Towards a Specific Chromophoric Substrate for α-Chymotrypsin

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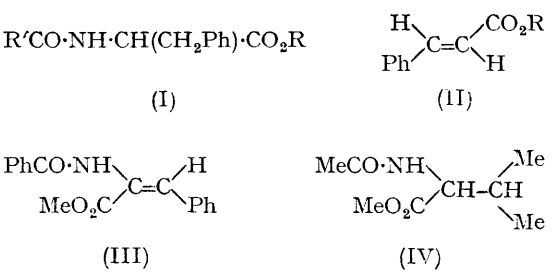
SPECIFIC substrates for α-chymotrypsin, such as *N*-acetyl-L-tyrosine ethyl ester, are derivatives of *N*-acylamino-acids which possess an aromatic ring attached to the carbon atom which is β to the carbon atom of the moiety undergoing hydrolysis [as in (I)]. Much information about the mechanism of action of α-chymotrypsin has been obtained by the study[1–5] of its catalysis of the hydrolysis of a series of esters (II) of *trans*-cinnamic acid, which are chromophoric but non-specific substrates for this enzyme. These substrates possess an aromatic ring on the β-carbon atom but lack the specific *N*-acylamino-side-chain. Bender[5] has pointed out that specific substrates of α-chymotrypsin are distinguished from non-specific substrates not by the magnitude of K_m(app.) but by their "kinetic specificity" which is reflected in k_{cat}'. Thus for *N*-acetyl-L-tyrosine ethyl ester,[6] K_m (app.) is $0{\cdot}7 \times 10^{-3}$M and k_{cat}' is 193 sec.$^{-1}$ and for methyl cinnamate,[2] K_m (app.) is $2{\cdot}05 \times 10^{-3}$M and k_{cat}' is $7{\cdot}3 \times 10^{-3}$ sec.$^{-1}$ All the catalytic constants referred to in this Communication were obtained at pH 7·9 and 25° (see reference 5).



We now report for the first time the α-chymotryptic hydrolysis of methyl α-benzamido-*cis*-cinnamate (III) a cinnamoyl ester which possesses the specific *N*-acylamino-side-chain.

To determine the Michaelis parameters for (III) the hydrolyses were carried out at 25·0° in phosphate buffer, $I = 0{\cdot}1$, which contained 4·8% v/v dioxan, under zero-order conditions. The enzyme concentration, determined by active-site titration with cinnamoylimidazole,[7] was maintained constant at $4{\cdot}05 \times 10^{-5}$M and the experiments were carried out at twelve concentrations of (III)

between $1{\cdot}41 \times 10^{-4}$ and $8{\cdot}44 \times 10^{-4}$M. The reaction was followed by observing the change in absorbance at 320 mμ of the reaction mixture in a Cary 15 recording spectrophotometer using the 0—0·1 absorbance slidewire. Under these conditions the absorbance/time profile consists in a rapid rise followed by a slower fall which eventually follows true zero-order kinetics. This type of absorbance/time profile is characteristic of the formation and subsequent decay of a highly absorbing enzyme–substrate intermediate which Bender and Zerner[2] have shown to be an acyl-enzyme in the case of the hydrolysis of unsubstituted cinnamoyl esters by α-chymotrypsin.

Lineweaver–Burk[8] treatment of the zero-order kinetic data by regression analysis using the least-squares procedure yields for (III) K_m (app.) = 24×10^{-3}M and $k_{cat}' = 100 \times 10^{-3}$ sec.$^{-1}$ The catalytic constant for (III) is greater than that for methyl cinnamate (see above) by at least an order of magnitude. Thus (III) represents, relative to methyl cinnamate, a step towards a specific substrate on the continuum of catalytic rate constants which Bender[6] has suggested exists for substrates, specific and non-specific, of α-chymotrypsin.

It is of interest that k_{cat}' for (III) is similar to that[9] for *N*-acetyl-L-valine methyl ester (IV) [K_m (app.) = 108×10^{-3}M and $k_{cat}' = 150 \times 10^{-3}$ sec.$^{-1}$] a substrate which possesses the *N*-acyl-amino-side-chain but not the aromatic ring on the β-carbon atom. This suggests that if the *N*-acyl-amino-side-chain of (III) binds to the enzyme, the geometry of (III) is such that the β-phenyl group cannot also be bound profitably by the enzyme. Support for this view is found in the fact that at high concentrations of (III) the Lineweaver–Burk plot deviates from linearity in the manner which is symptomatic of substrate inhibition.

Currently we are carrying out a detailed kinetic analysis of the α-chymotryptic hydrolysis of (III) and are examining the effect of changing the geometry about the β-carbon atom from *cis* to *trans* and from trigonal to tetrahedral.

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