CHYMOTRYPSIN-SUBSTRATE COMPLEXES. HYDROGEN ION EQUILIBRIA IN CHYMOTRYPSIN AND DIISOPROPYLPHOSPHORYL-CHYMOTRYPSIN.

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Evidence that the chymotrypsin (CT) catalyzed hydrolysis of p-nitrophenyl acetate and the formation of diisopropylphosphoryl (DIP)-CT are accompanied by conformational changes of the enzyme was presented in previous papers (Havsteen et al., 1962; 1963; Labouesse et al., 1962; Lumry and Parker, 1963). Kinetic studies of the reaction of CT with diisopropylphosphorofluoridate (DFP) showed the liberation of about 1 mole of H^+ per mole of enzyme at pH 7.0 (Jandorf et al., 1955). This liberation of H^+, however, decreases progressively between pH 7.0 and 9.5, although the formation of DIP-CT goes to completion (Moon, Sturtevant and Hess, 1962). Preliminary potentiometric titration experiments with CT and DIP-CT indicated that the kinetically observed decrease in H^+ liberation in the reaction of DFP with CT above pH 7.0 may be due to the uptake of H^+ by the enzyme during the reaction (Moon et al., 1962). In order to identify the ionizing group or groups in CT involved in this H^+ uptake, we have investigated the hydrogen ion equilibria of CT and DIP-CT.

Salt free, three times crystallized α-chymotrypsin from Worthington was used, and DIP-CT was prepared as previously described (Wootton and Hess, 1962). Before titration, the enzyme samples were exhaustively dialyzed at 4° C against 7×10^{-4} M HCl. Protein concentrations were determined spectrophotometrically, assuming a molecular weight of 25,000. Phosphorus analyses were performed before and after the titrations to check the DIP-CT preparation. For each titration, 90 ml of 4×10^{-5} M enzyme solution were used.

Potentiometric titrations of CT and DIP-CT were made in 0.15 M KCl and in 0.4 M KCl at 4°, 16° and 25° C in the pH range 1.6 to 11.5. The titration curves were reversible between pHs 2 and 10.7. Control experiments demonstrated that contribution of autolysis products to the titration data was negligible. Each titration curve was obtained by subtraction of a solvent titration curve from that of the enzyme solution at every pH and temperature. The pH dependence of the apparent heat of ionization ($\Delta H'$) was computed from the best fitting curves drawn through the experimental titration data at 4°, 16° and 25° C.

Titration curves of CT and DIP-CT at 4° in 0.15 M KCl are shown in Fig. 1. The same pattern was also observed at 4° in 0.4 M KCl and at 16° and 25° in 0.15 M and 0.4 M KCl. The titration curves (Fig. 1) coincide from pH 2.0 to about pH 6. Above pH 6 they diverge, more protons being bound by CT than by DIP-CT. This difference reaches a maximal value of 1 H^+ per enzyme molecule near pH 9.9. It should be noted that the titration curves are still paral-

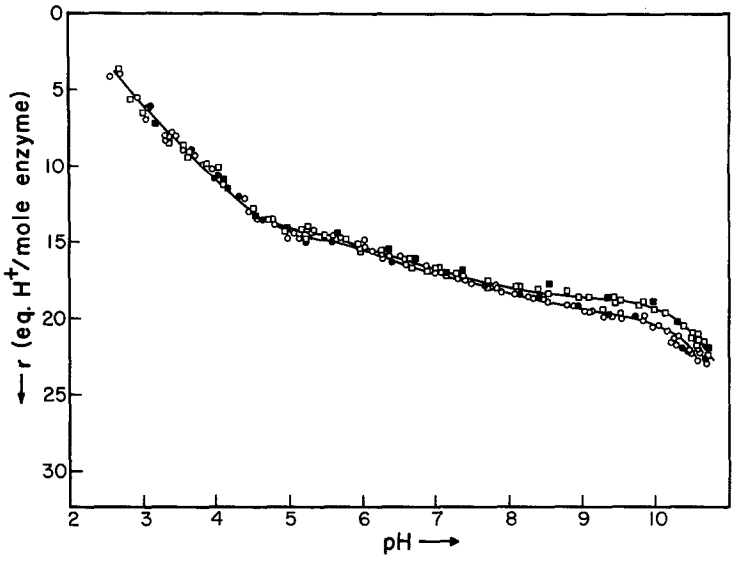

Figure 1. Electrometric titration of CT and DIP-CT at 4° C. in 0.15 M KCl. Enzyme concentration: 1 mg./ml. between pH 2.5 to 10.5; 4 to 13 mg./ml. between pH 2.0 to 2.5. CT: O ; DIP-CT: □ . Forward titration: open symbols; reverse titration: filled symbols. The experimental data between pH 2.0 to 2.5 are not shown in the Figure but were used for the calculation of r.

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lel at pH 10.2. This indicates a considerable increase of the pK of the ionizing group or groups perturbed in DIP-CT. It is known that buried groups in proteins can usually not be titrated until the molecule is denatured (Tanford, 1962). The experimental titration curves for both CT and DIP-CT gave an excellent fit to the theoretical titration curve in the pH region 2 to 5.0. The theoretieal curve was calculated using 13 carboxyl groups with a pK'_{int} of 3.5. Both the number of carboxyl groups (Wilcox, et al. 1957; Des-

nuelle, 1960) and the pK'_{int} (Tanford, 1962) are in agreement with previous data. Since chloride binding is usually maximal in the pH region 2 to 5, the fit of the experimental and theoretical titration curve indicates that the influence of ion binding on the data is probably small. Similarly, molecular aggregation and pH dependent conformational changes do not seem to have a decisive influence on the titration data from pH 2 to 5. Above pH 5, however, the experimental titration curves can no longer be fitted to theoretical curves. Two unusual characteristics were also noted in $\Delta H'$ above pH 5.0: a marked anomally in $\Delta H'$ between pH 5 and 6 which appears in both CT and DIP-CT and a pronounced difference between the $\Delta H'$s of CT and DIP-CT in the pH range 8 and 10, the $\Delta H'$s of CT being higher. These results will be discussed in a subsequent paper.

The difference between the titration curves of CT and DIP-CT accounts for the decrease in H^+ release above pH 7 observed in the kinetic investigation of the formation of DIP-CT. Since previous spectrophotometric titrations demonstrated that the tyrosyl residues in CT and DIP-CT have identical pK values (Havsteen and Hess, 1962), the data implicate the perturbation of α- or ε-amino groups in DIP-CT.

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