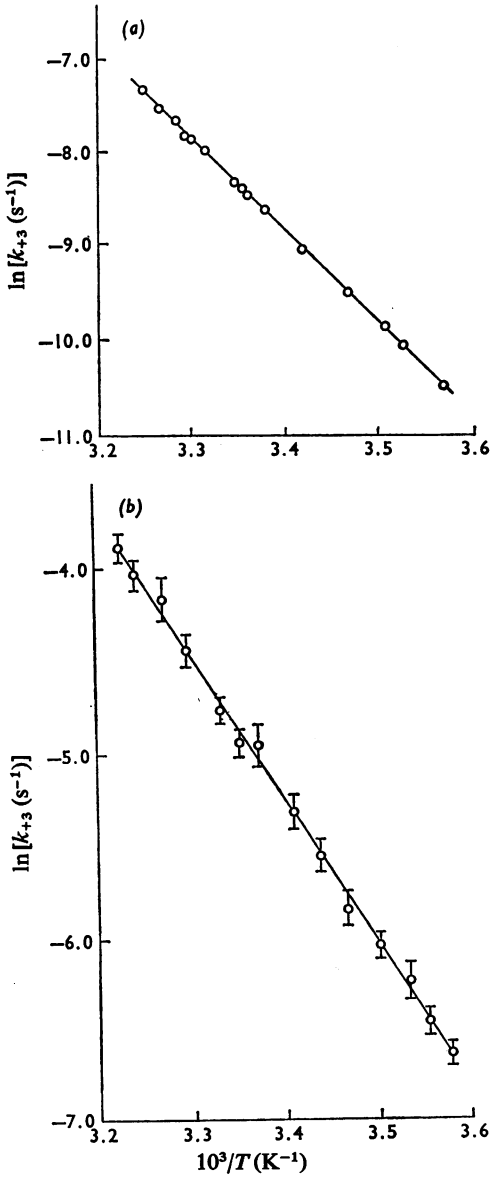

Fig. 8. *Arrhenius plots of the deacylation rate constant (k_{+3}) for the hydrolysis of p-nitrophenyl trimethylacetate (a) and p-nitrophenyl acetate (b) by α-chymotrypsin*
(*a*) pH 8.50; 90% confidence limits in the values of k_{+3} correspond approximately to the radii of the circles shown. (*b*) pH 7.40. Error limits correspond to 90% confidence limits in the rate constant.

Arrhenius and Van't Hoff plots of the rate and equilibrium parameters

To facilitate direct comparison of the results, data obtained at both pH values studied will be presented in graphical form.

Table 1. *Temperatures of anomalies observed in Arrhenius and Van't Hoff plots of kinetic and thermodynamic parameters, characterizing the α-chymotrypsin-catalysed hydrolysis of the acetate, propionate, butyrate and trimethylacetate of p-nitrophenol at pH 7.40 and 8.50*

Ester	pH	Kinetic parameter for which the anomaly is observed	Temperature of the anomaly (°C)
Acetate	8.50	K_s/k_{+2}	20.9
Propionate	8.50	K_s/k_{+2}	24.5
Butyrate	8.50	K_s/k_{+2}	29.3
Trimethylacetate	8.50	k_{+2}	26.2–28.0
Trimethylacetate	8.50	K_s	25.8
Acetate	7.40	k_{+2}	20.9
Acetate	7.40	K_s	21.8

Table 2. *Thermodynamic and pseudo-thermodynamic data for the α-chymotrypsin-catalysed hydrolysis of p-nitrophenyl esters at pH 8.50 and 7.40*
Error limits in enthalpies are 1 S.D. and in entropies are 95% confidence limits.

(*a*) Deacylation stage (pH 8.50)

Ester	ΔH^* (kJ/mol)	ΔS^* ($J \cdot K^{-1} \cdot mol^{-1}$)
Acetate	56.85±0.71	−94.6±1.7
Propionate	60.62±0.71	−80.2±1.9
Butyrate	67.49±1.9	−59.0±9.6
Trimethylacetate	78.1 ±0.6	−52.7±1.5
Acylation stage		
Trimethylacetate	75.4 ±4.6	0.0±17.16

(*b*) *p*-Nitrophenyl acetate (pH 7.40)

Pre-acylation stage (K_s)
279–293 K: $\Delta H = 40.4 \pm 0.84$ kJ/mol; $\Delta S = 84.9 \pm 6.7 J \cdot K^{-1} \cdot mol^{-1}$
296–309 K: $\Delta H = -16.3 \pm 1.1$ kJ/mol; $\Delta S = -107.2 \pm 7.1 J \cdot K^{-1} \cdot mol^{-1}$

Acylation stage (k_{+2})
279–293 K: $\Delta H^* = 85.3 \pm 1.5$ kJ/mol; $\Delta S^* = 56.1 \pm 5.4 J \cdot K^{-1} \cdot mol^{-1}$
296–309 K: $\Delta H^* = 1.42 \pm 0.66$ kJ/mol; $\Delta S^* = -229.0 \pm 2.5 J \cdot K^{-1} \cdot mol^{-1}$

Deacylation stage (k_{+3})
$\Delta H^* = 62.8 \pm 0.54$ kJ/mol; $\Delta S^* = -74.9 \pm 3.6 J \cdot K^{-1} \cdot mol^{-1}$

Arrhenius and Van't Hoff plots of the rate and equilibrium parameters obtained in this study, are shown in Figs. 5–8. In the case of Fig. 7(*a*), similar plots (showing sharp breaks) were obtained for the three substrates not included. The most noteworthy general point about these plots is, that although clear evidence of a sharp kinetic anomaly is observed in plots of the parameters characterizing the preacylation–acylation phase of the reaction (K_s, k_{+2} and K_s/k_{+2}), at both pH values studied, no evidence of anomalies is observed in any of the corresponding deacylation (k_{+3}) plots. The temperatures at which the anomalies are observed are given in Table 1, as can be seen, all anomalies lie in the range 20–30°C. The deacylation Arrhenius plots do not exhibit any evidence of the marked deviations from linearity observed by Talsky (1971) for many enzyme systems, when the temperature variation of the initial reaction rate if observed at small temperature intervals (2°C). This observation gives weight to the suggestion of Adams *et al.* (1976) that the anomalies observed by Talsky (1971) are due to the plotting of a complex rate parameter (initial rate) in place of true rate constants in the Arrhenius equation.

Thermodynamic and pseudo-thermodynamic activation parameters for the three stages of the reaction have been calculated from the rate–equilibrium constant data, and are shown in Table 2. The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ may be compared with other values of these parameters for the deacylation stage (Martinek *et al.*, 1972; Marshall & Chen, 1973).

Comparison of the activation parameters for the acylation and deacylation stages (below the anomaly temperature) shows that for both the acetate and the trimethylacetate the acylation stage is kinetically more specific than deacylation mainly due to more favourable $\Delta S^{\ddagger}$, the contribution to the rate enhancement being 65% due to $\Delta S^{\ddagger}$ for the acetate, and 85% due to $\Delta S^{\ddagger}$ for the trimethylacetate. This suggests a possible interaction (during activated complex-formation) of the *p*-nitrophenyl group with some locus in the active site. This would perhaps be similar to the hydrophobic interaction locus (B), proposed by Ingles & Knowles (1967) in their studies on specificity and stereospecificity in α-chymotrypsin catalysis.

Our results are consistent with the existence of a kinetic anomaly at approx. 25°C in the pre-acylation–acylation stage of the hydrolysis of non-specific ester substrates by α-chymotrypsin. In the case of specific ester substrates, limited available literature data for the deacylation stage only (Kaplan & Laidler, 1967; Glick, 1971) show that sharp kinetic anomalies occur at approx. 25°C in Arrhenius plots of k_{+3} for these substrates. Although the point will be discussed in more detail later in this paper, it is relevant here to point out that the occurrence or non-occurrence of anomalies in the deacylation stage of the hydrolysis seems to be related to the specificity of the deacylation stage. The more rapid the deacylation (i.e. the more kinetically specific), the greater the probability that kinetic anomalies will be observed.

The explanation of the kinetic anomalies in terms of rapid reversible denaturation of the enzyme (Kaplan & Laidler, 1967; Fersht & Requena, 1971) is not in accordance with the experimental evidence on deacylation kinetics. First, such denaturation would lead to a deacylation Arrhenius plot that showed the intersection of a straight line with a curve (e.g. Maier *et al.*, 1955), not (as clearly shown in Kaplan & Laidler, 1967) the sharp intersection of two straight lines. Secondly, if the mechanism of eqn. (1) is modified to include the equilibrium:

$$E_{active} \overset{K_{eq.}}{\rightleftharpoons} E_{inactive}$$

then it is easily shown that $k_{+3(obs.)} = k_{+3(true)}/(1+K_{eq.})$. The observed deacylation rate constant is therefore dependent only on the true rate constant and the equilibrium constant $K_{eq.}$. Obviously, if rapid reversible denaturation were a valid explanation of the observed anomalies, then the deacylation Arrhenius plots (for the substrates) studied in this work should also show deviations from linearity.

In connexion with these arguments, the results obtained by ourselves at pH7.40 are of particular relevance, since at this pH the amount of inactive enzyme, measured by Fersht & Requena (1971), is minimum. However, the Arrhenius plots for K_s and k_{+2} (Figs. 5 and 6*b*) still show clear evidence of a sharp kinetic anomaly.

Physical techniques (optical rotary dispersion, difference spectroscopy etc.) have been used to study the properties of α-chymotrypsin solutions with increasing temperature at low pH (Havsteen *et al.*, 1963; Biltonen & Lumry, 1969*a*,*b*). These studies show that a partial unfolding of α-chymotrypsin occurs between 20° and 50°C, and have been interpreted in terms of a two-state transition between different forms of α-chymotrypsin. Although direct comparison with our results is not possible, because of the differing pH values at which the studies were performed, several interesting parallels are observed.

First, the temperature at which the physical properties of the α-chymotrypsin solutions begin to change is approx. 25°C. Secondly, the studies by Havsteen *et al.* (1963) indicate that the temperature at which the physical property observed begins to change depends on the acyl-chymotrypsin studied, and varies between 20° and 30°C. Both of these observations are in agreement with our kinetic studies, particularly noteworthy being the fact that the start of the physically observed transition for acetyl-chymotrypsin occurs between 20° and 22°C [Havsteen *et al.* (1963), by two different methods], whereas the kinetic anomalies for the acetate obtained in this study lay between 20.1° and 21.5°C at

the two pH values studied. This constancy of the anomaly temperature strongly suggests that the kinetic transition is indeed related to the start of the unfolding process observed by physical techniques.

Other studies, on the rate of oxidation of surface and buried methionine residues in α-chymotrypsin (Hofmann & Wasi, 1973), indicate that the protein begins to unfold at approx. 25°C.

In the light of these results, the kinetic anomalies observed in this and other studies can reasonably be regarded as having their origin in the thermal unfolding of α-chymotrypsin in aqueous solution, the transition being virtually pH-independent [when our results are combined with those of Wedler *et al.* (1975), it is seen that sharp kinetic anomalies are observed between pH7.4 and 9.6, regardless of any possible relationship with the physical transitions observed between pH2 and 4].

Two points immediately arise on comparing anomalies observed by kinetic and physical techniques. First, the kinetic anomalies are 'sharp' (i.e. occur over a temperature range of <3°C), whereas the anomalies observed by physical techniques occur over a range of approx. 30°C. Secondly, the question arises as to what physical factor is responsible for the structural change in the protein between 20° and 50°C. The first point is explained by the consideration that the physical techniques used to study temperature-induced transitions observe structural changes in the molecule as a whole. In contrast, kinetic observations will be a specific indicator of changes occurring in the active site, and will be relatively unaffected by the unfolding of other regions of the protein structure. On this basis, it can be postulated that the active site of α-chymotrypsin undergoes some distortion of initiation of thermal unfolding of the protein at approx. 25°C; at temperatures above 30°C, however, the further major unfolding of the protein that occurs leaves the active site relatively unaltered.

Glick (1971) has speculated that the physical basis of the kinetic anomalies observed for α-chymotrypsin lies in some 'as-yet undiscovered thermally induced change near 290K' occurring in the properties of water. Since hydration plays a very considerable role in maintaining the structural stability of proteins in aqueous solution, this conclusion seems entirely reasonable. We would point out that several physical properties of water show anomalous behaviour between 15° and 35°C (e.g. surface tension and specific heat), which reflect changes in the forces between water molecules (Ling, 1967). Such changes are bound to have a considerable effect on the protein in aqueous solution, and it is therefore not surprising that temperature anomalies due to structural change are exhibited by α-chymotrypsin in this temperature range.

An interesting aspect of the results presented in the present paper is the remarkable inversion observed in the thermodynamics of binding of the substrate to the protein at both pH values studied (Figs. 5*a* and 5*b*). Some years ago Canady *et al.* (1969) observed non-linearities in Van't Hoff plots of the inhibition constant K_I, for the interaction of α-chymotrypsin with several inhibitors. In one case (benzoate anion) inversion was observed and in the case of all aromatic inhibitors non-linearity became apparent at approx. 25°C. It would appear, from the results presented by ourselves, that the major differences observed in the catalytic properties of α-chymotrypsin above and below the kinetic transition temperature arise from radical differences in the binding of the substrate to the enzyme. The fact that major changes in the overall protein structure are not detectable by physical techniques over the temperature ranges where gross kinetic anomalies are observed can be taken to indicate the sensitivity of the catalytic process to very small changes in the dimensions of the active site.

Regarding the absence of kinetic anomalies in the deacylation Arrhenius plots presented here, we would point out an apparent parallel that exists between the specificity of deacylation and the appearance of anomalies in deacylation Arrhenius plots, the more specific the deacylation process the greater being the probability that a kinetic anomaly will be observed. The elegant studies by Ingles & Knowles (1967) on the relationship between deacylation specificity in α-chymotrypsin catalysis, and the nature of the binding in the acyl enzyme, allow an interpretation of these observations consistent with the hypothesis presented by ourselves; namely that the kinetic anomalies arise from some small change in the dimensions of the active site. Basically, Ingles & Knowles proposed that in the most specific deacylation reactions (involving aromatic amino acid ester substrates of the '1' series) interaction between the acyl moiety and the enzyme occurred at three points, whereas in the least specific deacylations (e.g. with substrates of the type studied here) interaction occurred at only one point within the active site. If, without considering the detailed nature of the interactions, we now examine the effect of a small change in active-site dimensions on these binding situations, the possible cause of the occurrence or non-occurrence of anomalies become apparent.

When interaction occurs at only one point in the acyl-enzyme, the deacylation will be non-specific, owing to the possession of a relatively high degree of rotational freedom by the acyl moiety in the active site. Any change in the active-site dimensions will not alter the binding at the single interaction point, and therefore non-specific deacylation processes will not exhibit any marked anomalies in deacylation Arrhenius plots. On the other hand, an acyl moiety bound in the active site at three points is rigidly positioned for specific catalysis; change in active-site dimensions will now radically affect the ability to bind at all three points simultaneously, resulting in binding at two or

possibly only one point above the transition. The acyl moiety now possesses more degrees of freedom than when bound at three points, and deacylation will be less specific, resulting in an anomalous Arrhenius plot.

This type of argument can easily be extended to explain why anomalies are exhibited in the first two stages of the catalytic process for the substrates studied here, but not in the deacylation stage. Again, the participation of the *p*-nitrophenyl group in the binding of the substrate in the active site must be invoked, and it can be postulated that in the complex ES_1 binding occurs at two points, whereas in the acyl-enzyme binding only occurs at one point. The argument then parallels that given above.

Conclusions

We would summarize our conclusion by making the following points. (i) The kinetic transitions observed are best explained as having their origin in a thermally induced pH-independent transition in the enzyme at approx. 25°C. The transition is regarded as being between two forms of differing activity, and not between an active and an inactive form of the enzyme. (ii) Differences in the activity of the two forms arises from differences in the mode of binding of the substrate within the active site. The relationship between specificity and occurrence of kinetic anomalies can be adequately explained by extending the arguments of Ingles & Knowles (1967) on the relationship between number of binding interactions and deacylation specificity. (iii) The active-site transition may be regarded as a substate transition occurring within a major two-state thermal transition observed by physical techniques.

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