OXAZOLINONES AS ENZYME ACYLATING AGENTS

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Since the first saturated oxazolinone was prepared by Mohr and Geis (1908), oxazolinones have been studied firstly as activated intermediates in peptide synthesis (Greenstein and Winitz, 1961), and more recently in association with studies on racemization during peptide synthesis (Goodman and McGahren, 1965, and references therein).

Activated derivatives of N-acylamino acids have proved valuable substrates for mechanistic studies on hydrolytic enzymes (see, for example, Zerner, Bond and Bender, 1964). It has been shown (de Jersey, Kortt and Zerner, 1966; de Jersey and Zerner, unpublished results) that oxazolinones are intermediates in the hydrolysis of activated esters of N-acylamino acids. To assess results previously obtained with such activated esters, a knowledge of the reaction between oxazolinones and hydrolytic enzymes is required. Further, oxazolinones themselves are activated internal esters of N-acylamino acids and as such may be valuable as chromophoric substrates for mechanistic studies on hydrolytic enzymes.

In this communication we report on the reactions of two oxazolinones, 2-phenyl-oxazolin-5-one (PO)[1] and 2-phenyl-4,4-dimethyl-oxazolin-5-one (PDMO)[1]

(1) PO was purified by sublimation in vacuo and gave colourless crystals, m.p. 90-90.5°; PDMO was prepared by the action of dicyclohexyl carbodiimide on N-benzoylaminoisobutyric acid, and purified by sublimation in vacuo m.p. 46°; lit. m.p. 34°.

(Mohr and Geis, 1908) with α-chymotrypsin, trypsin, papain and ox liver carboxylesterase.[2]

A comparison was made between the α-chymotrypsin-catalysed hydrolysis of PO and p-nitrophenyl hippurate (PNPH) (de Jersey, Kortt and Zerner, 1966). Results and experimental detail are listed in Table I.

TABLE I

Hydrolysis of p-Nitrophenyl Hippurate and 2-Phenyl-Oxazolin-5-one by α-Chymotrypsin[a]

pH	k_{cat} ($sec.^{-1}$)		$K_{m(app)}$ (M)	
	PNPH	PO	PNPH	PO
6.97[b]	0.40 0.52[d]	0.53	2×10^{-5}	8×10^{-6}
5.08[c]	0.010[f]	0.011[e]	9×10^{-6}[f]	-

[a] 25.0°; 3% CH_3CN in reaction mixture; all results except (e) obtained by least-squaring data for Lineweaver-Burk plots, with $[S]_o >> [E]_o$; [b] 0.1 M phosphate buffer; [c] 0.1 M acetate buffer; [d] calculated using points at low substrate concentration; [e] by direct observation of the deacylation reaction at 250 mμ; [f] personal communication from Dr R. L. Blakeley.

Kinetic experiments were performed using a Cary 14 recording spectrophotometer fitted with 0 - 0.1 and 0 - 1.0 absorbance slide wires and a thermostatted cell compartment. The temperature was maintained at 25.0° ± 0.1°. The hydrolysis of PNPH was followed at 317 mμ or 400 mμ depending on the pH, while the hydrolysis of the oxazolinones was followed at 250 mμ. "Burst" experiments

(2) α-Chymotrypsin, trypsin and papain were obtained as twice- or thrice-crystallized products from Worthington Biochemicals Corporation. The purification and titration of ox liver carboxylesterase ("slow enzyme") will be reported elsewhere. α-Chymotrypsin solutions were routinely titrated with N-trans-cinnamoylimidazole (Schonbaum, Zerner and Bender, 1961).

were performed at pH 5 to determine the significance of the values of k_{cat}. Substitution of results in equation 1 (Ouellet and Stewart, 1959) established that for both PO and PNPH, $k_{+2} >> k_{+3}$ and therefore that k_{cat} is a measure of k_{+3}.

$$[P_1]_{burst} = \left[\frac{k_{+2}}{k_{+2} + k_{+3}} \cdot \frac{[S]_o}{[S]_o + K_{m(app)}}\right]^2 \cdot [E]_o \quad \ldots (1)^{3,4}$$

Since the values of k_{cat} are about the same for both substrates at each pH, it is reasonable to conclude that both the oxazolinone and the ester react to give hippuryl-chymotrypsin whose decay is rate-limiting. Kinetic data for PNPH are uncertain because of the production of oxazolinone by spontaneous hydrolysis of the ester. Therefore, the best kinetic data are obtained under conditions where the enzymatic rate is much greater than the spontaneous rate (Table I), especially since PO acylates α-chymotrypsin more rapidly than PNPH ($[S]_o = 10^{-4}$ M; pH 5).

In the hydrolysis of PDMO by α-chymotrypsin, trypsin and papain, the acylation reaction is clearly observable. For the α-chymotrypsin-catalysed hydrolysis of PDMO, k_{+2} and k_{+3} have been determined directly. k_{+2} was determined at pH 5.06 under conditions where $[S]_o >> [E]_o$ (Kézdy and Bender, 1962). k_{+3} was determined at pH 6.96 by direct observation of the deacylation reaction at 250 mμ with $[S]_o < [E]_o$. Under these conditions, an initial rapid decrease in absorbance at 250 mμ (corresponding to acylation), is followed by a much slower, smaller, first-order decrease in absorbance (corresponding to deacylation).

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(3) This equation is derived from the kinetic scheme

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \xrightarrow{k_{+2}} ES' \xrightarrow[+ P_1]{k_{+3}} E + P_2.$$

Evidence has been obtained that this scheme operates for all four enzymes investigated. The present results support this formulation.

(4) For oxazolinones, there is no P_1. $[P_1]_{burst}$ refers to the initial rapid decrease in substrate concentration, measured spectrophotometrically at 250 mμ.

Table II compares the values obtained for PDMO with results previously obtained for p-nitrophenyl trimethylacetate (PNPTMA). It can be seen that the k_{+2}/k_{+3} ratio for PDMO is very large, indicating the high reactivity of oxazolinones as acylating agents. By substitution of k_{+2}, k_{+3} and $K_{m(app)}$ in equation 1, with $S_o = 4.49 \times 10^{-5}$ M, $[P_1]_{burst} = 0.99_9 [E]_o$, establishing that a measure of the size of the burst at 250 mμ gives a direct measure of the concentration of active sites in the solution. $\Delta\epsilon^{250\ m\mu}_{PDMO \rightarrow acyl\text{-}enzyme}$ has been determined as 9430 ± 100. A comparison of the PDMO titration with the N-trans-cinnamoylimidazole titration at pH 7 shows agreement of the two methods within experimental error.

TABLE II

Hydrolysis of 2-Phenyl-4, 4-dimethyl-oxazolin-5-one and p-Nitrophenyl Trimethylacetate by α-Chymotrypsin[a]

Substrate	k_{OH^-} (M^{-1} $sec.^{-1}$)	k_{+2} ($sec.^{-1}$)	k_{+3} ($sec.^{-1}$)	k_{+2}/k_{+3}
PDMO	~180	180[b]	0.0011	1.6×10^5
PNPTMA	0.5[d]	0.18[c]	0.00007[d]	2.6×10^3

[a] 25.0°, 0.1 M phosphate buffer, pH 6.96; [b] k_{+2}, measured at pH 5.06, was 3.6 $sec.^{-1}$; [c] Bender and Hamilton (1962); [d] Bender (1962).

PDMO could also be used to titrate trypsin and papain. The acylation reaction for these enzymes is definitely slower than for α-chymotrypsin at the same substrate concentration. However, a valid titration can still be achieved even if $[P_1]_{burst}$ does not equal $[E]_o$, if the correction factor can be determined accurately. The acylation of papain[5] by PDMO was followed in two ways: (1) by direct observation of the burst at 250 mμ, and (2) by using benzyloxycarbonylglycine p-nitrophenyl

(5) Papain was activated by 2.5×10^{-4} M cysteine, in pH 6.06, 0.05 M phosphate buffer containing 5×10^{-4} M EDTA.

ester as assay substrate to determine the percentage of free enzyme remaining at various times after the addition of PDMO to the enzyme solution. When [PDMO] = 1.35×10^{-4} M, ca. 8% of the enzyme remains in the free state when a steady state has been reached. Therefore, $[P_1]_{burst} \doteqdot 0.92\,[E]_o$, and PDMO could be used to give an accurate titration of papain concentration.

PDMO proved to be a good substrate for ox liver carboxylesterase. Table III shows a comparison between the α-chymotrypsin- and ox liver carboxylesterase-catalysed-hydrolyses of PDMO and PNPTMA. Results indicate that all the values of k_{cat} in Table III reflect the deacylation rate constants. In the deacylation step, then, α-chymotrypsin does not favour the N-acylaminoacyl group much more than does the carboxylesterase. A factor of five may be explained in terms of the inductive effect of the N-acylamino group. With both substrates, the carboxylesterase is enormously more efficient than the proteinase.

The work reported here and earlier establishes

(1) that oxazolinones are highly activated internal esters of N-acylamino acids;

(2) that they are very efficient and relatively non-specific acylating agents for hydrolytic enzymes;

TABLE III

Hydrolysis of 2-Phenyl-4, 4-dimethyl-oxazolin-5-one and p-Nitrophenyl Trimethylacetate at 25° [a]

Enzyme	k_{cat} ($sec.^{-1}$) PDMO	PNPTMA	k_{cat} PDMO / PNPTMA
α-Chymotrypsin	1.1×10^{-3}	7×10^{-5}	16
Ox liver carboxylesterase	24.7	~5	5
k_{cat} Carboxylesterase / α-Chymotrypsin	2.3×10^{4}	7×10^{4}	

[a] pH 6.96, 0.1 M phosphate buffer

(3) that even simple oxazolinones have chromophoric properties suitable for spectrophotometric investigations;

(4) that they are, in principle, available for the titration of hydrolytic enzymes, as exemplified by the titration of α-chymotrypsin by PDMO;

(5) that unequivocal determinations of k_{+3} are accessible where (say) for steric reasons, a nitrophenyl ester may not have deacylation totally rate-limiting;

(6) that kinetic results obtained with N-acylamino acid activated esters should be treated with caution.

The logical extension of these observations on oxazolinones continues under active investigation in this Laboratory.

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