ACYL DERIVATIVES OF ALPHA-CHYMOTRYPSIN

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The advantages of using cinnamoylimidazole (Bender et al, 1962) indoleacryloylimidazole and furylacryloylimidazole (Bernhard et al, 1965) in the elucidation of enzyme reaction mechanism have been demonstrated in recent years. Alpha-chymotrypsin reacts rapidly with these acyl imidazoles to form stable acyl chymotrypsins whose absorption spectra have been reported. The λ_{max} of these acyl chymotrypsins all show an as yet unexplained shift towards shorter wavelengths upon denaturation (see Table I). In view of the close correspondence in λ_{max} between the denatured acyl chymotrypsin and synthetic O-acyl serine, the acyl moeity was presumed to reside on the hydroxyl group of a serine residue in the enzyme molecule (Bernhard et al, 1965). Similar conclusions were also inferred on the basis of amino acid analyses of the acyl peptide derived from chymotrypsin (Oosterbaan & Van Andrichem, (1958)) and subtilisin (Noller and Bernhard, 1965). Whilst λ_{max} of absorption of the model serine O-acyl derivative and that of the denatured acyl enzyme are identical there is a large difference (>20%) between the ε_{max} of the model compound and that of the denatured acyl enzyme. These observations have not been explained but the suggestion was made in the case of the cinnamoyl chymotrypsin in a footnote to a table (Bender et al, 1961), that it might be due to an effect of the protein on the chromophoric

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group. However, the additivity of molar absorptivities of covalently bound chromophoric groups in proteins has been established (Wetlaufer, 1962).

In view of these observed differences in the ε_{max} of O-acyl serines as compared to denatured acyl enzymes, experiments were undertaken to find a model compound whose spectral properties would be identical with those of the denatured acyl enzyme. Because of the similarities between cycloserine catalysed and alpha-chymotrypsin catalysed reactions reported earlier (Viswanatha, 1963) the current investigations deal with the reaction of cycloserine-diketopiperazine (DKP) with acyl imidazoles.

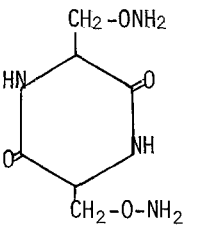


FIG. 1

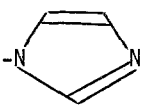
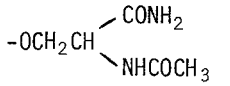
MATERIALS AND METHODS

DKP (Fig. 1) was prepared by the dimerization of cycloserine (D-4-amino-3-isoxazolidone) in solution at neutral pH. The DKP was crystallised from 60% alcohol and showed the presence of a single titratable group (pKa = 4.0) and m.p. 190°C (decomp) (Hidy, et al, 1955). Cinnamoylimidazole (CI) was prepared according to Bender et al, 1962, and had a m.p. 133°C. Furylacryloylimidazole (FAI) and indoleacryloylimidazole (IAI) were prepared according to Bernhard et al, 1965, FAI, m.p. 110°C; IAI, m.p. 191°C.

The acyl chymotrypsins were prepared by treating a 3×10^{-5} M solution of 3x recrystallised α-chymotrypsin, in 0.05 M acetate buffer at pH 4.95, with 100 µl of the acyl imidazole in acetonitrile so as to obtain a final concentration of acyl chymotrypsin of 2.7×10^{-5} M. The

TABLE I

λ_{max} (mμ); ε_{max} (M^{-1} cm^{-1} x 10^{-4}) of various

-X	R - C -					
	CINNAMOYL		FURYLACRYLOYL		INDOLEACRYLOYL	
	307 (307) (307)	2.34 (2.38[a]) (2.5[c])	340 (340)	2.61 (2.7[c])	378 (378)	2.62 (3.0[c]) †
	(281) (281)	(2.43[b]) (2.4[c])	(309)	(2.5[c])*	(335)	(2.7[c])*
-Chymotrypsin (Native)	292 (292) (292)	1.78 (1.76[a]) (1.7[c])	320 (320)	1.98 (1.95[c])	360 (359) (359)	1.78 (1.75[c]) ∓ (2.0[c])
-Chymotrypsin (Denatured)	281 (282[c])	1.78 -	310 (309[c])	2.00 -	335 (335[c])	1.90 -
-DKP	281	1.74**	309	2.00	335	1.79

The values in parentheses are those reported by

(a) Bender et al, 1961; (b) Bender et al, 1962; and
(c) Bernhard et al, 1965.

† The higher ε_{max} IAI value reported by Bernhard et al may be related to the indole acrylic acid (IAA) sample used, with a quoted $\varepsilon_{max} = 2.5 \times 10^4$. The IAA used in these studies had an ε_{max} 1.84×10^4 in agreement with the observations of McClure and Neurath, 1966.

* Based on an analogous model of Bernhard et al, 1965.

∓ Bernhard's data recalculated using the ε_{max} IAI = 2.62×10^4

** Twice recrystallized CI-DKP had a m.p. 178°C. ε_{max} = 1.77×10^4 λ_{max} 281 mμ.

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difference spectra of acyl chymotrypsins versus chymotrypsin were recorded using a Cary Model 14 Spectrophotometer. The spectra of the

reversibly denatured acyl chymotrypsins were obtained by heating the solutions to 51°C (Mercouroff and Hess, 1963). The results are presented in Table I.

The reaction of the DKP with acyl imidazoles was studied by treating a 2.5×10^{-2} M solution of DKP in phosphate buffer at pH 6.85 with 100 µl of the appropriate acyl imidazole solution in acetonitrile so as to obtain a final concentration of acyl DKP of 2.7×10^{-5} M. The apparent psuedo first order rate constants at 25°C for the hydrolysis of the various acyl imidazoles by DKP are CI: $6 \times 10^{-1} min^{-1}$, FAI: 3.5×10^{-1} min^{-1}, IAI: 7×10^{-2} min^{-1}. After the completion of the reaction the spectrum of the acyl DKP was determined by scanning the sample against untreated DKP solution. The results are presented in Table I.

DISCUSSION

The data presented in Table I show that acyl derivatives of DKP correspond more closely to the denatured acyl enzymes with regard to their spectral features, than does the O-acyl derivative of serine. These observations suggest that the attachment of the acyl moeity in the enzyme to an aminoxy ($-O-NH_2$) or some similar group is quite probable. Further it was found that neither λ_{max} of absorption nor the ε_{max} of the denatured furylacryloyl enzyme undergoes alteration upon extensive enzymatic degradation involving the action of pepsin at pH 2.0; trypsin and chymotrypsin at pH 8.0 followed by carboxypeptidases A and B. This observation indicates that in fact the conformation of the peptide chain has very little influence on the λ_{max} of the chromophore.

This correspondence observed in three different acyl derivatives of α-chymotrypsin and DKP renders the "O-N-acyl derivative" as a tenable alternative to the widely accepted hypothesis involving the O-acyl serine intermediate. Indeed the present observations strengthen Bernhard's suggestion (Bernhard et al, 1965) that alternative models involving "a

guanidoxy linkage", formed via serine and arginine (Viswanatha, 1964) or a ω-substituted arginine (Erlanger, 1960) at the active site of the enzyme should not be summarily rejected.

ACKNOWLEDGMENTS

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