α-CHYMOTRYPSIN: A STUDY OF THE PROMOTION OF THE DEACYLATION REACTION

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Summary

The effect of indole on the rate of deacylation of acetyl-chymotrypsin has been investigated in detail. In particular, the effects of indole concentration and pH on the magnitude of the increase in the deacylation rate constant have been determined. The deacylation of other acyl-chymotrypsins (formyl-, propionyl-, and *N*-formylglycyl-) in the presence of indole has been studied, and benzene, acetamide, and *N*-acetyltryptamine have been tested as modifiers of the deacylation of formyl-chymotrypsin. The largest rate enhancement observed was about threefold. Several possible mechanisms for the promotion of the deacylation of acyl-chymotrypsins by hydrophobic compounds are discussed in the light of the results obtained.

I. Introduction

α-Chymotrypsin (E.C. 3.4.4.5) has probably been studied more than any other enzyme and a reasonably comprehensive picture of its mechanism of action has emerged. Detailed three-dimensional structures of the enzyme and of its toluene-sulphonyl-derivative have been reported (Matthews *et al.* 1967; Blow 1969), and an X-ray crystallographic study is in progress on the structures of complexes of the enzyme with substrate analogues (Blow 1969). The α-chymotrypsin-catalysed hydrolysis of good substrates, as well as that of bad substrates, appears to proceed by the formation and subsequent deacylation of acyl-enzyme intermediates which are derivatives of Ser-195. Extensive specificity studies have shown that good substrates are derivatives of L-tyrosine, L-tryptophan, and L-phenylalanine in which the amino groups are acylated (Bender, Kézdy, and Gunter 1964; Ingles and Knowles 1968). Considering the deacylation reaction (for which the most accurate kinetic data are available), a typical good substrate has a deacylation rate constant of about 100 sec^{-1}. *p*-Nitrophenyl acetate, a poor substrate, has a deacylation rate constant of 0·007 sec^{-1}. Thus, a factor of about 10^4 exists between the deacylation rate constants of a good and a poor substrate. This factor must be explained largely by the interaction of the acyl group with the active site of the enzyme. More specifically, the presence of an α-acylamino group and a β-hydrophobic substituent is required for rapid deacylation rates to be achieved. The details of the interactions of these groups with the enzyme and the mechanism whereby these interactions result in rapid deacylation rates remain to be elucidated.

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One line of investigation has provided results which shed some light on the importance of the interactions of good substrates with enzymes. It has been found that in some instances, the rate of reaction of an enzyme with a *partial* substrate is accelerated by compounds related to good substrates. For example, the reaction of trypsin with *N*-acetylglycine ethyl ester is accelerated by alkylamines and alkylguanidines (Inagami and Murachi 1964; Inagami and York 1968). Several examples of the promotion of the reactions of α-chymotrypsin have been reported (Foster 1961; Ponzi and Hein 1966; Wallace *et al* 1966). In no case, however, has the mechanism of the observed promotion been investigated in any detail, and in all cases, the rate enhancements have been small. Foster (1961) showed that the deacylation of acetylchymotrypsin was accelerated by indole. In the present work, the observations of Foster have been extended to a number of acyl-enzymes and modifiers.

II. Materials and Methods

(*a*) *Materials*

The preparation and properties of *p*-nitrophenyl acetate, *N*-formylglycine *p*-nitrophenyl ester, and *N-trans*-cinnamoylimidazole have been described previously (de Jersey, Willadsen, and Zerner 1969). *p*-Nitrophenyl formate was prepared from formic acid and *p*-nitrophenol by the action of dicyclohexylcarbodiimide in tetrahydrofuran. Recrystallization from carbon tetrachloride gave colourless crystals, m.p. 72–73°C, lit. (Ohawa and Hase 1963) m.p. 72–74°C. *p*-Nitrophenyl propionate was prepared from propionyl chloride, *p*-nitrophenol, and pyridine in dry ether, and recrystallized from n-butanol–hexane, m.p. 63–64°C, lit. (Fife 1965) m.p. 62–63°C. Indole (British Drug Houses) was recrystallized several times from light petroleum. *N*-Acetyltryptamine was prepared from tryptamine hydrochloride and acetic anhydride, and recrystallized from ether–light petroleum, m.p. 74–77°C, lit. (Kralt and Moed 1963) m.p. 82°C. The *N*-acetyltryptamine was shown to be free of tryptamine by thin-layer chromatography on silica gel with methanol as solvent. Acetamide (Fluka AG, purum), benzene (British Drug Houses, Analar), and acetonitrile (Eastman Organic Chemicals, Spectro grade) were used without further purification. α-Chymotrypsin (crystallized three times) was obtained from Worthington Biochemical Corporation. Buffers were prepared using analytical grade reagents, the ionic strengths being adjusted to 0·2 by the addition of KCl.

(*b*) *Methods*

Spectrophotometric measurements were made using a Unicam SP.800 recording spectrophotometer fitted with an SP.850 control panel which allows scale expansions of 0·5–20. The spectrophotometer was coupled to a Heath servo-recorder (model EUW 20A). The cell compartment was thermostatted at 25±0·2°C, and all kinetic measurements were made at this temperature. Hydrolysis of the *p*-nitrophenyl esters was followed at 400 nm at pH's above 6·6, and at 347·5 nm at pH's below 6·6. Substrates and modifiers, with the exception of benzene, were made up in acetonitrile which has been shown to have no effect on the deacylation reaction at concentrations up to 20% (v/v) (Kézdy and Bender 1962). Two main procedures were used in setting up the reaction mixtures: aliquots (usually 100 μl) of modifier, substrate, and enzyme were added to 3 ml of buffer equilibrated in the cell compartment of the spectrophotometer; or aliquots of modifier and substrate were added to 3 ml of enzyme solution. Aliquots of substrate, modifier, and enzyme were added on flat-ended stirring rods. In each series of experiments, the acetonitrile concentration was kept constant and $\Delta\epsilon$ for the hydrolysis was determined using a standard solution of *p*-nitrophenol. Corrections for spontaneous hydrolysis of the esters had to be made but these were only significant for *p*-nitrophenyl formate and *N*-formylglycine *p*-nitrophenyl ester. The modifiers tested were found to have no effect on the spontaneous hydrolysis of the esters. Kinetic data were analysed using the HYPER program of Cleland (1967). Solutions of α-chymotrypsin were prepared by dissolving a known weight of enzyme in acetate or phosphate

buffer. The normality of enzyme solutions was routinely determined by titration with *p*-nitrophenyl acetate (Bender *et al.* 1966). The results of the titration with *p*-nitrophenyl acetate were found to agree within experimental error with the results obtained by titration with *N-trans*-cinnamoylimidazole (Schonbaum, Zerner, and Bender 1961).

III. Results

(*a*) *Deacylation of Acetyl-Chymotrypsin in the Presence of Indole*

Foster (1961) showed that 1 mM indole caused a 1·6-fold increase in the steady-state rate of the reaction of α-chymotrypsin with *p*-nitrophenyl acetate. In the present work, the steady-state reaction has been studied at a range of concentrations of indole (0–5 mM) and at a number of pH's from 6·6–7·7. Figure 1 shows representative results of the experiment carried out at pH 6·9$_4$. Under the conditions used, the size of the "burst"* was constant at all concentrations of indole. Hence, although indole inhibited the pre-steady-state reaction of α-chymotrypsin with *p*-nitrophenyl

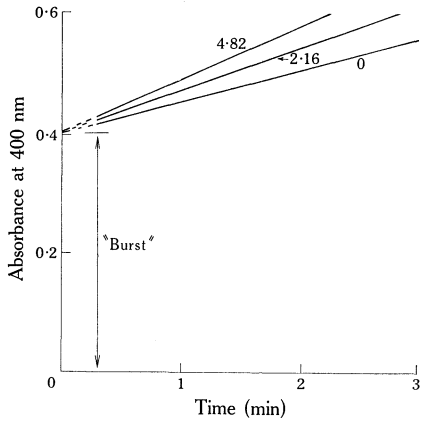


Fig. 1.—Hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin under steady-state conditions at three concentrations of indole (zero, 2·16, and 4·82 mM) in 0·05M phosphate buffer, pH 6·9$_4$; $[S]_0$ = 1·21 mM; $[E]_0 = 3{\cdot}6 \times 10^{-5}$M; 6·1% (v/v) acetonitrile; 25°C.

acetate (Foster 1961), k_{+2} (the acylation rate constant) is much greater than k_{+3} (the deacylation rate constant) and $[S]_0$ is much greater than K_m at all concentrations of indole, according to the equation of Stewart and Ouellet (1959). Figure 2 shows a plot of $k_{cat.}$ ($= V/[E]_0$) versus indole concentration for the reaction at pH 6·9$_4$.

The constant size of the burst indicates that only the deacylation reaction need be considered in any kinetic scheme to explain the results. Such a kinetic scheme is given in equation 1:

$$\begin{array}{lll} ES' & \xrightarrow{k_{+3}} & E+P_2 \\ K_{In} \Big\| \text{ indole} & & \\ ES' \text{ . indole} & \xrightarrow{k^{In}_{+3}} & E+P_2+\text{indole} \end{array} \qquad (1)$$

where ES' is the acyl-enzyme, E is the free enzyme, P_2 is the product acid, and K_{In} is

* The term "burst" refers to the rapid reaction which occurs when enzyme and substrate are mixed, during which the concentration of the acyl-enzyme (ES') increases to its steady-state concentration. The size of the burst is taken as the amount of *p*-nitrophenol released in this rapid reaction.

the dissociation constant and k_{+3}^{In} the deacylation rate constant of the complex of indole with the acyl-enzyme. From equation (1), the following relationships may be derived:

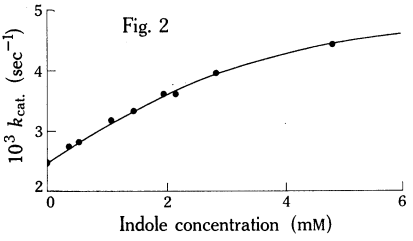
$$V = [E]_0(k_{+3}K_{In}+k_{+3}^{In}[\text{indole}])/(K_{In}+[\text{indole}]).$$

If V_0 = velocity when indole concentration is zero, then

$$V-V_0 = [E]_0(k_{+3}^{In}-k_{+3})([\text{indole}])/(K_{In}+[\text{indole}]).$$

i.e.
$$[E]_0/(V-V_0) = K_{In}/\{(k_{+3}^{In}-k_{+3})([\text{indole}])\}+1/(k_{+3}^{In}-k_{+3})$$
$$= 1/(k_{cat.}-k_{cat.}^0),$$

where $k_{cat.}^0$ is the value for $k_{cat.}$ at zero indole concentration. Therefore a plot of the reciprocal of $(k_{cat.}-k_{cat.}^0)$ against the reciprocal of the indole concentration should



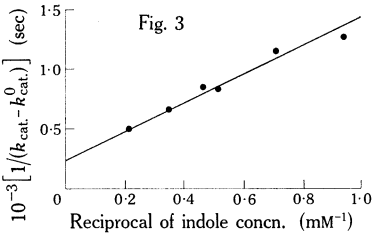


Fig. 2.—Effect of indole concentration on the rate of deacylation of acetyl-chymotrypsin at pH $6{\cdot}9_4$; experimental details as for Figure 1.

Fig. 3.—Double reciprocal plot of the data in Figure 2; slope = $K_{In}/(k_{+3}^{In}-k_{+3})$; intercept = $1/(k_{+3}^{In}-k_{+3})$. The graph shows the line of best fit as determined by computer evaluation of the data ($k_{+3}^{In} = 6{\cdot}5\times10^{-3}$ sec^{-1}; $K = 4{\cdot}9$ mM).

be linear and allow the determination of k_{+3}^{In} and K_{In}. Figure 3 shows such a plot, in which the data of Figure 2 were used. Values of k_{+3}, k_{+3}^{In}, and K_{In} determined at a number of pH values are listed in Table 1.

TABLE 1

KINETIC CONSTANTS FOR THE EFFECT OF INDOLE ON THE DEACYLATION OF ACETYL-CHYMOTRYPSIN AT DIFFERENT pH VALUES

Reaction measured in 0·05M phosphate buffers; 6·1% (v/v) acetonitrile; $[S]_0 = 1{\cdot}21$ mM; $[E]_0 = 2\text{–}4\times10^{-5}$M

pH	K_{In} (mM)	$10^3k_{+3}^{In}$ (sec^{-1})	10^3k_{+3} (sec^{-1})	k_{+3}^{In}/k_{+3}
$6{\cdot}6_4$	5·3±0·1	4·1±0·4	1·5	2·73
$6{\cdot}8_4$	10±2	7·5±0·9	2·1	3·57
$6{\cdot}9_4$	4·9±0·6	6·5±0·3	2·4	2·71
$7{\cdot}0_0$	10±5	9·4±2	2·9	3·24
$7{\cdot}5_1$	7±2	17±1	5·2	3·25
$7{\cdot}6_8$	6±2	19±1	6·1	3·12

(*b*) *Deacylation of Formyl-Chymotrypsin in the Presence of Modifiers*

p-Nitrophenyl formate is relatively unstable in alkaline solution, having a $k_{(OH^-)}$ about 70 times that for *p*-nitrophenyl acetate. Hence, the reaction with α-chymotrypsin was studied at pH $4\cdot8_0$ and pH $5\cdot9_5$. The experiments were performed and the results evaluated in the same way as has been described for those involving *p*-nitrophenyl acetate. The size of the burst was again found to be quantitative and independent of the concentration of the modifier. A similar experiment was carried out at pH $4\cdot7_4$ using benzene as modifier. The results of these three experiments are recorded in Table 2.

TABLE 2

EFFECT OF INDOLE AND BENZENE ON THE DEACYLATION OF FORMYL-CHYMOTRYPSIN AT 25°C

Modifier	pH	k_{+3} (sec^{-1})	$k_{+3}^{mod.}$ (sec^{-1})	$k_{+3}^{mod.}/k_{+3}$	$K_{mod.}$ (mM)
Indole[a]	$4\cdot8_0$[b]	0·010	0·031 ±0·001	3·03	1·72±0·10
	$5\cdot9_5$[c]	0·128	0·237 ±0·004	1·85	0·46±0·07
Benzene[d]	$4\cdot7_9$[e]	0·0084	$0\cdot017_7\pm0\cdot001_2$	2·11	7·8 ±1·3

[a] 0–5 mM. [b] The reaction mixture contained 3 ml of enzyme in 0·02M acetate buffer, 100 μl indole in acetonitrile, and 75 μl *p*-nitrophenyl formate in acetonitrile; $[S]_0 = 4\cdot1\times10^{-4}$M; $[E]_0 = 3\cdot89\times10^{-5}$M; 5·5% (v/v) acetonitrile. [c] In 0·025M phosphate buffer; $[S]_0 = 1\cdot0\times10^{-4}$M; $[E]_0 = 8\cdot07\times10^{-6}$M; 5·5% (v/v) acetonitrile. [d] 0–6·6 mM. [e] In 0·02M acetate buffer; $[S]_0 = 4\cdot0\times10^{-4}$M; $[E]_0 = 3\cdot77\times10^{-5}$M; 2·4% (v/v) acetonitrile

The deacylation of formyl-chymotrypsin was also observed in the presence of acetamide and *N*-acetyltryptamine, since an α-acylamino group is present in good substrates. 10 mM acetamide and 3·8 mM *N*-acetyltryptamine were found to have no detectable effect on the rate of deacylation of formyl-chymotrypsin in 0·02M acetate buffer, pH $4\cdot7_5$ ($[S]_0 = 4\cdot0\times10^{-4}$M; $[E]_0 = 3\cdot7\times10^{-5}$M; 5·5% (v/v) acetonitrile).

(*c*) *Effect of Indole on the α-Chymotrypsin-Catalysed Hydrolyses of* p-*Nitrophenyl Propionate and* N-*Formylglycine* p-*Nitrophenyl Ester*

The effect of indole on the deacylation of propionyl-chymotrypsin was determined at pH $7\cdot0_0$. Experimental details and results are given in Table 3. $k_{cat.}$ appears to be proportional to indole concentration in the accessible concentration range. This observation, together with the smallness of the effect, makes K_{In} and k_{+3}^{In} inaccessible.

The α-chymotrypsin-catalysed hydrolysis of *N*-formylglycine *p*-nitrophenyl ester was studied under steady-state conditions at indole concentrations of 0, 2·26, and 4 mM, in 0·05M phosphate buffer, pH $6\cdot9_2$. Figure 4 shows plots of $k_{obs.}$ ($= v/[E]_0$) against $[S]_0$. Indole clearly inhibits the reaction at low substrate concentrations and accelerates the reaction at high substrate concentrations. Experiments carried out at pH 4·8 showed that at a substrate concentration of $7\cdot94\times10^{-4}$M ($[E]_0 = 3\cdot61\times10^{-5}$M), the burst of *p*-nitrophenol ($[P_1]_{burst}$) is equal to $[E]_0$ in the absence and in the presence of 4 mM indole. However, with $[S]_0 = 1\cdot98\times10^{-4}$M, $[P_1]_{burst} = 0\cdot87[E]_0$ in the absence of indole, and $0\cdot75[E]_0$ in the presence of 4 mM

indole. Two conclusions may be drawn: the limiting values of $k_{obs.}$ in Figure 4 are measures of k_{+3}; and indole inhibits the acylation reaction. The effect of indole on k_{+3} for the deacylation of *N*-formylglycyl-chymotrypsin is small, since 4 mM indole causes an increase of only about 30%. Further, there is no evidence for saturation of the acyl-enzyme with indole in the accessible concentration range. The different effects of indole on the acylation and deacylation reactions result in non-linear double reciprocal plots in the presence of indole.

TABLE 3

EFFECT OF INDOLE ON THE DEACYLATION OF PROPIONYL-CHYMOTRYPSIN AT 25°C

The reaction mixture contained 3 ml of enzyme in 0·05M phosphate buffer, pH 7·0$_0$, 100 μl indole solution, and 100 μl *p*-nitrophenyl propionate solution; $[S]_0 = 1{\cdot}24$ mM; $[E]_0 = 4{\cdot}53 \times 10^{-5}$M; 6·3% (v/v) acetonitrile

[Indole] (mM)	No. of Runs	$10^3 k_{cat.}$ (sec^{-1})	$k_{cat.}/k^0_{cat.}$
0	3	3·52±0·01*	1·00
2·26	3	3·73±0·01	1·06
2·94	3	3·81±0·01	1·08
4·00	3	3·87±0·01	1·10

* Fife and Milstien (1967) reported a $k_{cat.}$ of $4{\cdot}8 \times 10^{-3}$ sec^{-1} at pH 7·28.

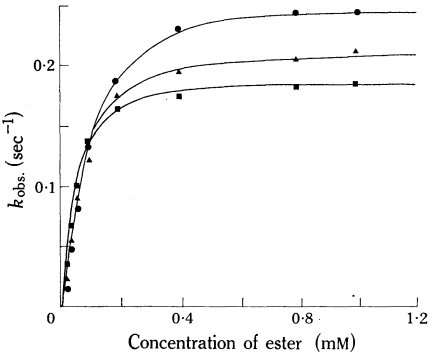


Fig. 4.—Hydrolysis of *N*-formylglycine *p*-nitrophenyl ester by α-chymotrypsin at three concentrations of indole: zero (■); 2·26 mM (▲), and 4·0 mM (●); 0·05M phosphate buffer, pH 6·9$_2$; 6·3% (v/v) acetonitrile. $[E]_0 = 9{\cdot}0 \times 10^{-6}$M.

IV. DISCUSSION

(*a*) *Quantitative Significance of the Observed Promotional Effects*

The deacylation rate constant for a specific acyl-chymotrypsin exceeds that for a non-specific acyl-chymotrypsin by a factor of about 10^4. The maximum acceleration of the deacylation of a non-specific acyl-chymotrypsin by a hydrophobic compound which has so far been observed is about threefold (Tables 1 and 2). Considering the factor of 10^4, this threefold increase does not seem to be of great significance.

However, the complex of indole or benzene with acetyl-chymotrypsin is not a satisfactory model for a specific acyl-chymotrypsin, since the α-acylamino group is absent. Two attempts were made to achieve better approximations to specific acyl-chymotrypsins. The arrangement of groups in a complex of *N*-acetyltryptamine with formyl-chymotrypsin should resemble the arrangement in *N*-acetyl-L-tryptophanyl-chymotrypsin. Similarly, a complex of indole with *N*-formylglycyl-chymotrypsin should resemble *N*-formyl-L-tryptophanyl-chymotrypsin. The results obtained indicate that such complexes do not form readily, presumably because the active site of the enzyme is unable to accommodate both the acyl group and the modifier. These observations are consistent with the K_{In} values obtained. K_{In} for formyl-chymotrypsin (Table 2) agrees well with the value of 0·8 mM obtained for indole as a competitive inhibitor by Wallace, Kurtz, and Niemann (1963), showing that acylation of Ser-195 does not change the structure of the hydrophobic site. K_{In} for acetyl-chymotrypsin is considerably higher than that for formyl-chymotrypsin, showing that steric hindrance to complex formation is appreciable. As expected, the larger acyl groups of propionyl-chymotrypsin and *N*-formylglycyl-chymotrypsin restrict complex formation even further.

The complex of indole with acetyl-chymotrypsin deacylates with a k_{+3}^{In} of about 0·02 sec^{-1} (Table 1). The experiments with benzene and formyl-chymotrypsin suggest that the complex of benzene with acetyl-chymotrypsin would deacylate at a similar rate. The reported deacylation rate constant for β-phenylpropionyl-chymotrypsin is *c.* 0·15 sec^{-1} (Ingles and Knowles 1968). Thus, the complex deacylates more slowly than the acyl-enzyme which it most closely resembles by an order of magnitude.

(*b*) *Possible Mechanisms of the Promotional Effects*

Several mechanisms may be envisaged whereby the presence of indole or benzene at the hydrophobic site could promote the deacylation reaction. In considering possible mechanisms, it will be assumed that a precise orientation of the catalytic groups of the enzyme *and* a precise orientation of the carbonyl group of the acyl-enzyme with respect to these groups are required for rapid rates of deacylation.

Inagami and York (1968), in a comprehensive study of the effect of alkylamines and alkylguanidines on the trypsin-catalysed hydrolysis of *N*-acetylglycine ethyl ester, showed that part of the observed increase in rate constant resulted from a change in the pK_a' of the catalytically important imidazole residue. The effect of indole on the deacylation of acetyl-chymotrypsin was found to be independent of pH within experimental error (Table 1), ruling out a pK_a' change as the explanation of the observed accelerations.

The promotion of the deacylation of acetyl-chymotrypsin by indole (Foster 1961) has been considered as evidence for the "flexible" enzyme theory (Koshland and Neet 1968). It may be postulated that binding of the modifier produces a conformational change resulting in a more active form of the enzyme, due to a more efficient orientation of the catalytic groups. X-ray crystallographic studies of the complex between α-chymotrypsin and *N*-formyl-L-tryptophan (cited by Hartley 1969) showed that this ligand does not produce a marked change in the conformation of the active site. Further, the magnitude of the effects observed here

argues against a substrate-induced conformational change being the major contributor to the factor of 10^4 in deacylation rate constants between specific and non-specific acyl-chymotrypsins.

Another possible mechanism involves the direct effect of the modifier on the freedom of rotation of the acyl group. Thus, the presence of indole at the active site may restrict the movement of the acetyl group of acetyl-chymotrypsin by the action of van der Waal's forces or steric hindrance. This restriction of movement could well result in the small increases in deacylation rates observed in the presence of modifiers. Two experimental results argue against this explanation. Indole increases the rates of deacylation of formyl- and acetyl-chymotrypsins by similar amounts. If the acceleration were due to a direct interaction of indole with the acyl group, the effect on the deacylation of the acetyl-enzyme might be expected to be greater than the effect on the formyl-enzyme. Secondly, indole and benzene have similar effects on the deacylation of formyl-chymotrypsin. If the increased rates were due to steric hindrance to the rotation of the formyl group, indole might be expected to have a greater effect than the smaller benzene molecule.

A further possibility is that the acyl-enzyme-modifier complex is less flexible than the acyl-enzyme, and that the increased rigidity results in increased catalytic efficiency. Glick (1968) found that the binding of neutral ligands to α-chymotrypsin results in the lowering of the pK'_a of a group of pK'_a 6·7 (the imidazole group of His-57) and the raising of the pK'_a of a group originally with pK'_a 8·6 (the α-amino group of Ile-16). These changes were taken to indicate the strengthening of the hydrogen bond between His-57 and Ser-195, and the ionic bond between the α-amino group of Ile-16 and the carboxylate ion of Asp-194, thus making the complex less flexible than the free enzyme. The rigidification mechanism seems to be the simplest and most reasonable explanation for the observed promotion of the deacylation reaction.

Finally, the present studies have done little to explain the difference in efficiency between α-chymotrypsin and liver carboxylesterases in the hydrolysis of esters such as *p*-nitrophenyl acetate (Stoops *et al.* 1969; Zerner 1969). For the reaction of the carboxylesterases with formate and acetate esters, the rapid rates of deacylation cannot be explained by the interaction of the acyl group of the substrate with the enzyme. If the carboxylesterases and α-chymotrypsin operate by the same mechanism, as the current evidence seems to indicate, and if specific substrates for α-chymotrypsin do not induce a conformational change in the enzyme, then it must be concluded that the arrangement of the catalytic groups in α-chymotrypsin is suboptimal for catalysis.

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