Direct Spectrophotometric Observation of an Acyl-Enzyme Intermediate in Elastase Catalysis

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SUMMARY

An acyl-enzyme intermediate in the catalytic action of the pancreatic serine protease, elastase, was spectrophotometrically observed. 4-Dimethylaminocinnamoyl-elastase was prepared by the reaction of 4-dimethylaminocinnamoylimidazole with a 20 fold excess of elastase for 0.5 hr in pH 5.2 buffer. The visible absorption spectrum of this acyl-enzyme is similar to that of the analogous aldehyde but is red-shifted when compared to that of the denatured acyl-enzyme or a model ester. Our results are the first direct evidence for the intermediacy of an acyl-enzyme in elastase catalysis.

Introduction

The mechanistic pathway in serine protease catalysis is thought to involve an acyl-enzyme intermediate, ES', equation 1 (1). The intermediacy of this acyl-enzyme is based upon indirect kinetic evidence and upon the direct spectrophotometric observation of chromophoric acyl-enzymes such as cinnamoyl-chymotrypsin (2) and cinnamoyl-trypsin (3). However, recent kinetic data has indicated that an acyl-enzyme intermediate may not be involved in catalysis by the pancreatic serine protease, elastase (4,5). For example, it has been found that k_{cat} for a series of elastase ester substrates derived from the same acid but from different alcohols increased as a function of the leaving group ability of the alcohol (4,5).
This is in contrast to the case of chymotrypsin and trypsin where ester substrates derived from the same acid but from different alcohols undergo

enzyme-catalyzed hydrolysis with the same k_{cat} due to the rate-determining deacylation of the common acyl-enzyme intermediate formed, ES'.

$$RCOR' + EnzOH \rightarrow \underset{ES'}{R\text{-}C(=O)\text{-}OEnz} + R'OH \xrightarrow{k_{cat}} R\text{-}COH + EnzOH \qquad (1)$$

One explanation offered (4) to explain the variation of k_{cat} with alcohol leaving groups is that the rate-determining step involves a nucleophilic displacement upon the tetrahedral intermediate, ET_1, by an enzymatic hydroxide ion equivalent followed by decomposition of a second tetrahedral intermediate, ET_2, to give the product acid without the intermediacy of an acyl-enzyme, equation 2.

$$RCOR' + EnzOH \rightarrow \underset{ET_1}{R\text{-}C(HO^-)(O^-)(OEnz)(OR')} \rightarrow \underset{ET_2}{R\text{-}C(OH)(O)(OEnz)} + R'OH \rightarrow RCOH + EnzOH \qquad (2)$$

In an attempt to determine which of these two pathways is involved in elastase catalysis, we have tried to spectrophotometrically detect intermediates in the catalytic pathway. Our results are discussed below.

Materials and Methods

4-Dimethylaminocinnamoylimidazole was synthesized in a manner similar to that used by Bernhard, Lau, and Noller (6). Imidazole and 4-dimethylaminocinnamic acid (K and K Labs) were coupled, using either dicyclohexylcarbodiimide or isobutyl chloroformate, in dimethylformamide-tetrahydrofuran (1: 4 by volume) to give 4-dimethylaminocinnamoylimidazole, m. p. 164-167. The halflife of 4-dimethylaminocinnamoylimidazole in pH 5.2 acetate buffer was 4. 5 hrs.

Elastase was obtained from a twice crystallized product of Sigma Chemical Co.

4-Dimethylaminocinnamoyl-elastase was prepared by the reaction of 4-dimethylaminocinnamoylimidazole with a 20 fold excess of elastase for 0. 5 hr at pH5.2 (0. 83% acetonitrile). The denatured acyl-enzyme was prepared by adding either 30 mg of solid sodium dodecyl sulfate to 3 ml of the native acyl-enzyme solution or by adding 1 ml of a 4% solution of sodium dodecyl sulfate to 3 ml of the native acyl-enzyme. The difference

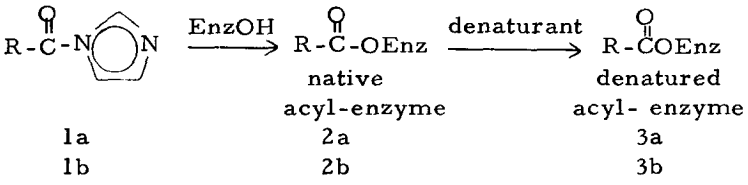
spectra were recorded by adding enzyme to both the reference and sample cells of a Cary 14 recording spectrophotometer and adding the acylating agent to the sample cell only.

Results and Discussion

If a common acyl-enzyme intermediate is involved in the mechanistic pathway, then elastase, trypsin,and chymotrypsin catalysis can be described by equation 3 where E is the enzyme, S is the substrate, E·S is the non-covalent Michaelis-Menten binding complex, ES' is the acyl-

$$E + S \rightleftarrows E{\cdot}S \xrightarrow[\text{acylation}]{k_2} ES' + P_1 \xrightarrow[\text{deacylation}]{k_3} E + P_2 \qquad (3)$$

enzyme intermediate, P_1 is the alcohol leaving group, and P_2 is the product acid. The accumulation of the acyl-enzyme intermediate, ES', which permits the spectrophotometric detection, is made possible by conditions wherein acylation and deacylation are both slow but deacylation is much slower than acylation ($k_2 >> k_3$). This can be achieved by the use of



	native acyl-enzyme	denatured acyl- enzyme
1a	2a	3a
1b	2b	3b

(4)

1a,2a,3a R=cinnamoyl
1b,2b,3b R=dimethylaminocinnamoyl

a chromophoric non-specific substrate (e. g. , a substrate which does not possess an α-acetamido functional group) which has a good leaving group. For example, cinnamoylimidazole, 1a, was found to stoichiometrically acylate chymotrypsin at low pH (2). However, deacylation was very slow, allowing direct spectrophotometric observation of the acyl-enzyme. The reaction of elastase with cinnamoylimidazole was slow, necessitating the use of a large excess of enzyme for acylation to compete favorably

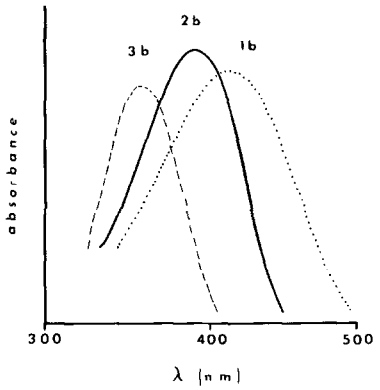


Figure 1. Absorption spectra of 4-dimethylaminocinnamoyl derivatives.
Curve 1b, 4 dimethylaminocinnmoylimidazole (. . . . .).
Curve 2b, native 4-dimethylaminocinnamoyl-elastase (————).
Curve 3b, denatured 4-dimethylaminocinnamoyl-elastase (------).

with buffer hydrolysis. However, the enzyme absorbs in the same spectral region as the cinnamoyl-enzyme, ~ 280 nm. Therefore, we used as an acylating agent, 4-dimethylaminocinnamoylimidazole, 1 b , which had absorption characteristics which permitted the observation of the visible spectrum of the acyl-enzyme (~ 400 nm) in the presence of a large excess enzyme. The absorption spectrum of 4-dimethylaminocinnamoyl-elastase, 2 b, is shown in Figure 1.

The indications that the acylimidazole, 2 a, forms an acyl-enzyme with the serine-195 of elastase are: 1) the enzyme catalyzes the hydrolysis of the acylimidazole; 2) the denatured acyl-enzyme had a similar visible spectrum to that of a model ester, Table I; 3) if a denaturant was added to the reaction mixture before acylation was complete, then the spectrum resembled that of a mixture of the denatured acyl-enzyme and the acylating agent, 2b; 4) the native acyl-enzyme exhibited a spectral red-shift, similar to that found for chromophoric acyl-enzyme derivatives of chymotrypsin (2,6).

TABLE I. Absorption Spectral Data of 4-Dimethylaminocinnamoyl Derivatives.

Derivative	λ_{max}	ϵ
4-Dimethylaminocinnamoylimidazole[a]	425	25200
Native 4-dimethylaminocinnamoyl-elastase[a]	401	27060
4-Dimethylaminocinnamaldehyde[a]	397	31575
Denatured 4-dimethylaminocinnamoyl-elastase[b]	370	23500
Ethyl 4-dimethylaminocinnamate[c]	369	25100
4-Dimethylaminocinnamic acid[d]	332	16300

a. pH 5.2 acetate buffer, 0.83% acetonitrile.
b. pH 5.2 acetate buffer, 0.83% acetonitrile, 1% sodium dodecyl sulfate.
c. pH 5.2 acetate buffer, 0.83% ethanol, 1% sodium dodecyl sulfate.
d. pH 5.2 acetate buffer, 0.83% dimethyl sulfoxide.

The most interesting spectral property of 4-dimethylaminocinnamoyl-elastase is that it is red-shifted relative to the denatured acyl-enzyme or a model ester, Table I. Two possible explanations based upon x-ray data and spectral models have been offered (7) to explain a similar spectral red-shift observed for chromophoric acyl-chymotrypsins: 1) the carbonyl group of the acyl derivative is hydrogen-bonded or protonated in the active site, or 2) an "out of the plane" acyl-enzyme is formed. In an "out of the plane" acyl-enzyme, the lone pair orbitals of the alkyl oxygen of the acyl-enzyme are perturbed in such a way that they cannot delocalize into the carbonyl. The net result is that the acyl-enzyme is the electronic equivalent of an aldehyde or ketone. The prediction that the chromophoric acyl-enzymes are similar to aldehydes is substantiated by a comparisons of the spectra of 4-dimethylaminocinnamoylelastase and 4-dimethylaminocinnamaldehyde, Table I.

In addition to acylating elastase, 4-dimethylaminocinnamoylimidazole can also acylate chymotrypsin, trypsin, and other proteases (8). Our results indicate that the elastase-catalyzed hydrolysis of non-specific substrates is mechanistically similar to that of chymotrypsin- or trypsin-catalyzed hydrolyses in that demonstrable acyl-enzyme intermediates are common to all three enzymes. We are now attempting to determine if a common acyl-enzyme is involved in the hydrolysis of specific substrates by these three enzymes.

Acknowledgement

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