ON THE ELUCIDATION OF THE PH DEPENDENCE OF CHYMOTRYPSIN CATALYZED REACTIONS AT ALKALINE PH[1]

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Previous kinetic investigations of reactions catalyzed by chymotrypsin (CT) have been confined mainly to the pH region below pH 8. The limited study which has been made of these reactions in the alkaline pH region has shown (Northrop, Kunitz, and Herriot, 1948) that the catalytic properties of CT decrease progressively above pH 8, and that an ionizing group with pK(app) $\sim$ 9 is implicated in this decrease (Bender et al., 1964). It has been assumed (Bender and Kézdy, 1965) that the ionization of this group controls the bond-breaking step of the catalytic reaction. In this paper we are reporting investigations of the pH dependence, above pH 8, of the α- and δ-CT-catalyzed hydrolysis of a specific amide substrate, N-acetyl-L-tryptophan amide (ATA). We present evidence that in this hydrolysis the decrease in catalytic properties of the enzyme above pH 8 is due to the effect of pH on the formation of enzyme-substrate complexes and not on the steady-state kinetic parameter, k_{cat}.

For the experimental work, three-times-crystallized chymotrypsinogen and α-CT were obtained from Worthington Corp.; δ-CT was prepared by activating

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chymotrypsinogen under conditions which lead essentially to the δ form of the enzyme (Jacobsen, 1947). The ATA, chromatographically pure with m.p. 192-193° and $[\alpha]_D^{23} = 19.1 \pm 1.2°$, was obtained from Cyclo Chemical Corp.

Amide hydrolysis was followed by a sensitive, continuous, automatic determination of ammonia released, with use of ninhydrin as color-developing reagent. The instrumentation and procedure were described in detail by Lenard et al. (1965). Parallel experiments in which the enzyme or the substrate was omitted were performed at all pH's; it was found that ammonia production arising from non-enzymic hydrolysis of substrate or autolysis of enzyme was insignificant. In several instances, the steady-state kinetic parameters obtained by this method were checked by a previously published spectrophotometric method (Bender et al., 1964). These comparative measurements were made with a Cary Model 14 spectrophotometer at a slit width of 0.27 mm.

In the kinetic experiments, the initial active enzyme concentration, E_o, varied from 2.5×10^{-6} M to 5×10^{-6} M in the experiments performed by the automatic ninhydrin method, and was 2×10^{-5} M in the spectrophotometric experiments. E_o was determined by the N-trans-cinnamoyl imidazole method (Schonbaum, Zerner, and Bender, 1961). The initial substrate concentration, S_o, ranged from 20×10^{-3} M to 2×10^{-3} M, with at least eight different S_o values used for measurements at each pH. All experiments were run at 25°.

The steady-state kinetic parameters k_{cat} and K_m(app) were calculated from the initial rate data by means of a digital computer program written for the Lineweaver-Burk form of the Michaelis-Menten rate equation (Lineweaver and Burk, 1934). Data weighting and calculation of the standard errors of the kinetic constants were performed as discussed by Wilkinson (1961). The values obtained in this way were used to construct the lines in Fig. 1, which shows representative kinetic data plotted according to the method of Eadie (1942).

Fig. 1 shows a comparison of the data obtained for the δ-CT-catalyzed hydrolysis of ATA at pH 8 and at pH 10. It can be seen that k_{cat} (the intercept of the ordinate) is the same at both pH's, while K_m(app) (the slope) is

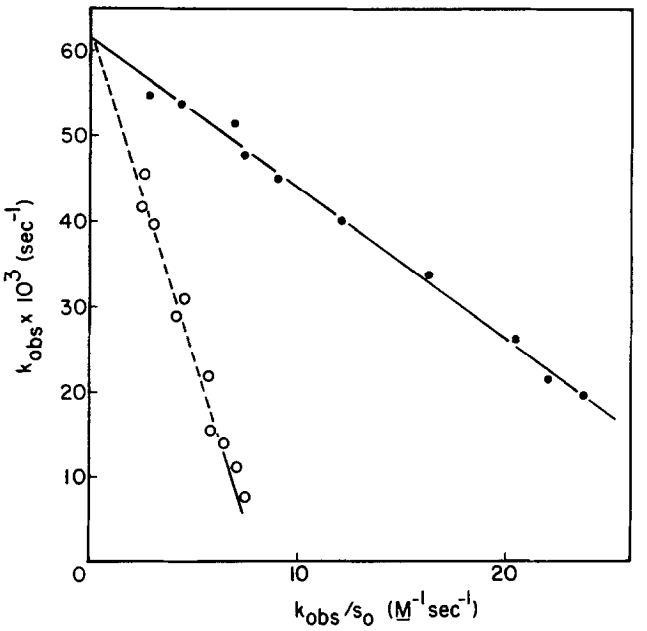

Figure 1

Eadie plots for the δ-CT-catalyzed hydrolysis of ATA at pH 8 (solid line, filled symbols) and at pH 10 (broken line, open symbols). Temperature 25°. The points are experimental and were obtained with use of the automatic ninhydrin method of Lenard et al. (1965). The lines represent the weighted least square fit to the data, calculated as discussed by Wilkinson (1961). For buffer composition, see legend of Fig. 2.

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greater at pH 10 than at pH 8. Similar data were obtained for the α-CT-catalyzed hydrolysis of the same specific amide substrate (Fig. 2): K_m(app) is again considerably larger at pH 10 than at pH 8, and k_{cat} is actually somewhat lower at pH 10 than at pH 8. A summary of the steady-state kinetic parameters for the α- and δ-CT-catalyzed hydrolysis of ATA in the pH region 8 to 10 is given in Fig. 2. It may be noticed in Fig. 2 that under comparable conditions, both the automatic ninhydrin method and the spectrophotometric method gave the same results.

The data presented in Figs. 1 and 2 demonstrate at least three important aspects of the CT-catalyzed hydrolysis of a specific amide substrate: (1) The pH dependence of this catalytic reaction at alkaline pH is adequately accounted for by the pH dependence of K_m(app). (2) The pH dependence of K_m(app)

implicates a group with pK(app) ~9, a group involved in all CT-catalyzed reactions. (3) There is no indication that an ionizing group with pK(app) ~9 affects k_{cat} of either the α- or the δ-CT-catalyzed reaction.

Evidence has been presented (Brandt and Hess, 1966) which indicates that in the α-CT-catalyzed hydrolysis of ATA, K_m(app) is a direct measure of the enzyme-substrate dissociation constant. It may be concluded, therefore, that the pH dependence of the α- and δ-CT-catalyzed hydrolysis of a specific amide substrate above pH 8 is due to the effect of pH on the formation of CT-substrate complexes.

Previous kinetic studies (Moon, Sturtevant, and Hess, 1965) of the stoichiometric phosphorylation of α-CT by its specific inhibitor diisopropyl phosphorofluoridate (DFP) are consistent with the data presented here and the conclusions reached. These studies indicated that the observed decrease in phosphorylation rate above pH 8 is due to the effect of hydrogen ions on a CT-inhibitor complex which is reversibly formed prior to the bond-breaking step. It was further shown (Oppenheimer, 1964; Oppenheimer, Labouesse, and Hess, 1966) that the pH dependence of this reaction above pH 8 is adequately accounted for by a pH-dependent equilibrium between active and inactive conformations of the enzyme, a group with pK(app) ~9 controlling both the decrease in the phosphorylation rate and the equilibrium between two conformations of the enzyme.

Therefore, the data obtained with both a specific CT inhibitor and a specific amide substrate indicate that the well known decrease of the catalytic properties of the enzyme above pH 8 (Northrop, Kunitz, and Herriot, 1948), is due to the effect of pH on the formation of reversible CT complexes which precede the bond-breaking step. Earlier experiments (Oppenheimer, 1964; Oppenheimer, Labouesse, and Hess, 1966) have indicated that a group with pK(app) ~ 9 controls the equilibrium between two conformations of the enzyme and thereby affects the formation of CT complexes. The conclusions reached from all these data are important because the presently accepted interpretation

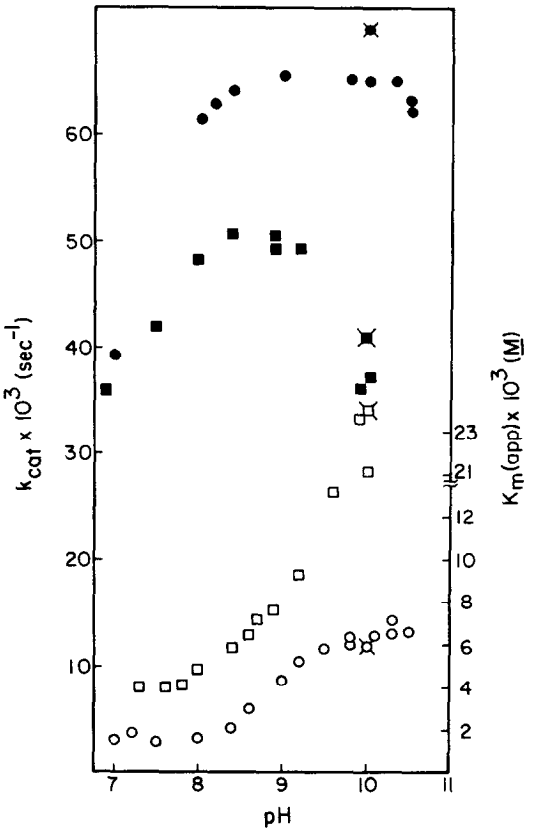

Figure 2

Summary of steady-state kinetic parameters for the α- and δ-CT-catalyzed hydrolysis of ATA in the pH region 7 to 10.5. Filled symbols, k_{cat}; open symbols, K_m(app); circles, δ-CT; squares, α-CT. Symbols that are crossed signify values obtained by the spectrophotometric method of Bender et al. (1964); all other values were obtained by the automatic ninhydrin method of Lenard et al. (1965). Buffers: pH 8.0 - 9.2, 0.02 M Tris-HCl; pH 9.2 - 10.5, 0.02 M K_2CO_3. Sufficient KCl was added to all solutions to give an ionic strength of 0.16 M. Temperature 25°.

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of the pH dependence of CT-catalyzed reactions is based on the assumption that the group with pK(app) ~ 9 affects k_{cat} (Bender et al., 1964). No evidence for this assumption was obtained.

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