INTERPRETATION OF THE ULTRAVIOLET SPECTRUM OF TRANS-CINNAMOYL-α-CHYMOTRYPSIN

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Recently, Bender et al. (1962) demonstrated that the enzyme-substrate compound, trans-cinnamoyl-chymotrypsin (CIN-CT) is an intermediate in the α-chymotrypsin (CT) catalyzed hydrolysis of CIN-esters. Kinetic evidence suggests (Bender et al., 1962) that CIN-CT is an ester rather than an imidazole derivative. So far, interpretations of the ultraviolet spectra of CIN-CT were ambiguous, since λmax of the CIN group of CIN-CT is at 290 mμ, of CIN-esters at about 280 mμ and of CIN-imidazole at 307 mμ.

Recently we observed (Mercouroff and Hess, 1963) a reversible, temperature induced conformational change of CIN-CT between 30° and 50° at pH 2.0. By means of this reversible conformational change it became possible to obtain direct spectrophotometric evidence that CIN-CT is an ester derivative and that λmax of the CIN group of CIN-CT depends on the conformation of the enzyme-substrate compound.

Three times recrystallized Worthington CT was used. CIN-CT was prepared according to the procedure of Bender et al. (1962). The M.W. of CT was taken as 25,000 (Wilcox et al., 1957).

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The ultraviolet difference spectrum of CIN-CT versus CT at pH 2.0 and 20° is shown in Fig. 1a. The spectrum is identical to that published by Bender et al. (1962) and is shown here only for comparison. The small peaks on top of the curve (shaded area) are identical to those observed (Wootton and Hess, 1961) in the difference spectra of enzyme-substrate compounds, containing non-chromophoric substrates, versus CT, and reflect differences in the spectral properties of the protein. After correcting for these differences, a λ max for the CIN-group of 290 mμ is obtained. The difference spectrum of CIN-CT versus CT at pH 2.0 and 51° is shown in Fig. 1b. The λmax of the CIN-group is now at 282 mμ, very similar to λ max of O-CIN-N-acetyl serineamide (O-CIN-N-ac-serNH_2) which is 281 mμ at pH 2.0 and 50°. On cooling the solutions to 21° the difference spectrum shown in Fig. 1a is again obtained and λmax is at 290 mμ. It has previously been observed (Mercouroff and Hess, 1963) that the transition of CIN-CT can be followed by measuring both the

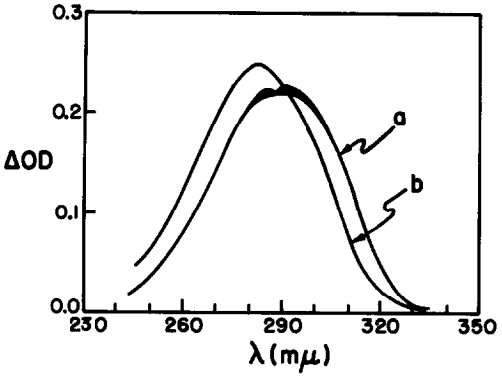

Figure 1

a - CIN-CT at 21° versus CT at 21°; 1.4 x 10^{-5} M, pH 2.0, 0.1% acetonitrile.
b - CIN-CT at 51° versus CT at 51°; 1.4 x 10^{-5} M, pH 2.0, 0.1% acetonitrile.

For each difference spectrum, the protein concentration is exactly the same in both samples. CIN-CT is prepared by adding to the enzyme solution at pH 5.0 a slightly smaller amount of trans-CIN-imidazole than the stoichiometry requires, letting the reaction take place for 1 minute, then acidifying to pH 2.0 and diluting to volume. Spectra are recorded on a Cary model 14 spectrophotometer.

changes in the absorption properties of the protein and the shift of the λ max of the CIN-group. Both methods gave the same Van't Hoff plot and the same transition parameters. This indicates an intimate relationship between the shift in the absorption spectrum of the CIN-group and the conformation of CIN-CT.

The experiment reported above also suggests that the CIN-group is no longer exposed to solvent in the enzyme-substrate compound. Recently, Herskovits and Laskowski (1962) have shown that it is possible to differentiate between chromophores of proteins which are exposed to solvent and those which are not. The method depends on the fact that if one changes the solvent in which a protein is dissolved, only the absorption spectra of the chromophores which are on the outside of the molecule can be perturbed.

Fig. 2 shows the difference spectrum of O-CIN-N-ac-serNH_2 in 20% ethylene glycol versus the same compound in water with a maximum at 305 mμ (curve a). Under these conditions native CIN-CT does not show spectral changes at 305 mμ or above (curve b) and gives a difference spectrum very similar to that obtained for native CT (curve c). For both enzymes the difference spectrum starts at 300 mμ, and corresponds mainly to the perturbation of the exposed tryptophans in the native state (Oppenheimer et al., 1963). On the contrary, when the tertiary structure of the CIN-CT is disrupted, it is possible to see a perturbation of the cinnamoyl chromophore in the presence of 20% ethylene glycol. The disruption of the tertiary structure is obtained here by the breaking of some covalent bonds by peptic hydrolysis at pH 2.0. After digestion the chromophore becomes accessible to the solvent and one observes a difference spectrum at 305 mμ between digested CIN-CT in 20% ethylene glycol and digested CIN-CT in water (curve d). In the case of digested CT no change in absorbancy is observed at 305 mμ (curve e). Thus, it seems clear that the cinnamoyl group is buried in the native state, being inaccessible even to "non-bulky solvents" such as ethylene glycol. Very similar results are obtained with 20% glycerol.

One can conclude from these experiments that the CIN-group is present as an ester in CIN-CT and that the chromophore becomes buried inside

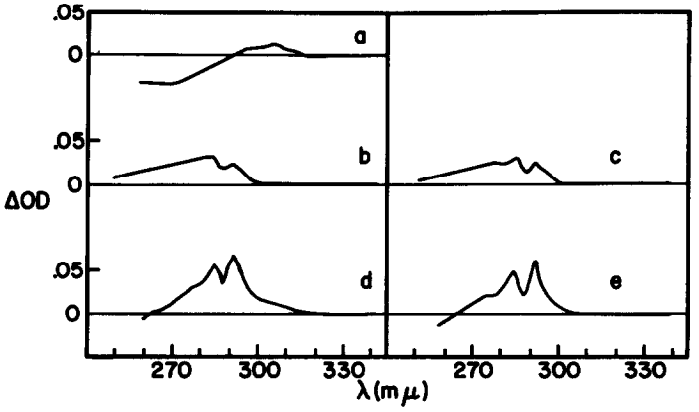

Figure 2

a - O-CIN-N-ac-serNH$_2$ in 20% ethylene glycol versus O-CIN-N-ac-serNH$_2$ in water; 2.6×10^{-5} M.
b - CIN-CT in 20% ethylene glycol versus CIN-CT in water; 2.6×10^{-5} M.
c - CT in 20% ethylene glycol versus CT in water; 3.2×10^{-5} M.
d - Digested CIN-CT in 20% ethylene glycol versus digested CIN-CT in water; 2.6×10^{-5} M.
e - Digested CT in 20% ethylene glycol versus digested CT in water; 3.2×10^{-5} M.

All spectra are obtained by use of tandem cells allowing correction for the solvent absorption (Herskovits et al., 1962). Spectra are recorded at room temperature. All samples are adjusted to pH 2.0 with HCl. CIN-CT is prepared by reaction of CT at pH 4.2 and room temperature with trans-CIN-imidazole, the excess of which is dialyzed out (dialysis against 10^{-3} M HCl). Digestions of enzymes are carried out at pH 2.0 and 38° for 12 hours, in presence of 10 μg/ml of crystalline pepsin. The final concentration of pepsin in the digested samples is 5 μg/ml and does not contribute detectably to the absorption spectra. The same digestion mixture is used for each pair of dilutions in ethylene glycol and in water.

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the protein molecule as a result of the conformational change which accompanies the formation of CT-substrate compounds (Wootton and Hess, 1961; Havsteen and Hess, 1962 and 1963; Labouesse, Havsteen and Hess, 1963; Havsteen, Labouesse and Hess, 1963; Mercouroff and Hess, 1963). As a result of the position of the CIN-group in the enzyme-substrate complex, its environment and hence its spectral properties are altered.

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