CONFORMATIONAL CHANGES ACCOMPANYING THE FORMATION OF CHYMOTRYPSIN-SUBSTRATE COMPLEXES. EVIDENCE FOR THE INVOLVEMENT OF AN N-TERMINAL α-AMINO GROUP IN THE ACTIVITY AND THE CONFORMATION OF THE ENZYME.*

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Data in the preceeding paper (Havsteen and Hess, 1963) suggested that the formation of diisopropylphosphoryl (DIP) chymotrypsin (CT) increases the pK of one or more amino groups. In this paper, evidence will be presented that formation of DIP-CT involves perturbation of the pK of the N-terminal α-amino group of isoleucine. Both the pH dependence of the specific rotation $[\alpha]$ of CT and the rate of catalyzed reactions at high pH appear to depend on the pK of this group.

Three times crystallized chymotrypsinogen (CTogen) from Worthington was allowed to react for 40 minutes in a pH stat, at pH 6.7, 3° C., with 0.1 M acetic anhydride. The product, acetylated CTogen, was fractionated by am-

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monium sulfate precipitation and chromatographed on a DEAE-cellulose column. All amino groups and 2 out of 4 tyrosines are blocked by acetylation (Table).

TABLE

Summary of Data

Material	CTogen	Acetylated CTogen	Acetylated CT	Acetylated DIP-CT
Free (a) amino groups (Van Slyke)	15.1	0.6	1.6	1.6
Free (b) amino groups (DNP)				
Lys	+ + +	0	0	
Cys	+	0	0	
Ileu	0	0	+	
Ala	0	0	0	
Tyrosine (c) residues	2 normal 2 abnormal	2 acetylated 2 abnormal	2 acetylated 2 abnormal	
Ionizable (d) groups		2.4	3.2	2.4
Specific (e) rotation		pH independent	pH dependent	

(a) In number of groups per molecule. CTogen has 14 amino groups (Chervenka and Wilcox, 1956). High values in proteins are attributed to unspecific reaction of guanidino groups with nitrous acid (Greenstein and Winnitz, 1961).
(b) Determined after reaction with DNFB of performic acid oxidized or guanidinium-HCl denatured proteins.
(c) Measured spectrophotometrically at 244 mu (Wetlaufer, 1962) at pHs 11.9 and 12.9. Abnormal tyrosines ionize slowly only at pH 12.9 (Havsteen and Hess, 1962).
(d) Reversible titration curves were obtained at 4° C., 0.15 M KCl, from pH 5.0 to pH 10.4. Groups are given between pHs 6 and 10.
(e) Specific rotation measured at 12° C. in 0.1 M KCl from pH 7.0 to pH 10.9 at 305, 310, 340 and 366mu. Solutions gave the same specific rotation at pH 7 before and after measurements at high pH.

Acetylated CTogen was activated with trypsin under conditions leading to δ-chymotrypsin. Acetylated CT re-

acts stoichiometrically with DFP and exhibits the same spectral characteristics as DIP-CT (Wootton and Hess, 1962). Sephadex G-50 chromatography and paper electrophoresis showed acetylated DIP-CT to be free from autolysis products. The specific activity of acetylated CT, using N-acetyl-L-tyrosine ethyl ester (ATEE) as substrate, is 85 % of the activity of activated CTogen. Acetylated CT has one free amino group by Van Slyke determination, and the only amino group detected after reaction with dinitrofluorobenzene was that of isoleucine (Table). N-terminal isoleucine is produced during activation of CTogen to δ-CT (Bettelheim and Neurath, 1955; Desnuelle et al., 1955).

Acetylated CTogen, acetylated CT and acetylated DIP-CT were titrated from pH 5.0 to 10.4. Because of insolubility, titrations below 5.0 were impossible. However, the titration curves of CT and DIP-CT are identical from pH 2.0 to 6.0 (Havsteen and Hess, 1963). The three titratable groups of acetylated CT in the pH region 6 to 10 account for the ionizing groups of two histidines and N-terminal isoleucine. The titration curves of acetylated CTogen and acetylated DIP-CT are superimposable; in the pH region 6 to 10, only two groups are titrated. The extra titratable group in acetylated CT has an apparent pK of 8.3. This indicates that the group which is not titrated in acetylated DIP-CT is the isoleucyl α-amino group produced by activation of acetylated CTogen.

The specific rotation of CTogen is pH independent, while large changes of $[\alpha]$ are observed with δ-CT in the

pH 8 to 11 region. Above pH 11, $[\alpha]$ for both proteins is the same (Neurath, Rupley and Dryer, 1956). Similar data are obtained with acetylated CTogen and acetylated CT. In the latter case, there is a correlation between the ionization state of the N-terminal α-amino group and the pH dependence of $[\alpha]$. When this group is unable to ionize between pHs 7 to 10, as in DIP-CT, $[\alpha]$ becomes pH independent (Havsteen and Hess, 1963; Lumry and Parker, 1963).

The catalytic rate constant (k_{cat}) of many CT catalyzed reactions decreases in the pH region 8 to 12 (Bender and Clement, 1963). In the acetylated CT catalyzed hydrolysis of ATEE and N-acetyl-L-tryptophan ethyl ester (ATrEE), a relationship between the pK of the single α-amino group and k_{cat} was observed. When acetylated CT is treated with methyl acetimidate (Hunter and Ludwig, 1962) in order to shift the pK of the α-amino group to about 12, k_{cat}, determined with ATrEE, becomes essentially pH independent between pHs 8 and 11. Blocking of the N-terminal isoleucyl group of acetylated CT with an acetyl group abolished catalytic activity.

Bender et al. (1963) proposed that above pH 8, the formation of acyl-CT is pH dependent and deacylation is pH independent. This can be explained by an increase in pK of the N-terminal α-amino group in CT-substrate complexes, as observed in acetylated DIP-CT. The experiments presented indicate that the pK of the N-terminal isoleucyl α-amino group of CT controls $[\alpha]$ and k_{cat}. Consequently the protonated α-amino group appears essential for maintaining CT in its catalytically active conformation.

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