THE α-CHYMOTRYPSIN-CATALYSED HYDROLYSIS OF 4-cis-BENZYLIDENE-2-PHENYL-2-OXAZOLIN-5-ONE. ABSENCE OF EVIDENCE FOR N→O TRANSFER.

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Evidence that the acyl-enzyme formed during the α-chymotrypsin-catalysed hydrolysis of esters is a serine ester is now little short of convincing, but the possibility of an N→O acyl shift during the reaction remains an attractive one (for a review, see Bender and Kézdy, 1964). The presentation of evidence for such a shift in an α-chymotrypsin-catalysed hydrolysis would constitute an important advance in our knowledge of the mechanism of the catalytic reaction.

Brocklehurst and Williamson (1967) have postulated an N→O shift to rationalise data obtained in a study of the α-chymotrypsin-catalysed hydrolysis of 4-cis-benzylidene-2-phenyl-2-oxazolin-5-one (PBO), the oxazolinone corresponding to α-benzamido-cis-cinnamic acid. Their principal observations may be summarized as follows:

(1) When PBO reacts with α-chymotrypsin (in molar excess), the absorbance due to the oxazolinone disappears rapidly, and new species are formed whose spectral characteristics depend on the pH. At pH 5, the new species has λ_{max} = 305 mμ (I), while at pH 8, a species with λ_{max} = 285 mμ (II) is formed.

(2) When the first-order decay of species I and II was followed by observing the decrease in absorbance at 310 mμ at various pH's, a discontinuous pH-rate profile was

obtained, which was analysed in terms of two sigmoid curves.

It was postulated that species I and II are both acyl-enzymes. In species I, it was proposed that a histidine residue is acylated (ES'_{His}), while in species II, it was proposed that a serine residue is acylated (ES'_{Ser}). It was further proposed that the discontinuous pH-rate profile is explained by a change in rate-limiting step in Eqn. (1):

$$ES'_{His} \xrightarrow{k_{+2}} ES'_{Ser} \xrightarrow{k_{+3}} E + Acid \qquad .....(1)$$

At pH 5, k_{+2} would be rate-limiting, while at pH 8, k_{+3} would be rate-limiting.

As part of a continuing study of oxazolinones as enzyme-acylating agents (de Jersey, Runnegar and Zerner, 1966), and in attempting to determine the significance of rate constants in an earlier paper of Brocklehurst and Williamson (1966), we considered PBO as a substrate for α-chymotrypsin. Our data on this system provide no evidence for an N→O acyl shift, and support an alternative explanation of the phenomena reported by Brocklehurst and Williamson (1967).

Our observations on the enzymatic and non-enzymatic hydrolysis of PBO are presented below (see Scheme I):

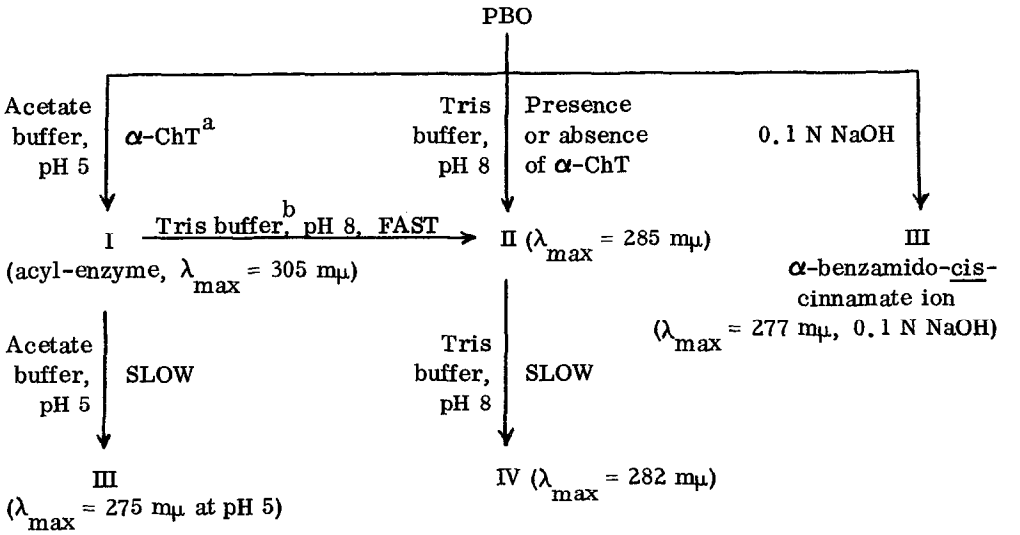
(1) The spontaneous hydrolysis of PBO at pH 7.97 in Tris-HCl buffer yields a species with λ_{max} = 285 mμ. This species (II) changes very slowly at 25° to species IV which has λ_{max} = 282 mμ.

(2) The α-chymotrypsin-catalysed hydrolysis of PBO at pH 7.97 in Tris-HCl buffer yields a species with λ_{max} = 285 mμ, when $[S]_o > [E]_o$ or $[E]_o > [S]_o$, under conditions where the enzymatic reaction is much faster than the spontaneous hydrolysis of the oxazolinone.

(3) Hydrolysis of PBO in 0.1 N NaOH leads directly to the formation of α-benzamido-cis-cinnamate ion (λ_{max} = 277 mμ).

(4) When PBO is reacted with α-chymotrypsin ($[S]_o > [E]_o$) to yield species II, the enzyme is fully active when assayed with N-benzyloxycarbonylglycine p-nitrophenyl ester.

SCHEME I



[a] α-Chymotrypsin. [b] Tris(hydroxymethyl)aminomethane-HCl buffer, 0.1 M or 0.02 M, containing 4.8% (v/v) dioxan, or 10% or 20% (v/v) acetonitrile.

Our observations establish that

(i) species II (λ_{max} = 285 mμ) is not an acyl-enzyme, and that it exists in solution as a separate entity, since it is formed in both enzymatic and non-enzymatic reactions;

(ii) the discontinuous pH-rate profile of Brocklehurst and Williamson (1967) refers to the slow reactions of Scheme I, and is not related to the scheme of Eqn. (1);

(iii) species I (λ_{max} = 305 mμ) is an acyl-enzyme. A "burst" experiment at pH 5.3 with PBO gave $[P_1]_{burst} = [E]_o$, establishing that acylation is much faster than deacylation under the conditions of the experiment. The λ_{max} of 305 mμ is not inconsistent with the identification of the acyl-enzyme as a serine ester (Bender, Schonbaum and Zerner, 1962).

An investigation of species II and IV revealed that species II was formed in the enzymatic hydrolysis of PBO in Tris buffer at pH 8,[1] but not in phosphate or N-ethyl-

(1) While not specifically stated in the relevant section of the text, it would appear that Brocklehurst and Williamson (1967) used a Tris buffer at pH 8.

morpholine buffers at the same pH. In these buffers, species III, α-benzamido-cis-cinnamate ion appeared to be the first product. Kinetic constants for the hydrolysis of PBO, catalysed by α-chymotrypsin, were determined in three buffers near pH 8, under zero-order conditions, by following the decrease in oxazolinone absorbance at 380 mμ (Table I). These results indicated a specific reaction of Tris with the acyl-

TABLE I

THE α-CHYMOTRYPSIN-CATALYSED HYDROLYSIS OF PBO[a]

Buffer[b]	pH	k_{cat}^{c}	K_{m}^{d}
0.1 M Tris-HCl	8.10	1.06	5.0×10^{-6}
0.02 M Tris-HCl	8.05	0.24	1.5×10^{-6}
0.1 M Phosphate	7.93	0.03_3	3.8×10^{-7}

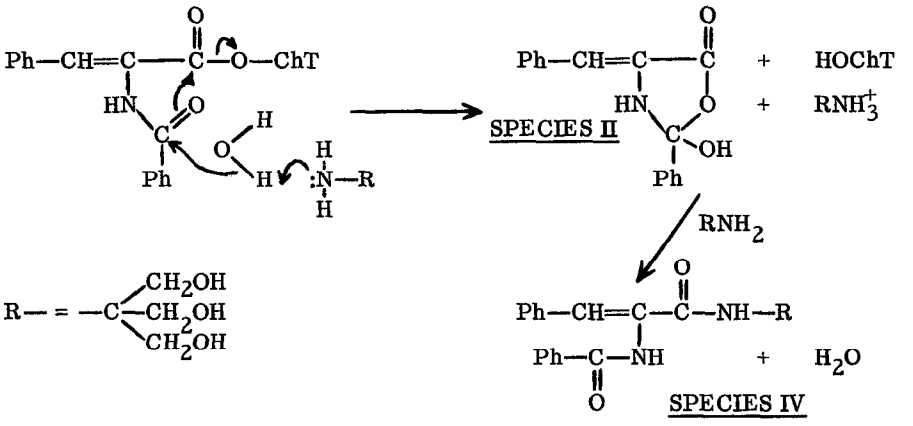
[a] 25°. [b] Buffers containing 10% (v/v) acetonitrile. [c] $sec.^{-1}$. [d] Molar.

enzyme. Consequently, an attempt was made to synthesize the Tris amide of α-benzamido-cis-cinnamic acid (V), by reaction of PBO with an equimolar amount of Tris in 85% (v/v) acetonitrile/water. From the reaction mixture a colourless compound was isolated in low yield, which after repeated crystallisation from chloroform/hexane melted with decomposition at 185° - 186°.[2] The λ_{max} of this compound in Tris buffer (containing 10% (v/v) acetonitrile) at pH 8 was found to be 282 mμ, indicating that species IV is likely the Tris amide of α-benzamido-cis-cinnamic acid.

The nature of species II is currently under investigation. Species II is formed from the acyl-enzyme (or from PBO) in a reaction which is catalysed by Tris. It is subsequently converted to species IV. The reaction mechanism shown in Scheme II is postulated for the enzymatic reaction.

(2) Analysis: Calculated for $C_{20}H_{22}N_2O_5$. - C, 64.85; H, 5.99; N, 7.56; O, 21.60
Found. - C, 64.21; H, 5.91; N, 7.50; O, 21.1

SCHEME II



In the first step, Tris, acting as a general base, forms species II from the acyl-enzyme. Species II is postulated to be a tetrahedral intermediate.[3] A similar tetrahedral intermediate was earlier suggested by Wenger, Urheim and Rottenberg (1962) to explain the enhanced rate of hydrolysis of hippurylcholine as compared with benzoylcholine. Furthermore, in order to account for the products of hydrolysis of N-benzoylaspartic acid, Capindale and Fan (1966) have again proposed a similar intermediate. In the second step, it is postulated that Tris, acting as a nucleophile on species II, forms the Tris amide of α-benzamido-cis-cinnamic acid (IV).

Catalysis by Tris of the steady-state hydrolysis of p-nitrophenyl acetate catalysed by α-chymotrypsin has recently been reported by Faller and Sturtevant (1966). Presumably also in Scheme II, in a less efficient reaction, Tris could yield the Tris amide directly from the acyl-enzyme.

The precise nature of species II described in this paper continues under active investigation in this Laboratory.

(3) It is likely that the species with λ_{max} = 285 mμ formed from the acyl-enzyme (λ_{max} = 305 mμ) at pH 1-2 (Brocklehurst and Williamson, 1967) is α-benzamido-cis-cinnamic acid ($pK_a^1 \sim 3.8$), λ_{max} = 285 mμ, and not species II.

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