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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

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°_{obsd}, ΔS°_{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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(2) Fulbright grantee, 1959–1962. A part of this work is abstracted from 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.

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(4) 1962–1963: Fulbright grantee and John Simon Guggenheim Fellow, Max Planck Institute for Physical Chemistry, Göttingen, Germany.

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the hydrolysis of specific substrates for CT most probably involves the same mechanism as the CT catalyzed hydrolysis of *p*-nitrophenyl acetate,[20] the data obtained in the studies reported here have an important bearing on CT catalyzed reactions in general.

Experimental

Materials. α-Chymotrypsin.—The enzymes used were salt free and obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Three times crystallized α-chymotrypsin, Lot 6027, referred to in this paper as CT, was used in most of the experiments. Lot 6026 (CT-26) was another three times crystallized preparation. Some experiments also were performed with chromatographically homogeneous (Amberlite CG-50) chymotrypsin, C.H.-CT. Assays of the lots, using the potentiometric method of Schwert, *et al.*,[21] and N-acetyl-L-tyrosine ethyl ester as the substrate at pH 8.0 and 25° in 10% acetone gave these values for k_3 in sec.$^{-1}$: CT, 115, CT-26, 95 and C.H.-CT, 120. The value for CT reported in the literature[22] using the same substrate at 25° is $k_3 = 115$ sec.$^{-1}$ at pH 7.8 in 30% methanol.

The enzymes were dialyzed against 7×10^{-3} *N* HCl before use in the transition experiments as described in the preparation of monoacetyl-CT. The enzyme activity of the preparations was checked frequently at the end of the experiments and no inactivation was found.

DIP-CT was prepared as described previously.[10] Phosphorus analysis by the method of Sumner[23] and the difference spectrum of DIP-CT *versus* CT[9,10] were used to check the preparation. DIP-CT was dialyzed before use as described below.

Monoacetyl-CT was prepared as described previously,[6] except the ether extraction step was omitted and 20 ml. of the enzyme solution was dialyzed at 4° against 4 changes (15 liters) of 7×10^{-3} *N* HCl for 24 hours. The pH of the solution then was adjusted to pH 2.0 with 0.1 *N* HCl and the transition experiments were started. The extent of acylation of the enzyme was determined as described previously.[6] The samples used were 90% acylated. Monoacetyl-CT also was assayed before and at the end of the experiments, using L-tyrosine ethyl ester as the substrate at pH 5.0 and 20°, to ascertain that the enzyme did not deacylate during the experiments. Preparations of monoacetyl-CT were routinely deacylated at pH 6.5, as described previously,[8] and assayed with N-acetyl-L-tyrosine ethyl ester at pH 8.0 and 25° to ensure that the preparation of this CT–substrate compound did not change its catalytic properties irreversibly. Finally, the preparations were checked for light scattering during the deacylation reaction.[8] No evidence for this was found with the preparations used.

Reagents.—Diisopropylphosphorofluoridate was obtained from K & K Laboratories, Inc., Jamaica, New York. N-Acetyl-L-tyrosine ethyl ester and L-tyrosine ethyl ester were purchased from Mann Laboratories, 136 Liberty Street, New York, N. Y. Crystalline pepsin was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, and crystalline trypsin was obtained from Worthington Biochemical Company, Freehold, New Jersey. All other reagents were reagent grade and were obtained from Mallinckrodt Chemical Works. All solutions were prepared with conductivity water.

Methods: Instruments.—A Cary Model 14 self-recording spectrophotometer was employed for all spectral measurements. A Radiometer pH meter, Type TTT1, with a type B glass electrode was used for pH measurements which are in reference to Beckman pH 4.0 and pH 7.0 standard buffers, and 0.01 *M* borax at pH 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[24] for all enzymes. Previous experiments[10] 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.[25]

Enzyme Assays.—The enzymes were assayed using the potentiometric method of Schwert, *et al.*[21] At pH 8.0 and 25°, 1.0 ml. of enzyme solution was added at zero time, to a solution of the following composition: 8 ml. of pH 8.0 buffer (4×10^{-3} *M* Tris, 0.1 *M* $CaCl_2$) and 4 of ml. 5×10^{-2} *M* N-acetyl-L-tyrosine ethyl ester in 28% (v./v.) aqueous acetone. At pH 5.0 and 20°, 1 ml. of enzyme solution was added, at zero time, to a solution of the following composition: 8 ml. of pH 5.0 buffer (1×10^{-3} *M* sodium acetate, 1×10^{-3} *M* $CaCl_2$) and 4 ml. of 18×10^{-3} *M* L-tyrosine ethyl ester.

The pH 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.

The Digestion of the Proteins by Proteolytic Enzymes.—Five ml. aliquots of the protein solution at pH 2.0 (about 2 mg./ml.) were added to two (10) ml. volumetric flasks. Crystalline pepsin was added to one solution to give a final pepsin concentration of 0.01 mg./ml. After two hours at 30° (in the case of CT) or 32° (DIP-CT) the solutions were adjusted to about pH 6.8 (as determined in parallel experiments) with 1 *M* potassium phosphate buffer and crystalline trypsin then was added to give a final concentration of 0.01 mg./ml. and the digestion was continued for 40 minutes at 40°. The pH of this solution then was adjusted to pH 2.0 (as determined in parallel experiments) and the pH was checked on aliquots of the sample solution. The experimental flask and the control flask were then adjusted to volume and the difference spectrum was obtained. Control experiments with trypsin and pepsin indicated that the contribution of the absorbancy of these enzymes to the difference spectrum was insignificant under the experimental conditions.

Polarimetric Determinations.—The Rudolph photoelectric polarimeter Model 200 S, equipped with a quartz monochromator and an oscillating polarizer, was used for the optical rotation measurements. The light source was a zirconium compact arc lamp with a useful range of 300 to 700 mμ. 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 a fused quartz end plate and a diaphragm of 2 mm. diameter, made by the Optical Cell Co. (Kensington, Maryland). Water was circulated through the jacket from a water bath kept at a constant temperature within 0.1°. The temperature of the solution in the polarimeter tube was determined with a thermocouple and calibrated against the temperature of the bath which was checked with a standard thermometer (NBS).

After allowing 30 minutes for thermal equilibrium, the optical rotation was then measured at 365 mμ. The temperature of the water bath then was changed slowly until another desired temperature was reached. Thirty minutes later the optical rotation was measured at that temperature again. The enzyme solutions were brought repeatedly from the high temperatures to lower temperatures and their optical rotation was again determined to ensure that thermal equilibrium had been reached at the lower temperatures. At the end of the experiments, the solutions were brought to 20° and their optical rotation was measured again. No evidence of irreversible changes in their specific rotation was observed. A protein concentration of 0.8 to 1.4 mg./ml. was used.

The optical rotation of the blank was determined with each experiment and subtracted from the observed rotation of the protein solution. The specific rotation $[\alpha]$ then was calculated.

Spectrophotometric Determinations.—A Cary Model 14 self-recording spectrophotometer and 1 cm. silica cells were used to measure the ultraviolet difference spectra of the proteins. Both cells contained protein at the same concentration. Constant temperature water, flowing through specially constructed blocks in which the reference cell and sample cell, respectively, were located, kept the temperature of the solutions within 0.1°. The temperature of the reference cell was maintained at 18°. The temperature of the sample cell was varied from 10 to 60°. The temperature of the solutions inside the cells and in the constant temperature water bath were calibrated as described in the polarimetric experiments. Temperature equilibrium and reversibility were ascertained as described in the previous section. At each temperature the difference spectra were recorded from 340 to 240 mμ. Small changes in zero values at 340 mμ between runs were corrected for in the calculations. A protein concentration of 0.6 to 1.1 mg./ml. was used.

Results

Effect of Temperature on the Specific Rotation at 365 mμ.—Optical rotation measurements were made of solutions of CT, DIP-CT and monoacetyl-CT at pH 2.0 and 0.01 *M* ionic strength. A low protein concentration of 0.8 to 1.4 mg./ml. was chosen to avoid aggregation of the enzymes. The measurements were found to be independent of protein concentration in the range used. The stability of monoacetyl-CT was ascertained at the end of the experiments as described in the Experimental section, and no evidence of deacylation was

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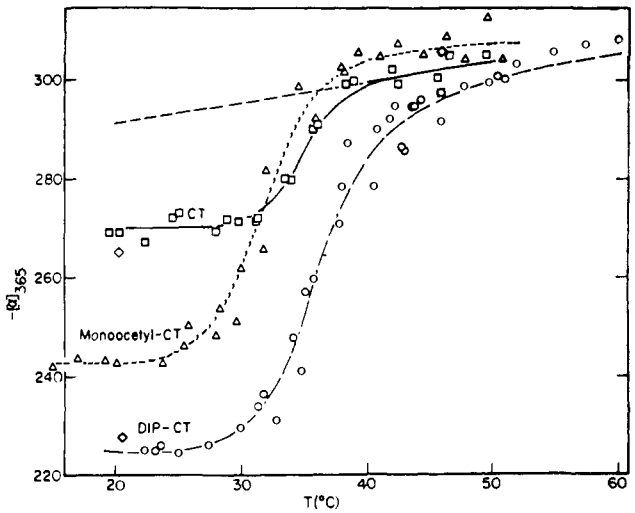

Fig. 1.—Optical rotation of CT, □; C.H.-CT, ◊; DIP-CT, O; C.H.DIP-CT, ◊; monoacetyl-CT, Δ, as a function of temperature. The dashed line is the linear extrapolation of the high temperature values of $[\alpha]_{365}$ for CT toward lower temperatures. Similar lines, not shown, were constructed for DIP-CT and monoacetyl-CT. The extrapolation lines were used for computation of $\Delta[\alpha]_{max}$ as a function of temperature (see text). The values determined by these lines are taken to be $[\alpha]_{365}$ of the high temperature forms of the proteins at any given temperature.

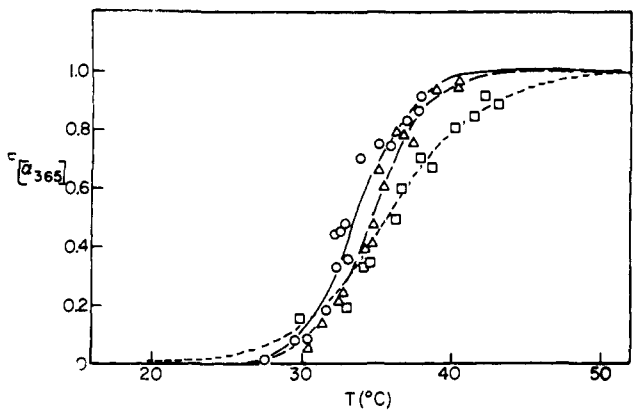

Fig. 2.—A plot of $F[\alpha]$ *versus* temperature for CT, O; DIP-CT, □; and DIP-CT in 0.6% (v./v.) 2-propanol, Δ; at pH 2.0; μ = 0.01 *M*. The coördinates defining the curves were computed from the lines of the corresponding van't Hoff plots which were determined by the method of least squares.

found. The change in rotation with temperature of all three proteins was found to be perfectly reversible. The solutions could be passed up and down through the temperature range used (18–55°) without observable hysteresis.

The specific rotation data of CT, DIP–CT, and monoacetyl–CT are shown in Fig. 1. It may be noted that the specific rotations of the three enzymes differ markedly at low temperatures. A statistical analysis of the slopes and intercepts of the high temperature extrapolation lines of the three proteins (Fig. 1) reveals significant differences in the slopes and/or intercepts. The high temperature extrapolation line (shown for CT in Fig. 1) gives the temperature dependence of $[\alpha]_{365}$ of the high temperature form of the molecule if the transition did not take place. The maximum change in $[\alpha]$ at any temperature, $\Delta[\alpha]_{max}$, is defined as the difference between the value obtained from the high temperature extrapolation line and the low temperature limiting value of $[\alpha]$. With the values of $\Delta[\alpha]_{max}$ thus determined, it is possible to compute a value of $F[\alpha]$, the

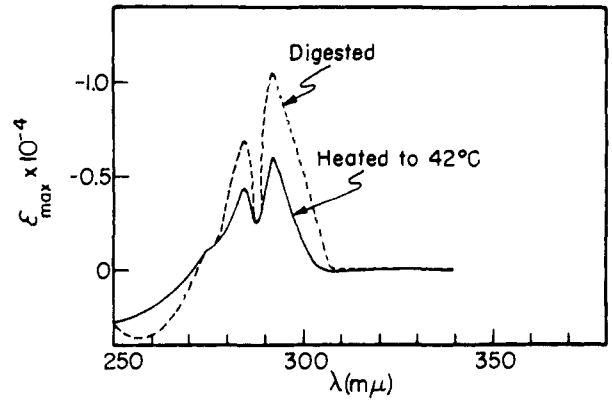

Fig. 3.—Ultraviolet difference spectra at pH 2.0 of CT (20°) *versus* CT (42°) and CT *versus* digested CT at 20°. The digestion of CT by proteolytic enzymes is described in the Experimental section.

fraction converted to the high temperature form, where $F[\alpha]$ is defined as

$$F[\alpha] = \Delta[\alpha]/\Delta[\alpha]_{max} \quad (1)$$

where $\Delta[\alpha]$ is the increment at any temperature over the low temperature limiting value of $[\alpha]$.

The values of $F[\alpha]$ from equation 1 for CT, DIP-CT in 0.6% (v./v.) 2-propanol, and DIP–CT in aqueous medium are plotted in Fig. 2. It can be seen that the curves for CT and DIP–CT in 0.6% (v./v.) 2-propanol are similar and quite different from DIP–CT in aqueous medium. The values of $F[\alpha]$ for all proteins are compared to $F(\Delta D)$, obtained from spectrophotometric measurements, in Fig. 6.

Effect of Temperature on Difference Spectra.—The temperature dependence of ΔD_{292} was obtained with solutions of CT, DIP–CT, and monoacetyl–CT at pH 2.0 and 0.01 *M* ionic strength, and are in reference to control solutions of the same protein at 18°. This temperature was chosen for the reference solutions, because at lower temperatures the temperature dependence of a negative absorption peak with a maximum near 290 mμ interferes with the measurements at 292 mμ. The absorbancy changes at low temperatures are still under investigation. A protein concentration of 0.6 to 1.1 mg./ml. was used. The stability of monoacetyl-CT was ascertained as described in the Experimental section. The change in optical density at 292 mμ with temperature of all three proteins was found to be perfectly reversible. The solutions could be passed up and down through the temperature range used (18–60°) without observable hysteresis.

In Fig. 3 the ultraviolet difference spectrum of CT at 20° *versus* CT at 42° (the transition of CT is complete at this temperature) is compared with the difference spectrum of enzymatically hydrolyzed CT *versus* CT, both at 20°. The hydrolysis of CT by means of pepsin and trypsin is described in the Experimental section. It is immediately apparent from Fig. 3 that only a part of the molecule is unfolded in the reversible transition at pH 2.0. The absorbancy at 292 mμ increases by a factor of 2 over the absorbancy change observed during the transition when the protein is more completely unfolded by partial enzymatic hydrolysis. Similar partial unfolding in the pH 2.0 transition is obtained with DIP–CT and monoacetyl–CT. This can also be seen in Table I, where the limiting absorbancy changes at high temperature are listed.

The differences in the ΔE_{max} values at 45° indicate that the high temperature forms of the molecules are not identical and that DIP–CT and monoacetyl–CT are less unfolded during the transition at pH 2.0 than CT. This also can be seen in Fig. 4, which records the dif-

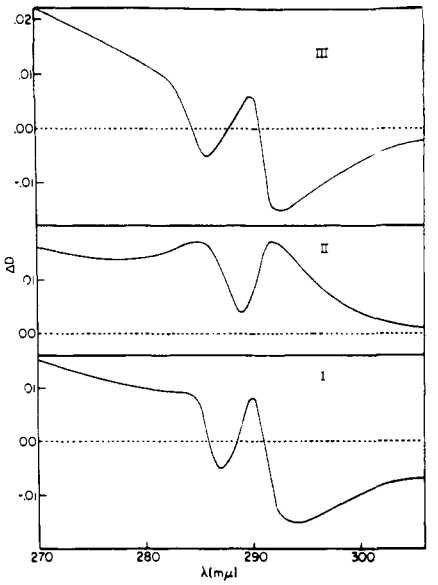

Fig. 4.—Ultraviolet difference spectra at pH 2.0; μ = 0.01 M, of DIP-CT *versus* CT. At 20°, curve I; at 45°, curve II; the same after cooling to 20°, curve III.

ference spectra of DIP–CT *versus* CT at 20, 45°, and after cooling the 45° solution to 20°. Curves I and III in Fig. 4 show the typical difference spectra of DIP–CT *versus* CT with an absorption maximum at 290 mμ, indicating the reversibility of the 290 mμ absorption peak. The difference spectrum of the high temperature forms of DIP–CT *versus* CT gives a typical denaturation spectrum with an absorption maximum at 292 mμ[15] indicating that the high temperature form of CT is more unfolded than the high temperature form of DIP–CT.

TABLE I

MAXIMUM ABSORBANCY CHANGES OBSERVED AT 292 mμ DURING PROTEIN TRANSITIONS AT pH 2.0

Protein	$-\Delta E_{max} \times 10^{-3}$ (45°)	$-\Delta E_{max} \times 10^{-3}$ (20°)
CT[a]	6.6	(5.3)[c]
DIP-CT[a]	5.9	(4.7)[c]
Monoacetyl-CT[a]	6.2	(5.0)[c]
Digested CT[b]		11.3[d]
Digested DIP-CT[b]		11.3[d]

[a] Reference cell contained the same protein at 20°. [b] Reference cell contained the same protein in the native state. The digestion procedure is described in the Experimental section. [c] These values were obtained from the extrapolation line of the high temperature form to 20°. [d] The effect of newly formed ionized end groups contributes to $-\Delta E_{max}$.

As can be seen from Fig. 5, Beer's law holds through the whole transition range and both the slopes and intercepts of the high temperature extrapolation lines are proportional to protein concentration (inset, Fig. 5). Although the data in Fig. 5 were obtained with CT, obeyance of Beer's law was also ascertained with DIP–CT and monoacetyl–CT, for which similar curves were constructed.

The maximum change in ΔD_{292} at any temperature, $\Delta D_{292(max)}$, is defined as the difference between the absorbancy at 292 mμ of the control solution at 18° and the value obtained from the high temperature extrapolation line (Fig. 5). With the values of $\Delta D_{292(max)}$ thus determined, it is possible to compute a value of $F(\Delta D)$, the fraction converted to the high temperature form, where $F(\Delta D)$ is defined as

$$F(\Delta D) = \Delta D_{292}/\Delta D_{292(max)} \qquad (2)$$

where ΔD_{292} is the increment at any temperature over the absorbancy at 292 mμ of the control solution kept at 18°.

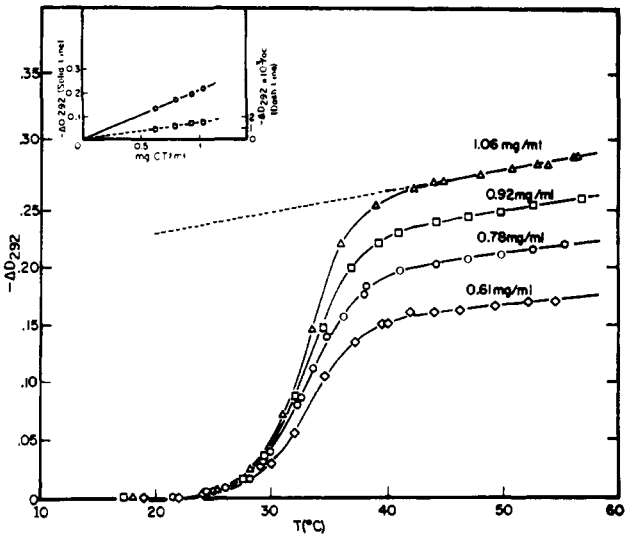

Fig. 5.—Ultraviolet difference spectra of CT at varying temperatures *versus* CT (18°) as a function of protein concentration. The dotted line is the linear extrapolation of the high temperature values of ΔD_{292} for CT (1.06 mg./ml.) to low temperatures. Similar lines, not shown, were constructed for all protein concentrations used. These lines were used for computation of ΔD_{292max} as a function of temperature (see text). The values determined by these lines are taken to be ΔD_{292} of the high temperature forms of the proteins at any given temperature. Inset: A plot of protein concentration *versus* the intercepts of the high temperature extrapolation line, O, solid line; protein concentration *versus* the slope of the high temperature extrapolation line, □, dashed line.

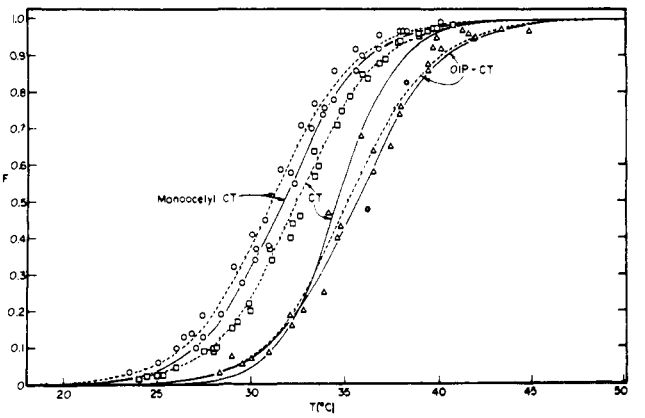

Fig. 6.—Comparison between the polarimetric and spectrophotometric methods. The fraction of the molecules converted to the high temperature form as determined from ΔD_{292} (symbols and broken lines) and from $[\alpha]_{365}$ (solid lines) are plotted against temperature. The coördinates defining the solid and broken curves were computed from the lines of the corresponding van't Hoff plots (Fig. 7). The latter were determined by the method of least squares.

The values of $F(\Delta D)$ from equation 2 are plotted in Fig. 6. The coördinates of the solid lines in Fig. 6 are defined by the values of $F[\alpha]$ from equation 1, and were obtained from polarimetric measurements. It may be noted that the changes in optical density and in optical rotation parallel each other for the transitions of DIP–CT and monoacetyl–CT, but not for the CT transitions. Qualitative differences between the transition curves of the three proteins are readily discernible in Fig. 6.

The Thermodynamic Parameters of the Transitions.—It is possible to define an equilibrium constant, K_{obsd}, using eq. 3.

$$K_{obsd} = F/(1 - F) \quad (3)$$

Values of K_{obsd} may then be computed for the transitions of CT, DIP–CT, and monoacetyl–CT from both the polarimetric and spectrophotometric experiments. These are plotted as log K_{obsd} *versus* $1/T$ in Fig. 7.

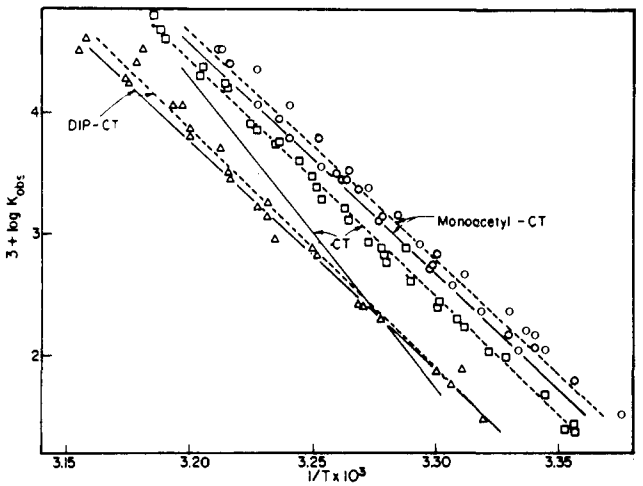

Fig. 7.—The logarithm of the equilibrium constant for the pH 2.0 transition, determined from ΔD_{292} (symbols and broken lines) and from $[\alpha]_{365}$ (solid lines) is plotted against the reciprocal of the absolute temperature. Each broken line represents three independent experiments and about 50 measurements of ΔD_{292}. Each solid line represents three independent experiments and 40 measurements of $[\alpha]_{365}$. The coördinates of the lines were computed by the method of least squares.

The coördinates of the broken lines were computed by the method of least squares using the spectrophotometric experiments. Each line represents 3 independent experiments and about 50 independent measurements. The coördinates of the solid lines also were computed by the method of least squares using the polarimetric measurements. They consist of 9 independent experiments and 112 independent measurements. As can be seen from Fig. 7, straight lines are obtained with all proteins throughout the whole transition range.

The heat of reaction may be computed from the slopes of the straight lines in Fig. 7.

$$\Delta H^0_{obsd} = -R \frac{d \ln K_{obsd}}{d(1/T)} \quad (4)$$

The transition temperature, T_{Tr}, defined as the temperature at which half of the protein has been converted to the high temperature form, can be obtained from the abscissa of the graph when log $K_{obsd} = 0$, and the free energy term vanishes. Then

$$\Delta S^0_{obsd} = \Delta H^0_{obsd}/T_{Tr} \quad (5)$$

The thermodynamic parameters may thus be computed by means of equations 4 and 5. The transition parameters for CT, DIP–CT, and monoacetyl–CT (as obtained from both polarimetric and spectrophotometric measurements) are recorded in Table II.

It can be seen that the agreement of the two methods for the transition parameters of monoacetyl–CT and DIP–CT is excellent. A ΔH^0_{obsd} value for the DIP–CT transition of 70 kcal./mole has been reported previously by Lumry.[26] The ΔH^0_{obsd} value for the CT transition obtained from the temperature dependence of $[\alpha]_{365}$ is in excellent agreement with the ΔH^0_{obsd} value for the CT transition obtained by Schellman[14] (ΔH^0_{obsd} = 110 kcal./mole) from the temperature dependence of $[\alpha]_D$ at pH 2.0; and with the value obtained by Brandts and Lumry[27] (ΔH^0_{obsd} = 110 kcal./mole) from the temperature dependence of the optical rotatory dispersion parameters, a_0 and b_0.

(26) R. Lumry, Biochemistry Seminar, Cornell University, December 8, 1961.

TABLE II

THERMODYNAMIC PARAMETERS OF TRANSITIONS OF CT, DIP-CT, AND MONOACETYL-CT AT pH 2.0[a]

Protein	Method	ΔH^0_{obsd} (kcal./mole)	ΔS^0_{obsd} (e.u.)	T_{Tr} (°C.)
CT	$[\alpha]_{365}$	117 ± 8	381 ± 29	34.7 ± 0.9
	ΔD_{292}	90 ± 2	295 ± 7	32.5 ± 0.4
Monoacetyl-CT	$[\alpha]_{365}$	87 ± 8	284 ± 26	31.6 ± 1.2
	ΔD_{292}	86 ± 2	283 ± 7	31.1 ± 0.6
DIP-CT	$[\alpha]_{365}$	86 ± 7	278 ± 24	35.6 ± 1.4
	ΔD_{292}	90 ± 4	292 ± 13	35.3 ± 0.9

[a] The protein solutions were dialyzed for 24 hours against 7×10^{-3} *N* HCl at 4°. Before the experiments the pH of the solutions was adjusted to 2.0 with HCl using a Radiometer pH meter, model TTT1. A statistical analysis of the data, taking into consideration the uncertainty of the high temperature extrapolation lines, was obtained with the help of a Burroughs 220 computer.

A statistical analysis of the data using appropriate methods for the testing of unpaired observations with unequal variances[28] gave the results listed in Table III, where a plus sign signifies a significance level of better than 0.05.

TABLE III

STATISTICAL ANALYSIS OF THE PARAMETERS OF THE pH 2.0 TRANSITIONS[a]

Proteins compared	Method	Parameter and significance ΔH^0_{obsd}	ΔS^0_{obsd}	T_{Tr}
CT and DIP-CT	$[\alpha]_{365}$	+	+	−
	ΔD_{292}	−	−	+
CT and mono-	$[\alpha]_{365}$	+	+	−
acetyl-CT	ΔD_{292}	−	−	−
DIP-CT and	$[\alpha]_{365}$	−	−	+
monoacetyl-CT	ΔD_{292}	−	−	+
CT by $[\alpha]_{365}$ and	$[\alpha]_{365}$ and			
CT by ΔD_{292}	ΔD_{292}	+	+	+

[a] Each comparison involves 6 independent experiments and a minimum of 90 independent measurements. A + sign means a significance level greater than 0.05. A method for testing unpaired observations with unequal variances was used.[28]

It may be seen in Table III that both methods indicate that the transition temperature T_{Tr}, is different for DIP–CT and monoacetyl–CT. Similar significant differences in the transition temperature are found by the spectrophotometric methods for CT and DIP–CT. Polarimetric measurements demonstrate significant differences in ΔH^0_{obsd} and ΔS^0_{obsd} between CT and the CT-substrate compounds, but the spectrophotometric method does not. It is also obvious from Table III that the two methods measure two different processes in the CT transition, since there is a significant difference between all parameters.

As in previous experiments,[9,10,29] we attempted to repeat some of the critical experiments with a chromatographically homogeneous preparation of α-chymotrypsin (C.H.–CT). However, this preparation did not exhibit a readily reversible phase transition at pH 2.0 even at a very low protein concentration of 0.6 mg./ml. As mentioned in a previous publication,[29] C.H.–CT and its DIP-derivative apparently have the same dispersion parameters as CT. Similarly, the low temperature points of the transition of C.H.–CT and DIP–C.H.–CT fall on the curves established by CT and DIP–CT (Fig. 1). A few reversible high temperature points could also be obtained with C.H.–CT and DIP–C.H.–CT by

(27) J. Brandts and R. Lumry, *J. Am. Chem. Soc.*, **83**, 4290 (1961).

(28) R. G. D. Steel and J. H. Torrie, "Principles and Procedures of Statistics," McGraw-Hill, New York, N. Y., 1960, p. 81.

(29) B. H. Havsteen and G. P. Hess, *J. Am. Chem. Soc.*, **85**, 791 (1963).

heating this preparation directly to the desired temperature, waiting for 15 minutes, and cooling. Repeated heating, or heating for a longer time led to irreversibility.

The few reversible high temperature points of C.H.–CT and DIP–C.H.–CT also fell on the transition curves of CT and DIP–CT. Because of the large amounts of enzyme required for these experiments and the difficulty in obtaining chromatographically homogeneous CT, further experiments had to be abandoned, for the time being, with this preparation.

It is instructive, however, to look at the thermodynamic parameters of three times crystallized CT-26 which has a specific activity 20% lower than CT. These values appear in Table IV.

TABLE IV

THERMODYNAMIC PARAMETERS OF THE TRANSITIONS OF CT-26 AT pH 2.0[a]

Protein	Method	ΔH^0_{obsd} (kcal./mole)	ΔS^0_{obsd} (e.u.)	T_{Tr} (°C.)
CT	$[\alpha]_{365}$	109 ± 9	357 ± 29	33.7 ± 0.7
	ΔD_{292}	80 ± 2	262 ± 6	32.2 ± .6
Monoacetyl-CT	$[\alpha]_{365}$	74 ± 8	244 ± 25	31.7 ± .7
	ΔD_{292}	78 ± 2	255 ± 6	29.3 ± .7
DIP-CT	$[\alpha]_{365}$	66 ± 8	213 ± 25	35.5 ± 1.0
	ΔD_{292}	74 ± 5	241 ± 16	33.7 ± 1.2
DIP-CT (0.6% v./v. 2-propanol)	$[\alpha]_{365}$	104 ± 6	339 ± 19	34.6 ± 0.6

[a] Conditions and analysis of the data as in Table II.

It is readily apparent from a comparison of Table II and IV that although the absolute values obtained with the two enzyme preparations are different, the differences between the parameters obtained with CT and the CT-substrate compounds on one side, and CT-26 and the CT-26 substrate compounds on the other, are the same. The polarimetric and spectrophotometric methods agree again for the transition parameters of DIP–CT-26 and monoacetyl–CT-26, but not for those of CT-26. The conclusions to be reached by an analysis of the data for CT-26 and CT (Table III) are the same.

Discussion

It is well established that the specific rotation becomes more levorotatory as the native structure of the protein is unfolded by changes in pH, urea concentration, or temperature, and that it accompanies other physical measures of the conformational change.[30] That optical rotation follows the loss of enzymatic activity as well as the increase in viscosity is clearly illustrated in the urea denaturation of δ-chymotrypsin which was studied by Neurath, *et al.*[31] Similarly, the change in ΔD_{292} which accompanies the unfolding of CT has been well documented by Chervenka.[15] Therefore, the following interpretations of the data can be made.

I. The transitions observed at pH 2.0 do not lead to a complete unfolding of the molecules. This can be seen from the changes in specific rotation at 365 mμ in the transition of CT where a change of 35° is observed. This corresponds to a change in specific rotation at the sodium line, $[\alpha]_D$, of 12° (from −73 to −83°),[14] while CT denatured under more drastic conditions has an $[\alpha]_D$ value of about −120°.[14,31] The incompleteness of the unfolding of the proteins in the thermally induced transitions at pH 2.0 may also be inferred from the spectrophotometric data. The position of the peak in the difference spectra of the proteins at low temperature *versus* the proteins at high temperature at 292 mμ (Fig. 3) arises from the perturbation of tryptophyl residues,[15] and the sign of ΔD_{292} implies that tryptophan chromophores pass from a non-polar medium to an aqueous one during the transition. The solvent perturbation method of Herskovits and Laskowski[32] suggests that 3 of the tryptophyl residues of CT are exposed and 4 are buried.[33] As may be seen in Fig. 3 and Table I, ΔE_{max} at 292 mμ for CT *versus* partially hydrolyzed CT is about two times larger than the ΔE_{max} value observed in the protein transitions. The transition at pH 2.0 then presumably only involves 2 of the tryptophyl residues of CT (Fig. 3).

II. The spectrophotometric and polarimetric methods probably measure two different, but not necessarily unrelated, processes in the CT transition. ΔH^0_{obsd} from the temperature dependence of $[\alpha]_{365}$ was found to be 117 ± 8 kcal./mole while a ΔH^0_{obsd} value of 90 ± 2 kcal./mole was found from the temperature dependence of ΔD_{292}. As can be seen from Table III, there is a significant difference between all the transition parameters obtained by these two methods. It can also be seen in Fig. 6 that the transition of CT occurs at a lower temperature when observed by measurements of ΔD_{292} than when measured by $[\alpha]_{365}$. A similar phenomenon has previously been observed by Foss[34] in the thermally induced transition of lysozyme at pH 2.2. A polarimetrically observed transition was found at 80°, while the spectrophotometrically observed transition occurred at 55°. The observation of two discrete transitions suggested to the author[34] that the environment of tryptophyl side chains changes before helical breakdown takes place, so that the denaturation of lysozyme may involve the sequential disruption of tertiary and then secondary structure. However, a change of solvation around the side chains of proteins[35] and alterations in the orientation of vicinal groups about the asymmetric carbons should also cause changes of specific rotation. However, the main differences observed in the experiment reported here are in the ΔH^0_{obsd} values which most probably measure a series of events which occur during the transition of the proteins. Since probably only two out of seven tryptophyl residues participate in the phase transition, as discussed above, it may be argued that not all events observed by ΔD_{292} are observed to the same extent by $[\alpha]_{365}$ and *vice versa*. This conclusion is given some credibility by the transition experiments with DIP–CT in 0.6% (v./v.) 2-propanol (Fig. 2). The addition of a small concentration of 2-propanol changes the ΔH^0_{obsd} value of DIP–CT by 30 kcal. as measured by polarimetry (Table IV), but insignificantly when ΔH^0_{obsd} is measured by ΔD_{292}.

III. Both measurements, $[\alpha]_{365}$ and ΔD_{292}, reveal significant differences between the high temperature forms (unfolded molecules) of CT and the CT-substrate compounds, monoacetyl–CT and DIP–CT. The temperature dependence of $[\alpha]_{365}$ of the high temperature forms of all three proteins was shown to be different, while spectrophotometric measurements indicate differences between unfolded CT and the unfolded CT-substrate compounds (Table I). This also can be seen in Fig. 4, curve II, which shows that more tryptophyl residues of CT than of DIP–CT become exposed to solvent during the transition. This experiment also substantiates our previous conclusion,[9,10] based on

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

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

(32) T. T. Herskovits and M. Laskowski, Jr., *J. Biol. Chem.*, **235**, PC 56 (1960).

(33) R. J. Williams and M. Laskowski, Jr., Abstracts, 141st National Meeting of the American Chemical Society, Washington, D. C., March, 1962, p. 42-C.

(34) J. G. Foss, *Biochim. et Biophys. Acta*, **47**, 569 (1961).

(35) C. Tanford, *J. Am. Chem. Soc.*, **84**, 1747 (1962).

spectroscopic and chemical studies, that the formation of the CT-substrate compounds results in transfer of one or more tryptophyl residues from a more exposed position in CT to one inside the molecule in the CT-substrate compounds.

IV. Significant differences in the transition temperatures between CT and DIP–CT appear in the spectrophotometric measurements, while significant differences between CT and the CT-substrate compounds appear in ΔH^0_{obsd} and ΔS^0_{obsd} as determined by polarimetric measurements.

The ΔH^0_{obsd} values for the transition of the enzyme substrate compounds are about 30 kcal. lower than for CT. From the studies of Scheraga and co-workers[36] it can be deduced that the interaction of the two isopropyl groups of DIP–CT with non-polar residues of the molecule can contribute to the observed enthalpy changes by no more than 2 kcal. per mole and the methyl group in monoacetyl–CT considerably less. Hence, the presence of these groups *per se* cannot explain the data.

V. No conclusions can be drawn at present from the data about the nature of the conformational changes which accompany the formation of the CT-substrate compounds. The high temperature forms of the proteins are clearly different and the molecules are only partially unfolded. Therefore, the experimental data refer directly only to the parts of the molecule which participate in the pH 2.0 transition and no inference can be made from them about the over-all changes in the enzyme which occur in the formation of the CT-substrate compounds. While the differences in ΔH^0_{obsd} and ΔS^0_{obsd} between CT and the CT-substrate compounds are quite large, these changes may be balanced by transformations in the molecules which are not directly observed during the experiments.

ΔH^0_{obsd} is considerably lower in the transitions of the CT-substrate compounds than in the CT transition. This may be explained in a number of ways. A part of the molecule which participates in the transition of CT can become stabilized in the CT-substrate compounds and may not participate in the transition. Processes which have a negative heat of reaction, such as the rupture of electrostatic interactions or of hydrophobic bonds, may contribute to the ΔH^0_{obsd} values of the transitions of the CT-substrate compounds. A variety of effects are thus possible and it is speculation to assign the observations to any one cause. The electrostatic interactions are probably almost the same in the three proteins at pH 2.0 since preliminary transition experiments in salt solutions do not reveal a significant change in the transition parameters. Previous studies[9,10] have already indicated that the tryptophyl residues are in a less polar environment in the CT-substrate compounds than in CT, and presumably participate in hydrophobic interactions. In these studies it was observed that very small amounts of 2-propanol (0.6% v./v.) have a dramatic effect on the DIP-CT transition (Fig. 2). The effect of 2-propanol on the CT transition is not known since serious complications were encountered in the transition experiments and this problem is under further investigation. In the meantime it might be suggested that small amounts of 2-propanol can weaken hydrophobic interactions in the CT-substrate compounds. In the absence of 2-propanol, the rupture of these bonds may contribute a negative heat of reaction to the transition process of the CT-substrate compounds. The broadening of the transition, especially obvious in the case of DIP–CT (Fig. 6), also indicates additional stabilization[37] of the enzyme–substrate molecule and the breaking of additional bonds during the transition. A dramatic increase in the thermal stability of the CT-substrate compounds, as compared to CT, has previously been observed[13] at pH 3.8 and 4.0. This increased stability of the enzyme–substrate compounds is also illustrated by the experiment in Fig. 4 (curve II) which indicates that the high temperature form of DIP–CT is less unfolded than the high temperature form of CT. These observations suggest, though only tentatively, that a part of the molecule which participates in the CT transition has become stabilized to a varying degree by hydrophobic interactions in monoacetyl–CT and DIP–CT and that a partial rupture of these bonds in the transitions contributes to the observed enthalpy and entropy change. Although these suggestions are tenuous, they can be tested by further experiments.

Investigations of the aggregation behavior of CT and DIP–CT in a number of laboratories[38] indicated that with the protein concentrations used in these studies (0.8 to 1.4 mg./ml.) the aggregation of the proteins is negligible. Control experiments with various α-chymotrypsin preparations, including chromatographically homogeneous α-chymotrypsin, agreed with each other. These precautions make it likely that the data obtained in these studies reflect inherent properties of the protein molecules investigated.

The following evidence has been presented in previous papers that the formation of the chymotrypsin compounds, monoacetyl–CT and DIP–CT, is accompanied by conformational changes. (1) Kinetic experiments gave evidence for a structural rearrangement of monoacetyl–CT.[5,6] (2) Light scattering experiments and spectroscopic studies revealed differences in the pH dependent aggregation between CT and the CT-substrate compounds.[7,8] (3) Spectroscopic studies showed differences in the environment of tryptophyl residues between CT and the CT-substrate compounds.[9,10] (4) Chemical studies corroborated the spectroscopic evidence.[10] (5) Preliminary titration experiments[39] indicated differences between CT and DIP–CT in the environment of other ionizing groups. (6) Polarimetric studies[13,29] demonstrated that the formation of the CT-substrate compounds is accompanied by significant changes in the optical rotatory dispersion parameters, a_0 and b_0. (7) In this paper we have demonstrated significant differences by two independent methods, not only between CT and the CT-substrate compounds, but also between DIP–CT and monoacetyl–CT.

The results obtained by seven independent approaches and eight independent methods are self consistent. Therefore, the evidence is excellent that the formation of enzyme–substrate compounds is accompanied by conformational changes.

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