A Reversible Thermal Transition of the Extracellular Ribonuclease of *Bacillus amyloliquefaciens**

Robert W. Hartley

ABSTRACT: The extracellular ribonuclease of *Bacillus amyloliquefaciens* (strain H2, formerly considered a strain of *Bacillus subtilis*) undergoes a reversible thermal transition which has been studied spectrophotometrically, polarimetrically, and by gel filtration. The transition is highly cooperative and involves extensive disorganization of the native structure. The transition temperature is reduced by urea and the transition may be observed isothermally at 25° as the urea concentration is varied. The transition temperature, T_m, is 50.2° in 0.1 M Tris-HCl (pH 8.0) and 34.3° in the same buffer containing 3 M urea. Enthalpy changes, ΔH_{unf}, of 140 and 100 kcal/mole, respectively, were obtained by van't Hoff treatment of the data. Ribonuclease activity and resistance to proteolytic enzymes are lost above the transition. At concentrations above 0.25 mg/ml, without urea, a denatured precipitate forms on heating to the transition range (>48°).

The precipitate is in equilibrium with dissolved protein at the transition temperature but completely insoluble at room temperature. A fully succinylated derivative was inactive but maintained essentially the native conformation. Its T_m and ΔH_{unf}, without urea, were 39.8° and 110 kcal/mole.

A thermal transition of the extracellular ribonuclease of *Bacillus amyloliquefaciens* is reported here, together with some results which suggest that the transition involves a single, highly cooperative step from a compact "native" conformation to a completely unfolded, "random coil" form. Cooperative transitions produced by heat or denaturing reagents (*e.g.*, urea) have been reported for a number of proteins. In several cases (Foss, 1961; Hermans and Scheraga, 1961; Scott and Scheraga, 1963; Barnard, 1964a,b. Bigelow, 1964; Tanford, 1964; Brandts and Lumry, 1963; Sophianopoulos and Weiss, 1964), the transitions have been studied extensively. In all such cases, however, complications arise because of stable intermediates and/or alternate states (Barnard, 1964a,b; Tanford, 1964; Aune *et al.*, 1967). In relatively large proteins such effects could be due to independent or semiindependent unfolding of different portions of the molecule. In large or small proteins, portions of the molecule may be stabilized by disulfide bridges, as would appear to be the case with pancreatic ribonuclease. The ribonuclease discussed in this paper is small (mol wt 10,700, ~94 residues) and contains no sulfur (Hartley *et al.*, 1963; Lees and Hartley, 1966).

The bacterium which produces this enzyme (strain H2, a derivative of strain H, IAM 1521) is the same as the one we in this laboratory and others have previously designated a strain of *Bacillus subtilis*. Welker and Campbell (1967) have recently shown that strain H, as well as several other highly amylolytic strains, are genetically distinct from *B. subtilis*. They have proposed the designation *Bacillus amyloliquefaciens*, originally applied to a related strain by Fukumoto (1943a,b).

Materials and Methods

The enzyme was prepared from spent culture medium of strain H2. Simplification and improvement of the purification since our previous report (Lees and Hartley, 1966) is not relevant to this paper and will be reported elsewhere.[1]

Solvents containing urea were made up with an 8 M urea solution which had been passed through DEAE-cellulose (free base form). Succinylation of the enzyme was carried out at 0° with succinic anhydride, following Habeeb *et al.* (1958). Samples were dansylated (Gray and Hartley, 1963), hydrolyzed, and assayed for ε-amino-labeled lysine by thin-layer chromatography. When none could be observed succinylation was considered complete. Dansyl-*O*-tyrosine spots did not diminish noticeably on succinylation but quantitative determinations were not carried out. As will be seen, some heterogeneity was introduced by the succinylation. The succinylated material had no enzymatic activity and did not react with antibody to the natural enzyme.

Optical rotatory dispersion studies were made in a Cary Model 60 apparatus. For measurements made at the temperature of the instrument's sample chamber (29°), a standard 10-mm path cell was used. A jacketed 1.0-mm path cell was used for experiments in which it was necessary to vary the temperature. The temperatures reported are those of the circulating water bath.

* From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland 20014. *Received March 5, 1968.*

[1] A recent batch was obtained in crude form, adsorbed to phosphocellulose, from Worthington Biochemical Corp. The product of 750 l. of medium; this material yielded 210 mg of purified enzyme.

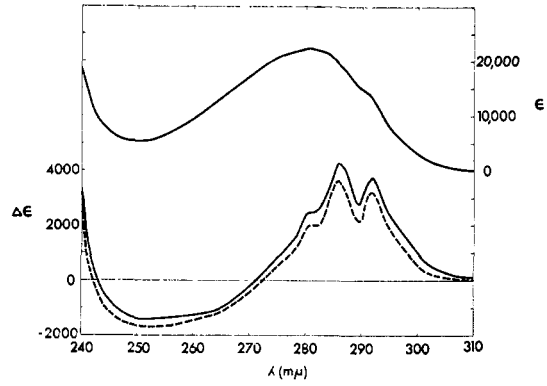

FIGURE 1: Transition difference spectra. Above, right-hand scale: spectrum of native enzyme in 0.1 M Tris-HCL (pH 8.0) expressed as molar extinction (optical density of 1 cm of 1 M solution). Below, left-hand scale: difference spectra. Solid line: difference spectrum between natural enzyme in 0.1 M Tris-HCl (pH 8), 3 M urea, 25° and same at 48°. Dashed line, same for succinylated enzyme except solvent is 0.1 M NH_4HCO_3 (pH 8).

Assays of the enzyme were carried out as reported previously (Rushizky *et al.*, 1963) except where the effect of varying conditions was under investigation. In a study of the effect of temperature on activity in 0.1 M Tris-HCl buffer at pH 8, the assays differed from the standard in (1) the assay temperature, (2) the fact that the enzyme and substrate were preincubated for 10 min at the assay temperature before mixing, and (3) that several digestion times were used at each temperature. For activity as a function of temperature in 3 M urea–0.1 M Tris-HCl (pH 8) the ethanol–lanthanum reagent recommended by Ambellan and Hollander (1966) was used to precipitate the undigested RNA.

Susceptibility to hydrolysis by trypsin and chymotrypsin was tested in 0.1 M Tris-HCl (pH 8.0) with or without urea as specified. The trypsin used was obtained from Dr. E. A. Barker. It had been treated with diisopropyl fluorophosphate to reduce chymotryptic activity, retaining 80% of its original tryptic activity. Chymotrypsin was three-times crystallized, obtained from Worthington Biochemical Corp. The extent of hydrolysis was

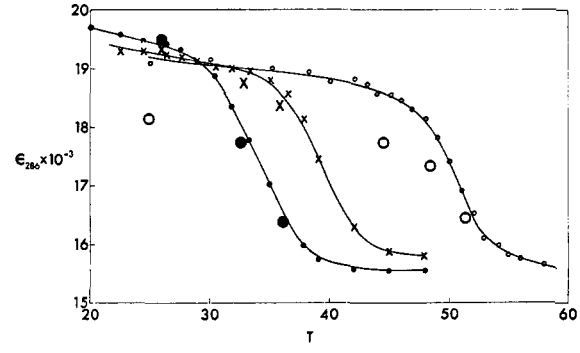

FIGURE 2: Melting curves as observed by absorbance at 286 mμ; (○) natural enzyme in 0.1 M Tris-HCl (pH 8); (●) same with 3 M urea; (×) succinylated enzyme in 0.1 M NH_4HCO_3 (pH 8). Large symbols represent points obtained on the cooling cycle.

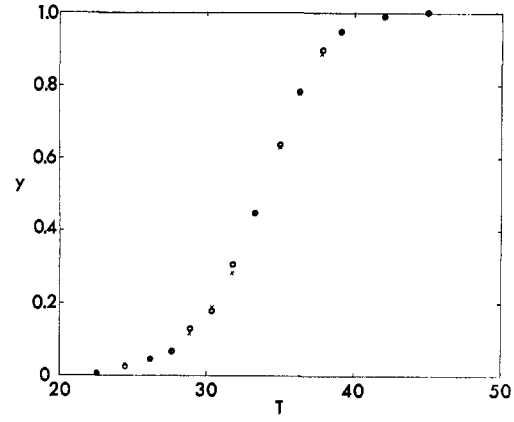

FIGURE 3: Comparison of spectrophotometric melting curves as observed at 286 (○) and 291 mμ (×). *y* represents the change as a fraction of the maximum change.

assayed as loss of ribonuclease activity and by chromatography on Sephadex G-50 as described below.

Analytical gel filtration with Sephadex G-75 (Pharmacia) was monitored fluorometrically as reported previously (Hartley *et al.*, 1963). In early work at room temperature, a Lucite column 183 × 0.63 cm was used. Later work requiring temperature control utilized a jacketed glass column 152 × 1.0 cm. The temperatures reported were read on a thermometer in the circulating stream near the jacket exit. The columns were filled completely and flow was upward. Solvent was applied with a gravity head from a Mariotte bottle and effluent volumes were recorded periodically as the effluent collected in a graduated cylinder. Samples applied contained about 30 μg of protein in 10 μl. A 100 × 0.63 cm column packed with Sephadex G-50 was used to assay extent of digestion by trypsin. The fluorescent (tryptophan-containing) components of a complete tryptic digest of the enzyme emerged as two peaks near the included volume.

Spectrophotometric measurements were made in a Zeiss Model PMQ II. Thermostated water was circulated through the cell holder, and the sample temperature was determined by a thermistor in a dummy cell placed in one of the four cell positions. All readings were made against air. Appropriate cell blanks were subtracted later as necessary.

A Spinco Model E ultracentrifuge was used for a meniscus depletion-type equilibrium centrifugation (Yphantis, 1964). Rayleigh interference optics were used with a conventional two-chamber cell. The solvent chamber contained 0.22 ml of 3 M urea–0.1 M Tris-HCl (pH 8) and the sample chamber 0.18 ml of 1.0 mg/ml of enzyme in the same solvent plus 0.01 ml of FC-43 fluorocarbon. The rotor temperature was controlled with the conventional RTIC unit. After running overnight at 30,000 rpm at 25°, the temperature was raised in 5° steps to 40°. Photographs were taken before and at 2 and 3 hr after each step.

The enzyme stock was maintained in solution at 5° over chloroform in 0.04 M NH_4HCO_3 (pH 8) at a concentration of about 3 mg/ml. Solutions made up from this stock by dilution, therefore, always contained small amounts of NH_4HCO_3. A few experiments in which

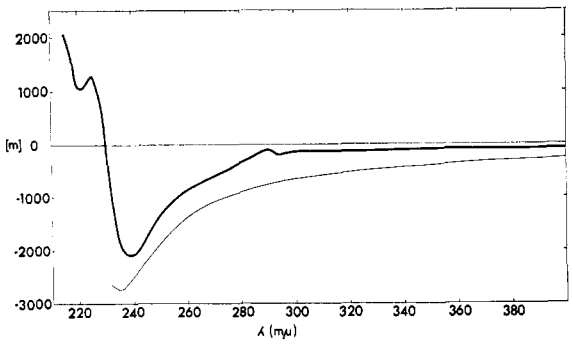

FIGURE 4: Optical rotatory dispersion of the natural enzyme at 29° in 0.1 M NaCl (heavy line) and in 0.1 M $NaHCO_3$–6 M urea (light line).

NH_4HCO_3 was excluded indicate that these amounts had no effect on the results and will therefore be ignored henceforth. It is not practical to remove the NH_4HCO_3 by evaporation, as a variable portion of the enzyme is converted by drying into a refractory solid, which can be recovered in solution only by drastic treatments such as succinylation. The succinylated enzyme is not subject to this difficulty and its solutions were made up from solid samples dried from NH_4HCO_3 solution.

Results

In Figure 1 are shown the difference spectra between the folded and unfolded forms of the natural and succinylated enzymes. There were no significant differences between the spectra of the enzyme with or without 3 M urea as long as the temperature was well below, or well above, the transition temperature (T_m) for each case. Also, the spectrum of the natural enzyme in 6 M guanidine hydrochloride, at any temperature, was not significantly different from that in 0.1 M Tris-HCl (pH 8.0) at 57° (above the T_m). The peaks in the difference spectrum at 281 and 286 mμ are similar to those appearing in the pancreatic ribonuclease transition (Sela *et al.*, 1957) and may be accounted for by a change in the environment of several of the tyrosines. The peak at 291 mμ may be attributed to similar changes in the tryptophan chromophores (Yanari and Bovey, 1960).

In Figure 2 is seen the effect of temperature, as reflected in the molar extinction at 286 mμ, for the natural enzyme with and without 3 M urea and the succinylated enzyme without urea. The large symbols in Figure 2 represent measurements made during the cooling cycle and demonstrate the reversibility of the transition. The incomplete reversal of the transition of the natural enzyme without urea is due to a secondary reaction which occurs slowly above about 55°. The transition is reversible (>95%) after exposure at 57° for 20 min. For the natural enzyme, with or without urea, the recovery of enzymatic activity is proportional to the recovery of the native spectrum. In all cases, the change in absorption at 291 mμ parallels that at 286 mμ. This is illustrated in Figure 3 (for the natural enzyme in 3 M urea). The fraction, y, of the maximum change for each wavelength is shown as a function of temperature.

Optical rotatory dispersion curves for the native and unfolded forms are shown in Figure 4. The solvents were 0.1 M NaCl and 0.1 M $NaHCO_3$–6 M urea. Both yield straight lines of a $\lambda^2[\alpha]$ *vs.* $[\alpha]$ (one-term Drude) plot between 320 and 440 mμ. The values of λ_c determined from the slopes of such plots are 195 mμ for the native form (no urea) and 212 mμ for the unfolded form (in 6 M urea). Moffit–Yang (1956) plots yielded, in both cases, values of b_0 near zero. The Cotton effect at 291 mμ, due, presumably, to oriented indole chromophores, disappears on unfolding.

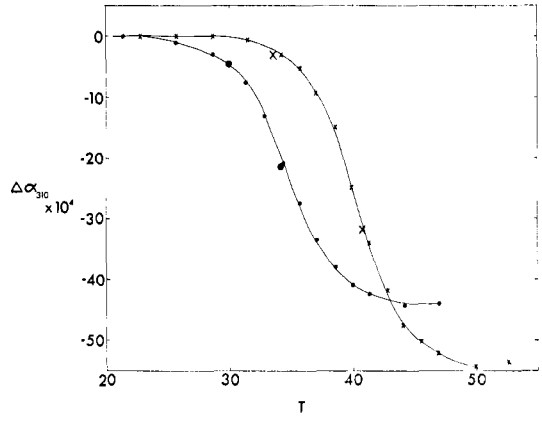

FIGURE 5: Melting curves as observed by optical rotation at 310 mμ (●) natural enzyme (0.175 mg/ml) in 0.1 M Tris-HCl (pH 8)–3 M urea and (x) succinylated enzyme (0.215 mg/ml) in 0.1 M NH_4HCO_3. Large symbols are points taken on the cooling cycle.

Melting curves based on optical rotation at 310 mμ are shown in Figure 5 for the natural enzyme in 3 M urea–0.1 M Tris-HCl (pH 8) and the succinylated enzyme in 0.1 M NH_4HCO_3 (pH 8).

In Figure 6 are shown the dispersion curves of the succinylated enzyme taken at various temperatures during a melting experiment on a more concentrated solution (12 mg/ml). The top and bottom curves were done at 25.3 and 52.2°, respectively, well below and well above the melting temperature of 42.3°. The intermediate curve (dashed) was done at 42.0°, on the cooling half of the cycle. The values represented by open circles were calculated from the two solid curves to show the

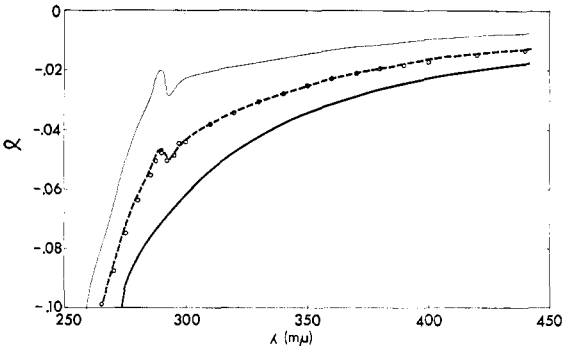

FIGURE 6: Optical rotatory dispersion of the succinylated enzyme (12 mg/ml) at 25.3° (thin line), 42.3° (dashed line), and 52.2° (heavy line). Solvent, 0.1 M NH_4HCO_3. The circles represent the dispersion of a hypothetical mixture containing 51% of the low-temperature form and 49% of the high-temperature form.

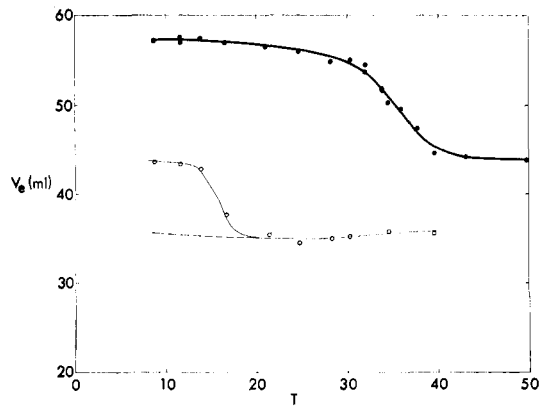

FIGURE 7: Melting curves as observed by gel filtration on Sephadex G-75. Column 152 × 1 cm. Solvent is 0.1 M Tris-HCl (pH 8)–3 M urea, throughout. V_2, elution volume. Each point represents a chromatographic run. (●) Natural enzyme; (⊙) succinylated enzyme. Dashed line indicates a splitting of the succinylated enzyme peak at low temperature; see Figure 8.

dispersion expected from a mixture containing 51% low-temperature (folded) form and 49% high-temperature (unfolded) form. The fit of these points to the curve is consistent with the absence of intermediate conformations. One-term Drude plots of these data, however, yield λ_c values of 207 and 220 mμ for the folded and unfolded forms, respectively, higher than the comparable parameters of the natural enzyme.

In Figure 7 is shown the effect of temperature on the elution volumes, V_e, of the natural and succinylated protein, on Sephadex G-75 equilibrated with 3 M urea–0.1 M Tris-HCl (pH 8.0). It is clear that the large changes in V_e are due to changes in the conformation or state of aggregation of the proteins, rather than changes in the

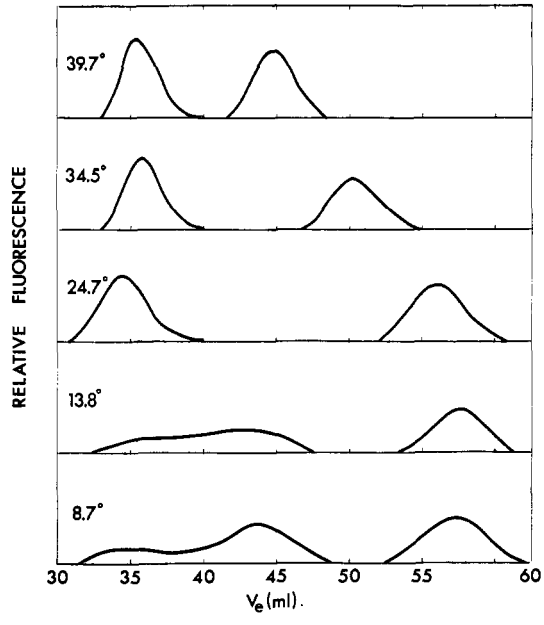

FIGURE 8: Representative chromatographic patterns. Gel filtration on Sephadex G-75. Solvent 0.1 M Tris-HCl (pH 8)–3 M urea. Temperatures as shown. Right-hand patterns are peaks of natural enzyme, left are those of succinylated enzyme.

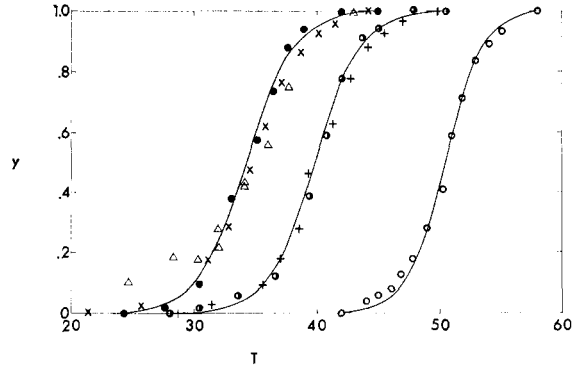

FIGURE 9: Comparison of melting curves as observed by absorption at 286 mμ, optical rotation at 310 mμ, and gel filtration. y represents the fraction of the maximum change in each parameter. Base-line corrections were made for the spectrophotometric data, using data outside the transition range. (○) OD_{286}, natural enzyme in 0.1 M Tris-HCl (pH 8.0); (●) OD_{286}, (x) α_{310}, (△) V_e natural enzyme in 0.1 M Tris-HCl (pH 8.0)–3 M urea; (◑) OD_{286}, (+) α_{310}, succinylated enzyme in 0.1 M NH_4HCO_3. Lines are theoretical curves for two-state reactions using the appropriate parameters from Table I.

Sephadex, since the transition temperatures of the two proteins are widely separated. Elution volumes were independent of the temperature history of the column. The dashed fork on the lower curve indicates a splitting of the succinylated enzyme peak in its transition range. Figure 8 shows the chromatographic patterns obtained in several of the individual runs. All of these patterns are invariant under a twofold change in the flow rate. The natural enzyme produces narrow, symmetrical peaks throughout (right-hand peak at each temperature), while the succinylated material is spread out, or split into two peaks, in its transition range. This heterogeneity, however, only involves differences in ease of unfolding. At lower urea concentrations a single peak (of folded molecules) is obtained at low temperatures. It may also be noted that in no case does preincubation of the sample in 8 M urea have any effect on the chromatographic pattern.

The possibility that the decrease in V_e with temperature involved aggregation rather than unfolding

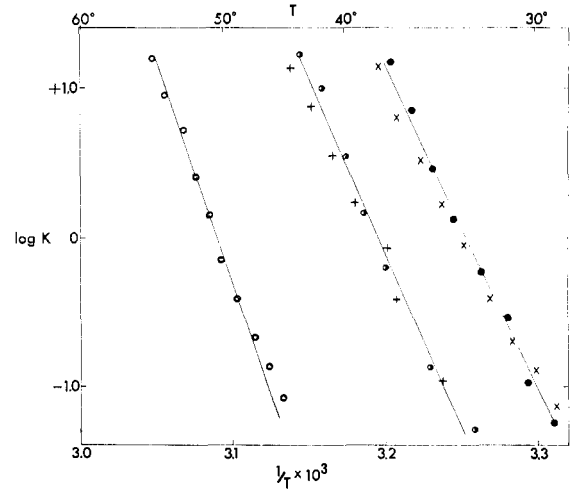

FIGURE 10: van't Hoff plots of spectrophotometric melting data. Same data and symbols as Figure 9. $K = y/(1 - y)$.

TABLE I: Thermodynamic Parameters of the Transition.

Material	Urea Concn (M)	Protein Concn (mg/ml)	Parameter Obsd	T_m (°C)	ΔH_{unf} (kcal)	ΔS_{unf} (eu)	$\Delta F_{unf}^{25°}$ (kcal)
Natural enzyme	0	0.16	OD_{286}	50.2	140	430	12
Natural enzyme	3	0.25	OD_{286}	34.3	100	330	2
		1.7	$[\alpha]_{310}$				
Succinylated enzyme	0	0.4, 2.1	OD_{286}	39.8	110	350	6
		2.1	$[\alpha]_{310}$				
Succinylated enzyme	0	12.0	$[\alpha]_{310}$	42.3	110		
Succinylated enzyme	3		V_e	15			

prompted an equilibrium ultracentrifugation experiment. Equilibrium distributions were obtained for the natural enzyme in 3 M urea–0.1 M Tris-HCl (pH 8) at 25, 30, 35, and 40°, covering the transition range in this solvent. Plots of ln C *vs.* X^2 were linear and within experimental error (<4%) identical in slope. The reduced molecular weight obtained was $M(1 - \bar{V}\rho) = 3.2 \times 10^3$. Using $\bar{V} = 0.7$ cm³/g and $\rho = 1.05$ g/cm³ yields a value for the molecular weight of 12,000. Considering the uncertainties in $\bar{V}$, this is in fair agreement with our previous value of 10,700 for the native enzyme (Hartley *et al.*, 1963). There is no doubt that the enzyme undergoes neither aggregation nor dissociation on "melting."

In Figure 9 the various parameters are plotted together as y, the fractional change, *vs.* temperature. For the spectrophotometric data, corrections were made for the variation with temperature outside of the transition range. It is clear that the same transition is being observed by the different parameters in each case. In Figure 10 are shown van't Hoff plots of the spectrophotometric and optical rotation data. Here $K = y/(1 - y)$ is the putative equilibrium constant of the simple unfolding reaction. The heat of unfolding may be determined from the slope of the van't Hoff plots, $\Delta H_{unf} = -R(d(\ln K)/d(1/T))$. At the transition temperature, T_m, the free-energy change, ΔF_{unf}, is zero and $\Delta S_{unf} = \Delta H_{unf}/T_m$. The thermodynamic parameters derived in this manner are given in Table I.

Increasing protein concentration has no effect on the transition up to at least 2 mg/ml. At 12 mg/ml, however, the succinyl enzyme is markedly stabilized.

The effect of varying urea concentration on V_e and OD_{286} are shown in Figure 11.[2] The 291-mμ extinction followed that at 286 mμ precisely, and both were virtually independent of urea concentration outside of the transition range. The slope of the V_e *vs.* urea concentration plot outside of the transition range is probably due to changes in the Sephadex. Again, however, elution volumes were independent of the column history. Similar plots of V_e for the succinylated enzyme and for bovine plasma albumin showed roughly parallel trends with sharp drops at 1.5 and 5.0 M urea, respectively. In Figure 12 is shown a plot of ln K *vs.* ln C for the absorption data of Figure 11. Here K is again the equilibrium constant and C is the urea concentration. The points fall on a straight line with a slope of 15.8. The significance of this number will be treated in the discussion.

In 0.1 M $NaHCO_3$ without urea, both the natural and succinylated proteins were unaffected by trypsin or chymotrypsin at room temperature. The natural enzyme was completely resistant even at 37°. At 25° the natural enzyme was unaffected in 2.5 M urea, but digested slowly at 3.0 M. The succinylated enzyme, at 25°, was unaffected at 1 M urea but digested rapidly at 2 M. It was digested without urea at 45°. By taking advantage of the change in OD_{286} with digestion and/or unfolding, one can follow the digestion with time in the lower end of the melting range. (Above the melting range there is little or no change in absorbance with digestion.) In Figure 13 are shown the results of such an experiment, illustrating the rapid rise in susceptibility as the transition temperature (34.3°) is approached from below. At 1000 sec each of the samples was warmed to 40°, whereupon the OD_{286} dropped to the value indicated by the dashed line, as would be expected regardless of di-

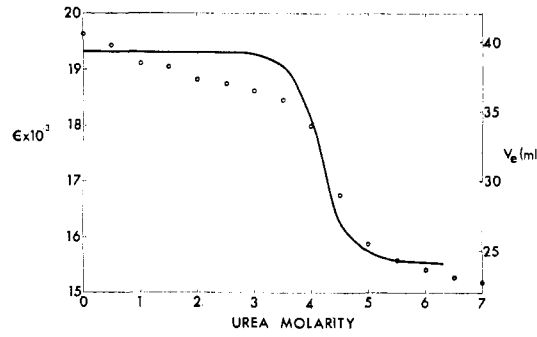

FIGURE 11: Isothermal transition of natural enzyme in urea. Solvent, 0.1 M Tris-HCl (pH 8) plus urea as shown on abscissa. Line, left-hand scale: absorption at 286 mμ, 25°, expressed as molar extinction. Circles, right-hand scale: elution volume from 183 × 0.63 cm Sephadex G-75 column, room temperature (~25°).

[2] In early gel filtration experiments of this type, $NaHCO_3$ buffer and untreated reagent grade urea were used. The transition urea concentration was found to be about 3 M, rather than the 4.2 M seen in Figure 11. The difference has been traced to the presence of 1.4 ppm of copper in the urea. The effects on the transition of copper and other reagents will be treated in more detail in a later publication.

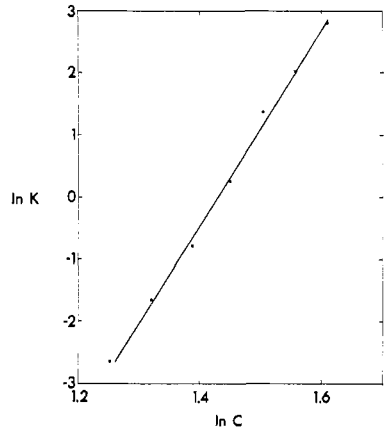

FIGURE 12: Ln K *vs.* ln C plot of Figure 11 spectrophotometric data. C is urea concentration and K, the equilibrium constant, is given by $y/(1 - y)$, where y is the fractional change in ϵ_{286} with respect to the maximum change.

gestion. After 30 min they were cooled to 25°. The OD_{286} values did not rise measurably, indicating that digestion had been completed at 40° in all cases. The number of peptide bonds which must be hydrolyzed to destabilize the molecule is not known, but other experiments suggest that the first split is rate limiting and that subsequent digestion to small peptides is rapid.

The effect of the transition on the ribonuclease activity of the enzyme was also investigated. In initial experiments, in which enzyme was incubated in 0.1 M $NaHCO_3$ for 10 min at the assay temperature before addition of the RNA, no hydrolysis of RNA occurred at temperatures above 45°. Subsequently, it was found that samples so incubated above 45° were inactive even at 37°, the standard assay temperature. As the enzyme concentrations involved were on the order of 10^{-5} mg/ml, it seems likely that this loss was due to irreversible adsorption of the unfolded form to the glass walls of the assay tubes. In 3 M urea this effect was negligible and assays through the melting range could be carried out successfully. Table II shows the OD_{260} values for 5- and 10-min incubations at four temperatures. As can be seen, the digestions were reasonably linear with time and yielded the relative activities also shown in Table II. The

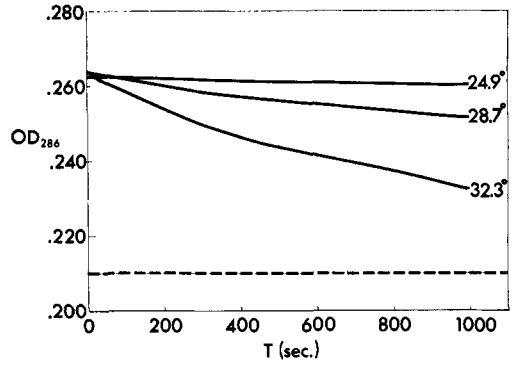

FIGURE 13: Digestion of the natural enzyme by trypsin in 0.1 M Tris-HCl (pH 8)–3 M urea. Ribonuclease concentration 0.155 mg/ml. Trypsin (0.005 mg/ml) added at $t = 0$. Temperatures as marked. Dashed line represents absorbance of complete digest.

TABLE II: Ribonuclease Activity in 3 M Urea.

Temp (°C)	OD_{260} 5 min	OD_{260} 10 min	Rel Act.
26	0.071	0.150	1.0
31	0.095	0.172	1.21
35	0.055	0.117	0.78
41	0.002	0.002	0.02

spectrophotometrically determined T_m for this solvent is 34.3°. It can be concluded: (1) that the unfolded form is inactive and (2) that there is no significant stabilization of the folded form by substrate RNA. The second point is in contrast to the behavior of pancreatic ribonuclease (Sela *et al.*, 1957; Barnard, 1964b).

When the enzyme, at a concentration above ~0.25 mg/ml (OD_{280} ~0.5) in 0.1 M $NaHCO_3$ or 0.1 M Tris-HCl (pH 8), is heated, a precipitate forms; slowly at 48°, immediately at 50° or higher. On cooling, the precipitate does not redissolve and may be removed by centrifugation. The activity of the supernatant solution is then independent of the original enzyme concentration and is equivalent to about 0.25 mg/ml of enzyme protein. The optical density at 280 mμ of these solutions is about 0.5 (equivalent to 0.25 mg/ml) for the lower starting concentrations, but increases to 0.6 with a starting concentration of 3.0 mg/ml. This reduction in specific activity is presumably due to concentration of a nonenzyme contaminant comprising 1–2% of the preparation.

The precipitate may be washed with buffer at room temperature without loss. Such a washed precipitate was resuspended in the same solvent and heated to 53°. On cooling, the supernatant fluid was again found to contain about 0.25 mg/ml of active enzyme. Thus, it would appear that the unfolded form has a low, but finite (0.25 mg/ml) solubility in the vicinity of 50° and that the portion which is in solution renatures on cooling. That all of the enzyme in the precipitate is in a nonnative conformation is indicated by the fact that it is rapidly dissolved and completely digested by trypsin or chymotrypsin at 37° in the absence of urea. Under these conditions the native enzyme is unaffected. The precipitate is readily dissolved in 3 M urea at room temperature, and subsequent dilution and assay reveals that all of the original activity is recovered.

Discussion

It may be argued from the foregoing results that the observed transition is: (a) essentially the same with or without urea and in both the natural and succinylated enzymes, (b) highly cooperative ("all or none"), (c) extensive, as from a tightly folded specific structure to a "random coil."

The close similarity of the changes in the absorption, rotatory dispersion, and effective radius through the transition for various cases strongly suggest the first con-

clusion. This is particularly true in view of conclusion c.

An all-or-none transition, for which all (or nearly all) molecules exist in one or the other of only two states, demands strictly parallel behavior of all parameters by which the two states may be differentiated. This requirement is satisfactorily met in this case by a wide variety of such parameters. Note, first the behavior of the absorptions at 286 and 291 mμ, as compared in Figure 3. The changes at these two wavelengths are presumed to derive from exposure of tyrosine and tryptophan, respectively (Yanari and Bovey, 1960), and the extent of the change at 286 mμ ($\Delta\epsilon_{286} = 4250$) suggests exposure of at least three or four of the tyrosines (Bigelow, 1964). This, of course, is why these particular wavelengths were used for the comparison but it should be added that the entire difference spectrum, including the other extrema at 250 and 281 mμ, appeared in synchrony through the transition. Similarly, the change in optical rotation was simultaneous at all wavelengths, as illustrated by the exercise in Figure 6, and accurately paralleled the spectrophotometric changes (Figures 9 and 10).

The change in the average effective radius, as determined by gel filtration, appears to follow the spectrophotometric changes very closely in both the temperature dependent and isothermal melting experiments (Figures 7 and 11). My use of the word "average," however, could be misleading and these results require further comment. While the optical techniques measure instantaneous averages over all of the protein molecules, gel filtration does not. Indeed, the finding of narrow peaks on gel filtration in the transition region implies that all molecules are identical, with intermediate effective radii. What is being measured, however, is clearly an average over time, since the residence time of the molecules on the column (10–20 hr) is some two orders of magnitude greater than the time constant of the reaction. In experiments with a short column with a high flow rate where the residence time of the protein was less than 20 min, it was found that the peaks were grossly broadened in the transition range but not above or below it. This would not be predicted on the basis of an actual intermediate conformation. Crude kinetic experiments (spectrophotometric) yielded a time constant on the order of 2–5 min, under the conditions of the chromatography. Thus, insufficient averaging would be expected from the all-or-none model for such short runs. The time constant is long compared with the residence time of a molecule in the vicinity of an individual gel bead, however, even in the 20-hr runs. If the flow rates were decreased to the point where this were no longer true, we would predict that the apparent transition temperature (or transition urea concentration at fixed T) would increase. This experiment has not been carried out.

The fit of the melting curves to straight lines on the van't Hoff plot (Figure 10) is consistent with the two-state model and the very high values of ΔH found make it difficult to involve anything less than the whole molecule in the reaction. The value of 140 kcal/mole obtained for the natural enzyme without urea at pH 8 amounts to 1.5 kcal/residue, which may be compared with the 0.95 kcal/residue involved in the unfolding of α-helical polybenzylglutamate (Ackerman and Neumann, 1967). Since this is a very small protein, the ΔH per *internal* residue must be higher.

I will not attempt to interpret the optical rotatory dispersion of the folded forms except to say that it is unusual and must reflect some specific structure. The change through the transition is great and the resulting dispersion curve is compatible with a completely unfolded random coil (Tanford *et al.*, 1967).

A rough calculation of the change in effective molecular volume may be made from the gel filtration data, using curves published by Laurent and Killander (1964). The increase on unfolding is nearly threefold for the natural enzyme and about twofold for the succinylated material. It must be noted, however, that the folded succinylated molecule appears to be about as large as the unfolded natural one. It appears that the succinyl groups protruding outward as they do from the surface have a much larger effect on the effective volume than the 10% or so that they add to the molecular weight.

The loss of activity and resistance to proteolytic attack through the transition are compatible with an all-or-none drastic unfolding although such is hardly necessary for these effects. The resistance to proteolytic attack of the succinylated protein below its transition does argue strongly for its native conformation, however.

Tanford (1964) has discussed the isothermal unfolding of various proteins in urea and has introduced the parameter

$$\nu = \frac{d(\ln K)}{d(\ln C)_{C=C^*}}$$

where C^* is the urea concentration for which $K = 1$. ν is a measure of the cooperativeness of the transition and the value of 15.8 determined in this work may be compared with that of 4.5 for pancreatic ribonuclease and 22 for β-lactoglobulin. The β-lactoglobulin is the only protein for which Tanford considers the parameter compatible with an all-or-none reaction. As ν should be proportional to the number of cooperating units in the folded structure and the molecular weight of the present enzyme is only about one-third of that of β-lactoglobulin, an all-or-none reaction is reasonable on this basis.

Aune *et al.* (1967) have recently shown that several proteins which undergo thermal transitions contain ordered regions above their transition temperatures. Thus secondary transitions could be observed polarimetrically on the addition of guanidine hydrochloride to solutions already above the transition temperature. An interesting question in regard to such ordered regions is the extent to which they represent residual native structures as against incidental folding subsequent to complete denaturation. Exactly analogous experiments have not been performed in the present case but the evidence suggests that secondary transitions of comparable extent would not be found.

The existence of a precipitate in equilibrium with both forms in solution at the transition temperature is of considerable interest. One wishes to know to what extent ordered forms are involved in the precipitate and how

its free energy at low temperatures compares with that of the native conformation.

References

Ackerman, T., and Neumann, E. (1967), *Biopolymers 5*, 649.

Ambellan, E., and Hollander, V. P. (1966), *Ann. Biochem. 17*, 474.

Aune, C. A., Salahuddin, A., Zarlengo, M. H., and Tanford, C. (1967), *J. Biol. Chem. 242*, 4486.

Barnard, E. A. (1964a), *J. Mol. Biol. 10*, 235.

Barnard, E. A. (1964b), *J. Mol. Biol. 10*, 263.

Bigelow, C. C. (1964), *J. Mol. Biol. 8*, 696.

Brandts, J., and Lumry, R. (1963), *J. Phys. Chem. 67*, 1484.

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

Fukumoto, J. (1943a), *J. Agr. Chem. Soc. Japan 19*, 487.

Fukumoto, J. (1943b), *J. Agr. Chem. Soc. Japan 19*, 634.

Gray, W. R., and Hartley, B. S. (1963), *Biochem. J. 89*, 379.

Habeeb, A. F. S. A., Cassidy, H. G., and Singer, S. J. (1958), *Biochim. Biophys. Acta 29*, 587.

Hartley, R. W., Rushizky, G. W., Greco, A. E., and Sober, H. A. (1963), *Biochemistry 2*, 794.

Hermans, J., and Scheraga, H. A. (1961), *J. Am. Chem. Soc. 83*, 3283.

Laurent, T. C., and Killander, J. (1964), *J. Chromatog. 14*, 317.

Lees, C. W., and Hartley, R. W. (1966), *Biochemistry 5*, 3951.

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

Rushizky, G. W., Greco, A. E., Hartley, R. W., and Sober, H. A. (1963), *Biochemistry 2*, 787.

Scott, R. A., and Scheraga, H. A. (1963), *J. Am. Chem. Soc. 85*, 3866.

Sela, M., Anfinsen, C. B., and Harrington, W. F. (1957), *Biochim. Biophys. Acta 26*, 502.

Sophianopoulos, A. J., and Weiss, B. J. (1964), *Biochemistry 3*, 1920.

Tanford, C. (1964), *J. Am. Chem. Soc. 86*, 2050.

Tanford, C., Kawahara, K., Lapande, S., Hooker, T. M., Jr., Zarlengo, M. H., Salahuddin, A., Aune, K. C., and Takagi, T. (1967), *J. Am. Chem. Soc. 89*, 5023.

Welker, N. E., and Campbell, L. L. (1967), *J. Bacteriol. 94*, 1124.

Yanari, S., and Bovey, F. A. (1960), *J. Biol. Chem. 235*, 2818.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.