# THE FLUORESCENCE OF α-CHYMOTRYSIN IN THE PRESENCE OF SUBSTRATES AND INHIBITORS*

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α-Chymotrypsin (CT) has a fluorescence spectrum similar to that exhibited by many proteins containing tryptophan residues but no prosthetic group. Teale (1960) found that the fluorescence yield of CT in 8 M urea is more than twice the value in aqueous solution. This effect, which is reversible, is to be attributed to conformational changes in the protein. We have observed much smaller fluorescence increases produced by various substrates and inhibitors, and have studied the kinetics of these changes.

## Experimental

CT and chymotrypsinogen (CTgen) from Worthington Biochemical Sales were used without further purification. Di-isopropylphosphoryl chymotrypsin (DIP-CT) was prepared by reaction of CT with excess di-isopropylfluorophosphate (DFP), and was dialyzed against dilute acid. [2] All other reagents were purchased either in chromatographically-pure form or were of analytical grade.

Measurements were made in a stopped-flow apparatus designed for both spectrophotometric and fluorometric observations. The small dimensions of the quartz observation tube (2 x 2 x 10 mm) and the need for fast response in the recording system necessitated the use of large slit widths in both the source and

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receiver monochromators in order to obtain adequate sensitivity to follow the small and rapidly changing fluorescence increases encountered in this work. The increases, which were observed at 334 mμ, are therefore probably smaller than would be observed under optimum conditions. The fluorescence was excited at about 293 mμ by a 100-watt Osram superpressure Hg arc powered by a closely regulated constant current supply. All experiments were performed at pH 8.0, 25°, in a 0.1 M tris (hydroxymethyl) aminomethane-HCl buffer, with enzyme concentrations of 1-2 mg. per ml.

## Results and Discussion

The results are summarized in Table 1. The third column in the table gives the concentration of reagent at which the fluorescence increase in the fourth column was observed. For L-tyrosine ethyl ester (L-TEE) and β-phenylpropionic acid (PPA) the fluorescence increase varied with concentration in the manner expected if a complex is formed with the indicated dissociation constant, and the fluorescence increase listed corresponds to saturation of the enzyme by the reagent. It is interesting that in the case of L-TEE, the dissociation constant is, within an experimental uncertainty of about 25%, the same as the Michaelis-Menten constant for this substrate as determined by kinetic experiments using the absorption change at 236 mμ. In the case of PPA, the dissociation constant is considerably less than the value 0.025 M reported by Foster and Niemann (1955). Isopropyl alcohol also showed a saturation effect, but it did not give data fitting the expression for a 1 : 1 complex.

Several interesting points appear in the table. N-acetyl-L-phenylalanine (L-AP) gave a barely detectable fluorescence increase while the D-isomer gave a six-fold greater increase. DFP produced a very much larger increase than any of the other reagents tested, although it does not introduce into the enzyme a group any larger than that introduced by a substrate such as L-TEE. The

Table 1

Changes in the fluorescence of α-chymotrypsin, chymotrypsinogen and DIP-chymotrypsin produced by various reagents

| Protein | Reagent* | Reagent molarity | Increase of fluorescence, % | Rate constant for fluorescence increase, $sec.^{-1}$ |
|---|---|---|---|---|
| CT | APEE | 0.005 | 5.2 | 1.2 ± 0.2 |
| | L-AP | .010 | .2 | -- |
| | D-AP | .010 | 1.2 | 1.5 |
| | L-TEE | -- | 3.9 (K = .0026M) | 1.1 ± 0.1 |
| | L-TA | .015 | 1.9 | 1.4 ± 0.2 |
| | PPA | -- | 5.0 (K = .010M) | 1.4 ± 0.1 |
| | Isopropyl alc. | -- | 3.5 | 1.6 ± 0.2 |
| | DFP | excess | 15.6 | 1.6** |
| CTgen | L-TEE | 0.010 | 0 | -- |
| | PPA | .025 | 0 | -- |
| | Isopropyl alc. | 5 % | .7 | -- |
| DIP-CT | APEE | 0.010 | 0.3 | -- |
| | Isopropyl alc. | 10 % | .7 | -- |

* See text for meaning of abbreviations.

** Rate constant is proportional to DFP concentration; this value is for 0.005M DFP.

enzymically inactive species CTgen and DIP-CT are affected very little by reagents which have a significant effect on the active enzyme, suggesting that the observed effects result from conformational changes in the vicinity of the active site.

In all cases where significant fluorescence changes were produced, it was possible to show that the changes follow apparent first order kinetics with good accuracy. In four cases, acetyl-L-phenylalanine ethyl ester (APEE), L-TEE, PPA and isopropyl alcohol, the rate constant was shown to be independent of enzyme and reagent concentrations over a considerable range. In

the case of L-TEE, in which there is a substantial fluorescence increase due to the hydrolysis of the substrate, the data reported refer to the initial "burst" of fluorescence increase from which the steady-state rate of increase has been deducted.

It is surprising that all the fluorescence increases take place at nearly the same rate, even with substrates having widely different turnover rates. Hammond and Gutfreund (1955) reported 160 $sec^{-1}$ as the turnover rate for APEE. We have found 8 $sec^{-1}$ for L-TEE, and if the assumption is made that the ratio of rates for L-TEE and L-TA is the same as that for acetyltyrosine ethyl ester and amide, we estimate a value of 0.007 $sec^{-1}$ for L-TA. Thus, two substrates differing in turnover rates by a factor of $2 \times 10^4$ have practically the same rate constant for fluorescence increase.

In a typical experiment with APEE the rate of the overall hydrolysis reaction was completely unaffected by the relatively slow change leading to the fluorescence increase, and the hydrolysis reaction was 75% complete before the fluorescence reached its maximum 3.5 secs. after mixing. When the hydrolysis was complete, about 7.5 secs. after mixing, 45% of the fluorescence increase still remained and underwent a slow decay.

It is necessary to conclude that the fluorescence changes reported here, although caused by substrates or inhibitors, have no direct connection with the reaction catalyzed by the enzyme, and in particular do not represent the formation of reaction intermediates. It appears that various reagents stimulate conformational changes in CT which lead to small increases in fluorescence but do not detectably affect the activity of the enzyme, and that these changes all take place at about the same rate, presumably all of them being rate-limited in a similar manner by the enzyme.

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