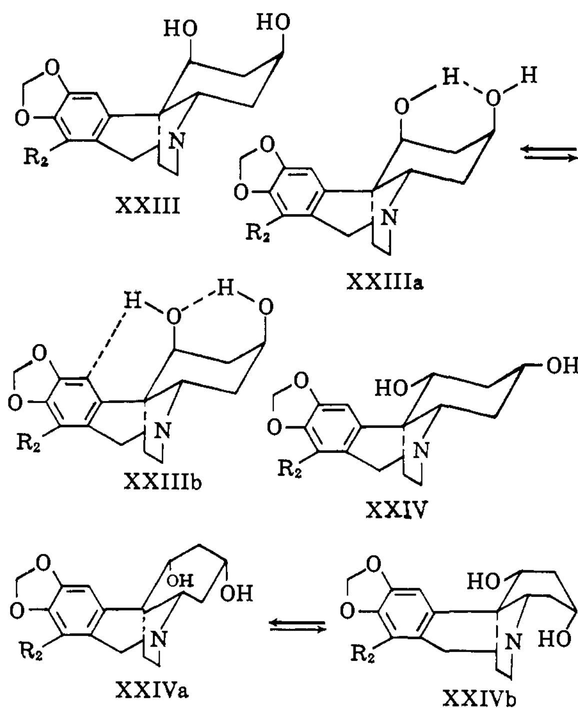
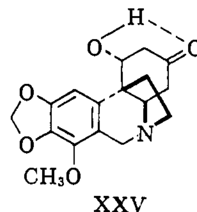


XVIa (3599 and 3584 cm.$^{-1}$, respectively) and may be assigned to the C_1-hydroxyl of XXIIIb. While conformation XXIVb permits the C_1-hydroxyl group to be within probable bonding distance of the aromatic nucleus, the actual distance appears to be considerably longer than that of XXIII and by analogy with XVe would give rise to a band near 3616 cm.$^{-1}$. In addition, conformation XXIVb would not exhibit 1,3-diaxial OH. . .O bonding at 3533 cm.$^{-1}$ nor would XXIVa exhibited the OH → π interaction mentioned above. Thus it becomes necessary to assume a comparable concentration of both conformations XXIVa and XXIVb in equilibrium in solution in order to explain partially the observed curve. However, the latter conformation (XXIVb) not only places the C_3-hydroxyl at a point in space occupied by a hydrogen atom on C_{11} of the ethano bridge but also causes loss of the powerful hydrogen bonding present in XXIVa. In contrast, all of the observed bands are neatly accommodated by the two rotational species XXIIIa and XXIIIb which do not require unlikely conformational changes of ring C.



Finally, XXV shows bands at 3618, 3590 and 3533 cm.$^{-1}$. The first two were expected by analogy with P-XVa or XXII. The last band is regarded as most unusual since it indicates that OH → carbonyl bonding is occurring although the distance is quite large (2.8 Å.).[26] It seems unlikely that the band is due to OH → π bonding with the carbonyl carbon because of its position and intensity which resembles that of the *cis*-diols XXIII and XXIII (no methoxyl). Such a bond also is unlikely considering the positive nature of the carbonyl carbon atom. Partial hydration of the carbonyl group was eliminated since the carbonyl band was unchanged on addition of a droplet of water or heavy water to the cell. Nor is the band an overtone of the carbonyl group[27] since the entire pattern was shifted to the 2700 cm.$^{-1}$ region on deuteration of the hydroxyl group. We are forced to conclude that hydrogen bonding is possible in axial 3-hydroxycyclohexanone systems.

(26) It is noteworthy that weak OH → O bonding is observed in P-XIIIe where the distance also is 2.8 Å.

(27) R. N. Jones and C. Sandorfy in "Technique of Organic Chemistry," Vol. IX, Interscience Publishers, Inc., New York, N. Y., 1956, p. 425.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY, ITHACA, N. Y.]

Evidence for Conformational Changes in α-Chymotrypsin-catalyzed Reactions. VI. Changes in Optical Rotatory Dispersion Parameters

BY BENT H. HAVSTEEN[1] AND GEORGE P. HESS[2]

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The optical rotatory dispersion parameters of α-chymotrypsin, diisopropylphosphoryl-α-chymotrypsin and monoacetyl-α-chymotrypsin were determined at *p*H 3.8 and 12°. The dispersion parameters, a_0 and b_0, of monoacetyl-α-chymotrypsin and diisopropylphosphoryl-α-chymotrypsin were found to be similar but significantly different from the optical rotatory dispersion parameters of α-chymotrypsin. Deacylation of monoacetyl-α-chymotrypsin results in a molecule which has the same a_0 and b_0 values as α-chymotrypsin, indicating that the changes in the optical rotatory dispersion parameters are reversible and intimately related to the formation and breakdown of this enzyme-substrate compound. Studies of the *p*H dependence of the specific rotation, $[\alpha]$, at 365 mμ and of the temperature dependence of $[\alpha]_{365}$ at *p*H 3.8 and 4.0 also revealed significant differences between the enzyme-substrate compounds diisopropylphosphoryl-α-chymotrypsin, monoacetyl-α-chymotrypsin and α-chymotrypsin. Previous evidence, in combination with the data presented here, indicates that the formation of the enzyme-substrate compounds is accompanied by conformational changes.

Two reactions of α-chymotrypsin (CT) are considered in this investigation: the stoichiometric reaction of CT with diisopropylphosphorofluoridate (DFP) to give diisopropylphosphoryl-α-chymotrypsin (DIP-CT) and HF[3]; and the CT-catalyzed hydrolysis of *p*-nitrophenyl acetate.[4] This latter reaction proceeds *via* an intermediate, monoacetyl-CT, which can be isolated below *p*H 5.0.[5] Above *p*H 6.0, monoacetyl-CT, isolated according to the procedure of Marini and Hess,[6] is kinetically the intermediate in the chymotrypsin-

(1) Fulbright grantee, 1959–1962. This work is a small part of a thesis submitted by B. H. Havsteen to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(2) Address 1962–1963: Fulbright grantee and John Simon Guggenheim Fellow, Max Planck Institute for Physical Chemistry, Göttingen, Germany.

(3) E. F. Jansen, M. D. F. Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189, 201 (1949); **185**, 209 (1950).

(4) B. S. Hartley and B. A. Kilby, *Biochem. J.*, **56**, 288 (1954); H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci. U.S.*, **42**, 719 (1956); *Biochem. J.*, **63**, 656 (1956).

(5) A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956).

(6) M. A. Marini and G. P. Hess, *J. Am. Chem. Soc.*, **82**, 5160 (1960).

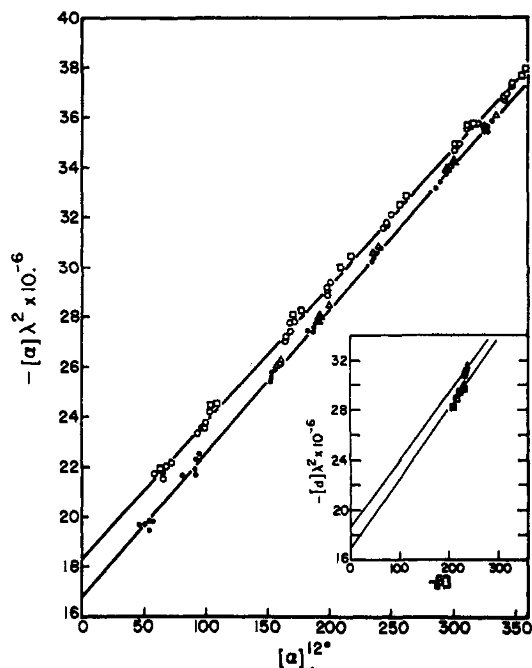

Fig. 1.—Drude plot of the rotatory dispersion of: CT, O; DIP-CT, ●; monoacetyl-CT, Δ; monoacetyl-CT after deacylation, □; pH 3.8, acetate buffer–KCl (μ = 0.14 M), 12°. Protein concentration was 0.8 to 1.4 mg./ml. The solid lines were computed as described in the text using the data for CT and DIP-CT, respectively. Inset: The solid lines and the conditions of the experiment are the same as in Fig. 1. Chromatographically homogeneous CT, O; CT, ⍿; CT in 1% (v./v.) 2-propanol, ●; DIP-CT prepared from chromatographically homogeneous CT, ⊡; DIP-CT, ■; and DIP-CT in 0.6% (v./v.) 2-propanol, ■; protein concentration 1.2 mg./ml.

catalyzed hydrolysis of p-nitrophenyl acetate.[6,7] It has been demonstrated that in this reaction and in the reaction of CT with DFP the same seryl hydroxyl group of CT becomes substituted.[8] Since the hydrolysis of specific substrates for CT most probably involves the same mechanism as the CT-catalyzed hydrolysis of p-nitrophenyl acetate,[9] the data obtained in the studies reported here may have an important bearing on CT-catalyzed reactions in general.

Earlier kinetic and equilibrium studies demonstrated that monoacetyl-CT can undergo a structural change in solution under conditions where CT cannot.[6,7,10,11] Subsequent studies of the CT-catalyzed hydrolysis of p-nitrophenyl acetate revealed changes in the spectrum of the enzyme at 290 mμ which are intimately related to the formation and decomposition of the monoacetyl-enzyme.[12,13] These absorbancy changes at 290 mμ have also been observed in the reaction of DFP with CT or trypsin,[12] and more recently by Bender, *et al.*,[14] in the formation of cinnamoyl-CT. Experiments[12,13] on the characterization of the spectral changes at 290 mμ suggested that they are due to reversible conformational changes of the enzyme brought about by acylation of its active site. To characterize these reversible, structural changes, initial investigations were concerned with the chemical reactivity of tryptophyl residues[13] and the hydrogen ion equilibria of tyrosyl residues in CT and DIP-CT.[15] Both chemical and spectroscopic studies indicated that the tryptophyl residues are not equivalent in CT and DIP-CT, while titration of the tyrosyl residues did not reveal differences between the molecules. This investigation is concerned with the optical rotatory dispersion and the pH and temperature dependence of the specific rotation of CT, DIP-CT and monoacetyl-CT. A preliminary report of a part of these investigations has appeared.[16]

Results

Optical Rotatory Properties.—The experiments were performed at pH 3.8 where monoacetyl-CT is stable and CT has some activity. A low protein concentration of 0.8 to 1.4 mg./ml. was chosen and an ionic strength of 0.14 M was used to avoid aggregation of the enzymes. The specific rotation, $[\alpha]$, was found to be independent of the protein concentration in the range used.

Optical rotatory dispersion curves for the proteins were plotted by two methods. The data in Fig. 1 were plotted according to a modified Drude equation[17]

$$\lambda^2[\alpha] = \lambda_c^2[\alpha] + K$$

The parameter, λ_c, was obtained from the slope of the straight line of a plot of $-\lambda^2[\alpha]$ *vs.* $-[\alpha]$, and K from the intercept. In order to compare these data with other data in the literature, and because a_0 and b_0 have not previously been reported for CT, the data were also plotted according to the Moffitt equation,[18] Fig. 2

$$[\alpha]_\lambda = \left(\frac{100}{M}\right)\left(\frac{n^2+2}{3}\right)\left(\frac{a_0\lambda_0^2}{(\lambda^2-\lambda_0^2)} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}\right)$$

where M is the average residue weight, taken here as 104, and n is the refractive index of the solvent. The indices of refraction, n, of the solvents used were obtained from the "International Critical Tables," and

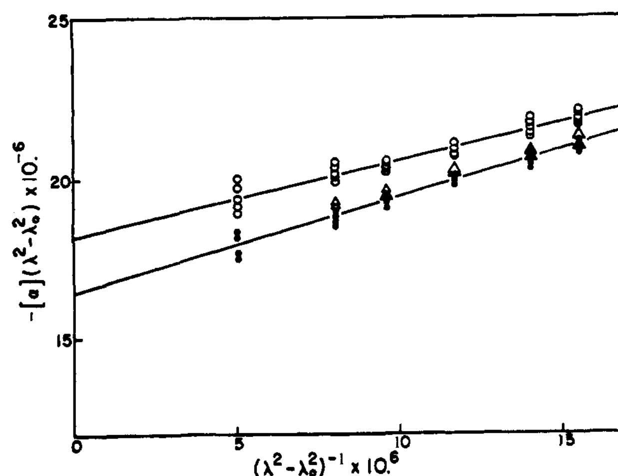

Fig. 2.—Moffitt plot of the rotatory dispersion of: CT, O; DIP-CT, ●; and monoacetyl-CT, Δ. All other conditions as in Fig. 1.

refer to 12°, the temperature at which the experiments were carried out. No correction of n for wave length

(7) G. P. Hess and M. A. Marini, "Abstracts, IV Intern. Congr. Biochem., Vienna," 1958, p. 42; M. A. Marini and G. P. Hess, *J. Am. Chem. Soc.*, **81**, 2594 (1959); *Nature*, **184**, 113 (1959).

(8) N. K. Schaffer, L. Simet, S. Harshman, R. R. Engle and R. W. Drisko, *J. Biol. Chem.*, **225**, 197 (1957); F. Turba and G. Gundlach, *Biochem. Z.*, **327**, 186 (1955); R. A. Oosterbaan and M. E. van Adrichem, *Biochem. Biophys. Acta*, **27**, 423 (1958); R. A. Oosterbaan, P. Kunst, J. van Rotterdam and J. A. Cohen, *ibid.*, **27**, 549, 566 (1958).

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(11) J. F. Wootton and G. P. Hess, *ibid.*, **83**, 4234 (1961).

(12) G. P. Hess and J. F. Wootton, *Fed. Proc.*, **19**, 340 (1960); J. F. Wootton and G. P. Hess, *Nature*, **188**, 726 (1960).

(13) J. F. Wootton and G. P. Hess, *J. Am. Chem. Soc.*, **84**, 440 (1962).

(14) M. L. Bender, G. R. Schonbaum and G. A. Hamilton, *J. Polymer Sci.*, **49**, 75 (1961).

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(16) B. H. Havsteen and G. P. Hess, *ibid.*, **84**, 491 (1962).

(17) J. T. Yang and P. Doty, *ibid.*, **79**, 761 (1957).

(18) W. Moffitt, *J. Chem. Phys.*, **25**, 467 (1956).

was made. The value of λ_0 was taken to be 212 mμ[19] in accordance with that found best for poly-γ-benzyl-L-glutamate[2] and for many proteins,[20] in a variety of solvents.[2] In the wave length range investigated, no deviation from linearity of the Moffitt plot could be detected. The parameter, b_0, was calculated from

$$-\frac{M}{100}\left(\frac{3}{n^2+2}\right)\Big/\lambda_0{}^4$$

times the slope of the plot in Fig. 2; and a_0, from the intercept times

$$-\frac{M}{100}\left(\frac{3}{n^2+2}\right)\Big/\lambda_0{}^2$$

Figure 1 is a modified Drude plot for CT, DIP-CT, monoacetyl-CT and deacylated monoacetyl-CT at 12° and pH 3.8. Figure 2 is a Moffitt plot for CT and DIP-CT at the same pH and temperature.

The two lines in Fig. 1 and 2 represent the dispersion of the specific rotation of CT and DIP-CT, respectively. The coördinates of the lines were computed by the method of least squares using an appropriate weighting factor (λ^{-2}) for the difference in magnitude of [α] obtained at different wave lengths.[21] The line for CT was established by 48 independent measurements and the line for DIP-CT, by 43 independent measurements. It can be seen in Fig. 1 that the dispersion of [α] for monoacetyl-CT falls on the line established by measurements for DIP-CT. When monoacetyl-CT is deacylated the resulting molecule has the same optical rotatory dispersion parameters as CT. This indicates that the changes in optical rotatory dispersion parameters are reversible and intimately related to the formation and breakdown of the enzyme-substrate intermediate, monoacetyl-CT. A summary of the optical rotatory dispersion parameters is given in Table I.

TABLE I

OPTICAL ROTATORY DISPERSION PARAMETERS OF α-CT, DIP-CT AND MONOACETYL-CT

Protien	λ_c, mμ	b_0, deg.	K, $(m\mu)^2 \times$ deg. $\times 10^{-6}$	a_0, deg.
α-CT	234 ± 1	− 93 ± 2	−18.4 ± 0.13	−335 ± 5
	234.9 ± 2[a]	− 84 ± 4[b]	−19.1 ± .1[a]	−336 ± 1[b]
DIP-CT (mono-acetyl-CT)	241 ± 1	−118 ± 1	−16.8 ± .13	−305 ± 3
		−109 ± 2[b]		−304 ± 1[b]

pH 3.8 acetate buffer, KCl, $\mu = 0.14\ M$, 12°. The optical rotatory dispersion parameters were not evaluated separately for monoacetyl-CT. [a] Reference 22. [b] Reference 23.

A statistical analysis of the data using appropriate methods for the testing of unpaired observations with unequal variances revealed that the dispersion parameters b_0 or λ_c and a_0 or K for CT are significantly different from those of either DIP-CT or monoacetyl-CT. The λ_c value for CT is in good agreement with the value obtained by Jirgensons[22] at pH 3.1, even though he used a protein concentration 10 times greater than the one used in the studies reported here. The differences between the dispersion parameter for CT and DIP-CT calculated by us, and by Foster and Leonard,[23] are the same. However, the absolute values differ. This most probably is due to the fact that we used the indices of refraction, n, of the actual solvents, but did not correct n for wave length. Foster and Leonard[23] used the refractive index of pure water and allowed for the dispersion.

(19) See ref. 23.

(20) W. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci. U.S.*, **42**, 596 (1956).

(21) J. A. Schellman, *Compt. rend. Lab. Carlsberg, Ser. Chim.*, **30**, 363 (1958).

(22) B. Jirgensons, *Arch. Biochem. Biophys.*, **85**, 532 (1959).

(23) Personal communication by Professor J. F. Foster, Department of Chemistry, Purdue University. We are grateful to Mr. W. Leonard and Prof. Foster for making these values available to us. They are based on application of their statistical method (M. Sogami, W. J. Leonard, Jr., and J. F. Foster, *J. Am. Chem. Soc.*, in press, for the evaluation of the best parameters of the Moffitt plot to our data and were computed on a Royal RCP-4000 computer. These authors also found a λ_0 of 212 mμ.

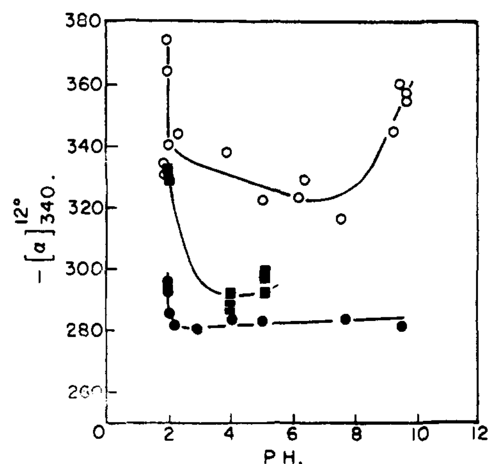

Fig. 3.—The pH dependence of the specific rotation at 340 mμ and 12° of: CT, O; monoacetyl-CT, ■; and DIP-CT, ●; protein concentration 1.2 mg./ml. For the buffer composition, see the Experimental section.

To eliminate the possibility that the differences in dispersion parameters between CT and DIP-CT or monoacetyl-CT are due to small impurities in the preparation, $[\alpha]_{365}$ was also determined for chromatographically homogeneous CT and its DIP-derivative. The values obtained in these experiments are compared to those obtained with three times crystallized CT (inset of Fig. 1). It can be seen from this figure that the data appear to be the same for both CT preparations.

Studies reported in an accompanying paper[24] demonstrate that 0.6% (v./v.) of 2-propanol has a dramatic effect on the reversible, thermally induced, phase transition of DIP-CT at pH 2.00. The deviation of the transition curve in 2-propanol from the one obtained in water is marked at high temperatures. It was therefore of interest to investigate the effect of 2-propanol on the specific rotation of CT and DIP-CT. The results of these experiments can be seen in the inset of Fig. 1. One per cent (v./v.) of 2-propanol, or a molar ratio of 2-propanol to CT of 4000:1, does not appear to affect the dispersion parameters of CT; and 0.6% (v./v.) of 2-propanol apparently has no effect on the dispersion parameters of DIP-CT, while 2% (v./v.) has (not shown).

The Effect of pH on the Specific Rotation.—The effect of pH on the specific rotation at 340 mμ and 12° of CT, DIP-CT and monoacetyl-CT can be seen in Fig. 3. The experiments were carried out with a protein concentration of 1.2 mg./ml. and with an ionic strength of 0.14 M to avoid aggregation of the enzymes. The curve for CT is almost identical with the curve for δ-chymotrypsin published by Neurath, Rupley and Dreyer,[25] except that the enzyme concentration employed by these authors was 40 mg./ml. The specific rotation of DIP-CT is essentially independent of pH. Because of the instability of monoacetyl-CT, measurements of [α] could not be extended beyond pH 5.0. Its specific rotation is more pH dependent than for DIPCT, but less than that of CT.

The Effect of Temperature on the Specific Rotation.—The effect of temperature on the specific rotation of α-CT, DIP-CT and monoacetyl-CT can be seen in Fig. 4. In the absence of other data, $[\alpha]_{340}$ can only

(24) B. Havsteen, B. Labouesse and G. P. Hess, *J. Am. Chem. Soc.*, **85**, 796 (1963).

(25) H. Neurath, J. A. Rupley and W. J. Dreyer, *Arch. Biochem. Biophys.*, **65**, 243 (1956).

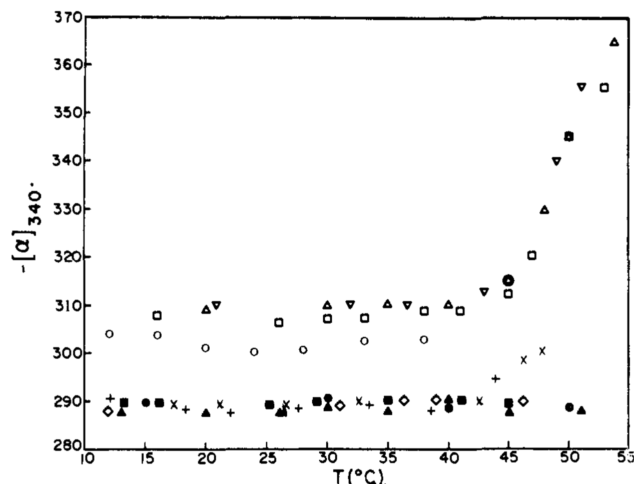

Fig. 4.—The temperature dependence of the specific rotation at 340 mμ, pH 4.0 (HCl), 0.10 M KCl; protein concentration in mg./ml. CT: 1.1, □; 1.4, ∇; and 1.5, ∇. DIP-CT: 1.1, ▲; 1.2, ●; and 1.4, ◊. Monoacetyl-CT: 1.4, (+); and 1.4 (x). At pH 3.8 (acetate buffer, μ = 0.14 M): 1.4 CT, O; and 1.4 DIP-CT, ■.

be considered as one indication of the thermal stability of the proteins. For each experiment the same protein solution was used throughout the temperature range. Measurements were made until the solutions became turbid. This occurred at a different temperature for each protein. It can be seen in Fig. 4 that the specific rotation of DIP-CT and monoacetyl-CT is considerably less temperature dependent than the specific rotation of CT.

Discussion

Although the equation used to evaluate the dispersion constants must be considered as empirical, they have been calibrated by studies of synthetic polypeptides in known conformation.[26] The conclusion of Moffitt and Yang[2] that the dispersion constants λ_c and b_0 are relatively independent of solvent has been substantiated in a large number of studies.[26,27] The interpretation of the optical rotatory dispersion parameters of myoglobin[28] has been supported directly by the crystallographic investigations of Kendrew, *et al.*[29] It can be seen from Table I that the formation of DIP-CT or monoacetyl-CT is accompanied by small but significant changes in λ_c or b_0. Possible explanations are an increase in right-handed helices, a decrease in left-handed helices, or a change in β-structure.[26]

The influence of solvent on the dispersion parameters of β-lactoglobulin and γ-globulin has recently been studied by Tanford and co-workers.[27] Even 40% ethylene glycol had only a small effect on a_0 but no effect on b_0. Figure 1 shows that 1% 2-propanol, or a molar ratio of 2-propanol to CT of 4000:1, does not have any observable effect on the optical rotatory dispersion constants of CT. Yet the introduction of a single diisopropylphosphoryl group into the active site of CT, or even more remarkably of a single acetyl group, changes K or a_0 by 10% (Table I). Whether this change in a_0 merely reflects the change in b_0 or also reflects some additional changes in the molecule such as changes in side group interactions or in β-structures is not known at present. Differences in specific rotation at 589 mμ between δ-chymotrypsin and DIP-δ-chymotrypsin have also been observed by Neurath, Rupley and Dreyer,[25] and in a_0 for CT and DIP-CT by Lumry and Parker.[30]

Although the interpretation of changes in the optical rotatory dispersion parameters is tenuous, the important point is that the data indicate that the formation of the CT-substrate compounds DIP-CT and monoacetyl-CT is accompanied by conformational changes.

Further evidence for the occurrence of conformational changes in the formation of CT-substrate compounds is the effect of pH on the specific rotation of CT, DIP-CT and monoacetyl-CT (Fig. 4). The similarity between the pH dependence of $[\alpha]$ of δ-chymotrypsin[25] and CT (Fig. 4) is striking. Neurath, Rupley and Dreyer[25] attributed the variation in rotation of δ-chymotrypsin with pH to pH-dependent structural changes of the enzyme. The observation that similar data are obtained with different enzyme concentrations (1 mg./ml. in these studies and 40 mg./ml. in the studies of Neurath, *et al.*[25]) probably excludes the possibility that these changes in rotation with pH are due to pH dependent aggregation of the enzymes. The observation that the specific rotation of CT is pH dependent and that the specific rotation of DIP-CT is pH independent again suggests differences in conformation of these proteins. It should be mentioned that in this respect DIP-CT resembles chymotrypsinogen in which the specific rotation also is essentially pH independent.[25] There is a striking change in the specific rotation of CT in the pH region 7–9 and a similarity in the pH independence of $[\alpha]$ of DIP-CT and chymotrypsinogen. It is interesting to note that preliminary titration studies suggest that CT has two more titratable ionizing groups (probably amino acid groups) than DIP-CT in this pH region.[31] Similarly, CT has two more amino groups than chymotrypsinogen.[32]

Previous reports by Cohen and Erlanger[33] indicated that diethylphosphoryl-CT is more stable than CT at 56° and pH 7.0, and the authors suggested on the basis of this experiment that the formation of phosphoryl-CT is accompanied by a change in conformation. However, CT can and probably does autolyze under these conditions, while the inhibited enzyme cannot do so. The data presented in Fig. 4 were obtained under less tenuous circumstances and they demonstrate the increased thermal stability of DIP-CT and monoacetyl-CT as compared to CT. This observation is treated more quantitatively in an accompanying paper,[24] in which we determine the thermodynamic parameters of the thermally induced transitions of CT, DIP-CT and monoacetyl-CT at pH 2.00.

The following evidence has been presented in previous papers supporting the thesis that the formation of the chymotrypsin-substrate compounds monoacetyl-CT and DIP-CT is accompanied by conformational changes: 1. Marini and Hess presented evidence that monoacetyl-CT can exist in two forms characterized by differences in the kinetics of the deacylation reaction.[6,7] 2. Subsequently, Wootton and Hess[10,11] noticed that monoacetyl-CT can rearrange in solution, under conditions where CT does not, to give a molecule

(26) For the most recent review see: P. Urnes and P. Doty, *Advan. Protein Chem.*, **16**, 402 (1961).

(27) C. Tanford, C. E. Buckley III, P. K. De and E. P. Lively, *J. Biol. Chem.*, **237**, 1168 (1962); C. Tanford, *J. Am. Chem. Soc.*, **84**, 1747 (1962).

(28) P. Urnes, K. Imahori and P. Doty, *Proc. Natl. Acad. Sci. U.S.*, **47**, 1635 (1961).

(29) J. C. Kendrew, R. E. Dickerson, B. E. Strandberg, R. G. Hart, D. R. Davies, D. C. Phillips and V. C. Shore, *Nature*, **185**, 422 (1960); J. C. Kendrew, H. C. Watson, B. E. Strandberg, R. E. Dickerson, D. C. Phillips and V. C. Shore, *ibid.*, **190**, 666 (1961).

(30) R. Lumry and H. Parker, *Fed. Proc.*, **21**, 246 (1962).

(31) A. Y. Moon, J. M. Sturtevant and G. P. Hess, forthcoming manuscript.

(32) W. J. Dreyer and H. Neurath, *J. Biol. Chem.*, **217**, 527 (1955); P. Desnuelle and M. Rovery, *Proc. IIIrd Intern. Congress Biochem.*, *Brussels*, 78 (1955).

(33) W. Cohen and B. F. Erlanger, *J. Am. Chem. Soc.*, **82**, 3928 (1960).

which rapidly aggregates in the *p*H region 5.5 to 8.0. 3. A difference spectrum between the enzyme-substrate compounds and CT was noticed.[12,13] The experiments suggested that this difference spectrum is due to a nonionic solvent effect arising from a change in environment of tryptophyl residues of CT in the formation of the CT-substrate compounds. 4. Differences between CT and DIP-CT in the chemical reactivity of tryptophyl residues was noticed.[12,13] 5. Preliminary data indicate the existence of differences in reversible titration curves between CT and DIP-CT, presumably due to the perturbation of amino groups in the formation of DIP-CT.[31]

In this paper we have demonstrated the following significant differences between CT and the CT-substrate compounds DIP-CT and monoacetyl-CT. 6. Differences in the optical rotatory dispersion parameters b_0 or λ_c and in a_0 or K at *p*H 3.8 and 12°. 7. Differences in the *p*H dependence of the specific rotation at 340 mμ and 12°. 8. Differences in the temperature dependence of the specific rotation at *p*H 3.8 and 4.0.

Previous control experiments with freshly activated, 12 times crystallized chymotrypsinogen[13] and experiments with chromatographically homogeneous CT reported in this paper make it unlikely that the observations reported are due to an impurity.

The observations that the measurements reported are independent of protein concentration, the experiments of Jirgensons,[22] who obtained the same λ_c value with a ten times higher protein concentration, and the qualitative agreement of the *p*H dependence in $[\alpha]$ of CT obtained in these studies and in experiments with δ-chymotrypsin[25] with a 40 times higher protein concentration all indicate that the observations reported here are not due to differences in the state of aggregation.

The evidence based on kinetic, chemical, spectroscopic and optical rotatory dispersion studies strongly indicates that the formation of CT-substrate compounds is accompanied by conformational changes of the protein. Experiments described in an accompanying paper,[24] in which we report the determination of the thermodynamic parameters of the thermally induced phase transitions at *p*H 2.00 of CT and the CT-substrate compounds, are in agreement with this conclusion.

Experimental

Materials. **α-Chymotrypsin.**—Salt-free, three-times crystallized α-chymotrypsin (CT) (Lot 6018-19), Worthington Biochemical Corporation, and chromatographically homogeneous (Amberlite CG50) α-chymotrypsin (Lot CDC 45) were used. Assays of the lots, using the potentiometric method of Schwert, *et al.*,[34] and N-acetyl-L-tyrosine ethyl ester as the substrate at *p*H 8.0 and 25° in 10% acetone gave the following values for k_3: CT, Lot 6018-19, k_3 = 105 sec.$^{-1}$; Lot CDC 45, k_3 = 120 sec.$^{-1}$. The value for CT reported in the literature[35] using the same substrate at 25° is: k_3 = 115 sec.$^{-1}$ at *p*H 7.8 in 30% methanol.

DIP-CT was prepared as described previously.[13] Phosphorus analysis by the method of Sumner[36] and the difference spectrum of DIP-CT *versus* CT[12,13] were used to check the preparation.

Monoacetyl-CT was prepared and checked for the extent of acylation as described previously.[6] Monoacetyl-CT was also assayed, before and at the end of each experiment, using L-tyrosine ethyl ester as the substrate at *p*H 5.0 and 20° to ascertain that the sample did not deacylate during the experiment. The samples used were 90% acylated. Preparations of monoacetyl-CT were routinely deacylated at *p*H 6.5, as previously described,[11] and assayed with N-acetyl-L-tyrosine ethyl ester at *p*H 8.0, 25°, to ensure that the preparation of this CT-substrate compound did not change its catalytic properties irreversibly.

Reagents.—Diisopropyl phosphorofluoridate was obtained from K & K Laboratories Inc., Jamaica, N. Y. N-Acetyl-L-tyrosine ethyl ester and L-tyrosine ethyl ester were purchased from Mann Laboratories, 136 Liberty St., New York, N. Y. Tris-(hydroxymethyl)-amino methane (Tris) was obtained from the Fisher Scientific Co. All other reagents were reagent grade and were obtained from Mallinckrodt Chemical Works. All solutions were prepared with conductivity water.

Methods. Instrument.—A Cary model 14 self-recording spectrophotometer was employed for all spectral measurements. A Radiometer *p*H meter, type TTT1, with a type B glass electrode was used for *p*H measurements which are in reference to Beckman *p*H 4.01 and 7.00 standard buffers, and 0.01 *M* borax at *p*H 9.18 and 25° (National Bureau of Standards). The instrument used in the optical rotatory dispersion studies is described in detail below.

Protein concentrations were determined by spectrophotometric measurements at 280 mμ, using a molar extinction coefficient of 50,000[37] for all enzymes. Previous experiments have demonstrated that the error in determining the concentration of DIP-CT or monoacetyl-CT at 280 mμ is negligible. The molecular weight of α-chymotrypsin was taken as 25,000.[38]

Enzyme Assays.—The enzymes were assayed using the potentiometric method of Schwert, *et al.*[34] At *p*H 8.0 and 25°, 1.00 ml. of enzyme solution was added at zero time to a solution of the following composition: 8 ml. of *p*H 8.0 buffer (4×10^{-3} *M* TRIS, 0.1 *M* $CaCl_2$) and 4 ml. of 5×10^{-2} *M* N-acetyl-L-tyrosine ethyl ester in 28% (v./v.) aqueous acetone. At *p*H 5.0 and 20°, 1 ml. of enzyme solution was added at zero time to a solution of the following composition: 8 ml. of *p*H 5.0 buffer (1×10^{-3} *M* sodium acetate, 1×10^{-3} *N* $CaCl_2$) and 4 ml. of 18×10^{-3} *M* L-tyrosine ethyl ester.

The *p*H was kept constant by the addition of 0.2 *N* KOH (CO_2 free). The addition of the base and the recording of the base added were performed by a Radiometer, type TTT1, automatic titrator and the Radiometer Titrigraph, type SBR 2b, driving a calibrated Agla syringe.

Optical Rotatory Dispersion.—The Rudolph high precision photoelectric polarimeter, model 200, equipped with a quartz monochromator and an oscillating polarizer, was used for optical rotatory dispersion measurements. The light source for the optical rotatory dispersion experiments and the experiments on the temperature dependence of the specific rotation at 340 mμ was a zirconium compact arc lamp with a useful range of 300 to 700 mμ. In the studies of the *p*H dependence of $[\alpha]_{340}$ a xenon compact arc lamp was used. The symmetrical angle was set at 5°.

The solutions to be measured were contained in a water-jacketed 20-cm. quartz polarimeter tube of 7-mm. bore with fused quartz end plates and a diaphragm 2 mm. in diameter, made by the Optical Cell Co. (Kensington, Md.). Water was circulated through the jacket from a water-bath kept at 12 ± 0.1° for the optical rotatory dispersion experiments and the experiments on the *p*H dependence of the specific rotation.

The indices of refraction, *n*, of the solvents used were obtained from the "International Critical Tables" and refer to 12°. No correction of *n* for wave length was made.

After allowing 30 minutes for thermal equilibrium, the optical rotation was then measured at the following wave lengths in mμ: 330, 340, 360, 385, 410, 490 and 589. At the end of each experiment the optical rotation was measured again at the same wave length used in the beginning of the experiment and no change in optical rotation was noticed.

The optical rotation of the blank was determined as a function of the wave length each time an experiment was run and averaged separately. The average solvent blanks were subtracted from the observed rotation of the protein solution. The specific rotation, $[\alpha]$, was then calculated.

In the experiments on the temperature dependence of the specific rotation at 340 mμ the same enzyme solutions were used throughout the temperature range and measurements were made until the solutions became turbid. Twenty minutes was allowed at each temperature for thermal equilibrium. The temperature of the water-bath was then changed slowly (about 1° per minute) until the next desired temperature was reached. Solvent blanks were measured at each temperature and subtracted from the observed rotation of the protein solution as described above.

The effect of *p*H on the specific rotation at 340 mμ of the proteins was studied in the following buffers, which were adjusted to an ionic strength of 0.1 *M* with KCl: *pH* 2.0 to 3.0 HCl; *p*H 3.8 to 5.0, 0.05 *M* acetate; *p*H 7.0 to 8.0, 0.05 *M* Tris; *p*H 9.0 to 10.0, 0.05 *M* borate. Solvent blanks were measured at each *p*H and subtracted from the observed rotation of the protein solution as described above. Assays of monoacetyl-CT with tyrosine ethyl ester at *p*H 5.0 before and after the experiments at *p*H 5.0 indicated that the deacylation during this experiment was insignificant.

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Evidence for Conformational Changes in α-Chymotrypsin Catalyzed Reactions. VII. Thermally Induced Reversible Changes of Conformation at pH 2.0[1]

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The reversible change of conformation in α-chymotrypsin(CT), monoacetyl-α-chymotrypsin, and diisopropylphosphoryl-α-chymotrypsin with varying temperature was studied with ultraviolet difference spectra and optical rotation measurements at pH 2.0. The increase in $-[\alpha]$ (indicating unfolding of the protein) and the decrease in absorbancy at 292 mμ observed in the difference spectra (indicating changes in the environment of tryptophyl residues) parallel each other in the denaturation of monoacetyl-α-chymotrypsin and diisopropylphosphoryl-α-chymotrypsin, but not in the transition of α-chymotrypsin. Both measurements, $[\alpha]_{365}$ and ΔD_{292}, indicate that the three unfolded proteins are not in the same conformation and that the molecules are only partially denatured in the transition at pH 2.0. Equilibrium data were obtained for the conformational changes of the three proteins. The parameters ΔH^0_{obsd}, ΔS^0_{obsd}, and the transition temperature, T_{Tr}, were evaluated for all three proteins, using both the temperature dependence of $[\alpha]_{365}$ and ΔD_{292}. Significant differences were found, not only between α-chymotrypsin and the enzyme–substrate compounds, but also between monoacetyl-α-chymotrypsin and diisopropylphosphoryl-α-chymotrypsin. The dramatic differences between α-chymotrypsin and the enzyme–substrate compounds in the thermodynamic parameters of the pH 2.0 transition cannot be explained, *per se*, by the introduction of a single diisopropylphosphoryl or a single acetyl group into the chymotrypsin molecule. Previous evidence, in combination with the data presented here, indicate that the formation of the enzyme–substrate compounds is accompanied by conformational changes.

Introduction

Earlier kinetic and equilibrium studies demonstrated that monoacetyl-α-chymotrypsin, the intermediate in the chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate, can undergo a structural change in solution under conditions where chymotrypsin cannot.[5–8] Subsequent studies of the chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate revealed changes in the spectrum of the enzyme at 290 mμ which are intimately related to the formation and decomposition of the monoacetyl-enzyme.[9,10] These absorbancy changes at 290 mμ also have been observed in the reaction of diisopropylphosphorofluoridate (DFP) with CT and trypsin[9] and more recently by Bender, *et al.*,[11] in the formation of cinnamoyl–CT. Experiments[9,10] on the characterization of the spectral changes at 290 mμ suggested that they are due to reversible conformational changes of the enzyme, brought about by acylation of its active site. To characterize these reversible structural changes, initial investigations were concerned with the chemical reactivity of tryptophyl residues,[10] the hydrogen ion equilibria of the tyrosyl residues in CT and diisopropylphosphoryl–CT (DIP–CT),[11] and the optical rotatory dispersion parameters of CT, DIP–CT, and monoacetyl–CT at pH 3.8.[13] This investigation is concerned with reversible, thermally induced conformational changes of CT, DIP–CT and monoacetyl–CT at pH 2.0.

The existence of reversible conformational changes in CT was inferred by Schellman[14] from the observation that the specific rotation at 589 mμ undergoes a reversible change with temperature at pH 2.0. It also has been shown[15] that the optical density of CT solutions decreases at 292 mμ under conditions which disrupt the integrated structure of this protein. This has been attributed to a change in the environment of the tryptophyl groups.[15] The data reported here show that the changes in optical rotation and in optical density, which occur when solutions of DIP–CT and monoacetyl–CT are heated at pH 2.0, parallel each other. However, the polarimetric and spectrophotometric methods do not give the same results for the CT transitions.

Two reactions of CT are considered in this investigation: the stoichiometric reaction of CT with DFP to give DIP–CT and HF[16]; and the CT catalyzed hydrolysis of *p*-nitrophenyl acetate.[17] This latter reaction proceeds *via* an intermediate, monoacetyl–CT, which can be isolated below pH 5.0.[18] Above pH 6.0, monoacetyl–CT, isolated according to the procedure of Marini and Hess,[6] is kinetically the intermediate in the chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate.[5,6] It has been demonstrated that in this reaction and in the reaction of CT with DFP the same seryl hydroxyl group of CT becomes substituted.[19] Since

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