protons exchange with rates which are influenced by the net molecular charge of bacitracin and the steric environment of each individual proton. Oxidized bacitracin exchanges with the same pH_{min} as poly-D,L-alanine, but bacitracin A has one less negative charge than oxidized bacitracin, and thus exchanges with a lower pH_{min}. Both bacitracins show the same slow and intermediate classes of hydrogens. The abnormally slow proton in bacitracin is either in a single intramolecular hydrogen bond in the macrocyclic ring or is the ϵ-*Lys* amide proton, which may be intrinsically slowly exchanging. If a hydrogen bond is present, it must allow a secondary structure with the tail compactly folded over the ring.

Acknowledgments

The authors gratefully thank Dr. T. P. King for the use of his laboratory in performing the ninhydrin assays. The authors are also indebted to Dr. T. Hettinger for samples of bacitracin derivatives and for valuable discussions concerning the interpretation of exchange data.

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Reversible Pressure–Temperature Denaturation of Chymotrypsinogen*

S. A. Hawley

ABSTRACT: The pressure–temperature-reversible transition surface for chymotrypsinogen at pH 2.07 has been determined by ultraviolet difference spectra over the temperature interval 8.5 to 70° between atmospheric pressure and 7000 atm. Assuming the transition involves two states, it is shown that the surface can be fit to a relatively simple equation of state which elicits approximately elliptical contours of constant free-energy difference on the pressure–temperature plane. Similar results are obtained by analyzing the recent ribonuclease data of Brandts *et al.* (*Biochemistry 4*, 1038 (1970)), and in both cases the equation of state is found to be compatible with known denaturation phenomena associated with pressure–temperature interactions, including the cold melting phenomenon. Values are calculated for the apparent thermodynamic transition parameters, *i.e.*, $\Delta\alpha$, $\Delta\beta$, ΔC_p, ΔS, ΔV, and ΔG.

In 1914 Bridgman observed that elevated hydrostatic pressure irreversibly denatures egg albumin. Since that time a number of investigations have revealed that pressure denaturation of proteins exhibits some unusual and interesting characteristics. It has been observed, for example, that while very high pressures (7500 atm) invariably produce protein denaturation, moderate pressures (1000 atm) may stabilize the native form, thereby increasing the temperature

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required for heat denaturation (Johnson and Campbell, 1946; Tongur, 1952; Suzuki *et al.*, 1958, 1959, 1962). On the other hand, investigation of the effect of pressure on urea-induced denaturation of ovalbumin at low temperature reveals moderate pressures destabilize the protein at 0°, increasing the rate of denaturation, but have little effect at 40° (Kauzmann, 1954). Although such evidence suggests that the effect of pressure on protein stability may be both an interesting and complex phenomenon, the available data from most relevant studies are not measurements of equilibrium, reversible properties and hence not well suited for thermodynamic characterization. More recent investigations of reversible ribonuclease denaturation at elevated hydrostatic pressure by Brandts *et al.* (1970) and Gill and Glogovsky (1965) indicate somewhat less complex behavior, with the apparent melting pressure increasing monotonically with decreasing temperature over the temperature range 0–65°. Investigations by Brandts and Hunt (1967) of the transition of ribonuclease at atmospheric pressure, however, predict the presence of a second melting temperature below 0°, involving the same denatured state. The presence of two transition temperatures, corresponding to heat and cold denaturation, has been discussed by Brandts (1968) and can be shown to be a consequence of an approximately quadratic relation between free-energy difference between states and temperature. The predicted cold denaturation phenomenon implies that the melting pressure cannot increase indefinitely as the temperature is lowered, but rather must ultimately decrease so that an atmospheric pressure-axis crossing is obtained below 0°. Thus the pressure–temperature transition surface for ribonuclease is expected to have interesting features not previously discussed but implicitly indicated in the data of Brandts *et al.* (1968, 1970).

In this report, initial experimental data defining the pressure–temperature (1–7000 atm, 8.5–70°) transition surface for chymotrypsinogen in aqueous solution (pH 2.07) will be presented and compared to some of the results of Brandts.

Throughout the analysis the transition will be treated as a two-state process and the transition surface discussed from an operational point of view. This is a somewhat less enlightened approach than is possible in light of recent advances in conceptual basis of protein transitions (Lumry *et al.*, 1966; Tanford, 1968). Nevertheless, it is justifiable from the standpoint that it provides a practical and direct scheme for ordering experimental information.

Experimental Section

The experimental technique employed to characterize the transition is similar to that employed by Brandts *et al.* (1970) in investigations of the pressure denaturation of ribonuclease. The relative concentration of the denatured species was determined from the difference in ultraviolet absorbance (292 nm) between the sample and a suitable reference containing chymotrypsinogen.

For measurements at pressures below about 3500 atm, the pressure vessel and sample holder described by Hawley and Chase (1970) was employed. At higher pressures, approaching 7000 atm, a modified sample holder was required (Hawley, 1970). In both cases, the sample was isolated from the pressure vessel and the hydraulic fluid by a flexible Teflon enclosure sealed between ultraviolet grade sapphire windows. The difference between the two-sample chambers is primarily in window strength as determined by the configuration of the high-pressure windows.

Elevated hydrostatic pressure was generated by a conventional hand pump–intensifier system developed by Bridgman, and in fact it is likely that the system is at least in part the one employed by Bridgman in his investigations of albumin denaturation. Pressure was determined from the change of resistance of a coil of manganin wire immersed in the pressure system, as measured by a modified Cary–Foster bridge (Bridgman, 1931). The maximum uncertainty associated with pressure determination is estimated as ±5 atm and attributed primarily to the drift of the slide-wire resistance over the course of an experiment.

Temperature of the sample was controlled by pumping thermostatic liquid through two annular regions surrounding the exterior of the pressure vessel. Temperature was measured with a quartz-thermometer probe imbedded in the exterior of the pressure vessel. Although resolution of temperature approached 0.001° over short time intervals, fluctuations of temperature during the course of an experiment were typically 0.05°. The reference solution was maintained at about 15° and at atmospheric pressure. The pressure vessel was rigidly mounted into the sample compartment of a Perkin-Elmer 350 spectrophotometer modified to provide digital readout of transmission. Instrumental bandwidth was adjusted to be about 1 nm at 292 nm.

The transition was mapped most often by following the absorbance along lines of constant temperature (8.5, 19.5, and 30.2°) to pressures exceeding 6000 atm, or along isobars at a number of pressures between 1 and 3500 atm over the temperature range 10–75°. On occasion, however, other lines on the *PT* plane were employed. In all, the transition surface was mapped from about 350 absorbance readings of which slightly over 100 occurred in the 10–90% apparent fraction denatured region.

The transition rate varied enormously over the range of pressure and temperature of this investigation. Along the atmospheric pressure isobar, for example, the transition occurred at least as quickly as the sample could be brought to thermal equilibrium—of the order of 15 min, while at 8.5° and in the middle of the transition (3100 atm) about 3 days were required for the transition to come to equilibrium. The criterion for equilibrium under such circumstances becomes somewhat arbitrary to the extent that there is a practical upper limit set by instrumental stability and the patience of the investigator. In the present investigation the transition was considered to be at equilibrium when the variation of absorbance became indistinguishable from instrumental fluctuations over a time interval ranging from five to ten apparent reciprocal rate constants to a maximum of 24 hr.

Reversibility as judged by spectrophotometric criteria, *i.e.*, return to initial absorbance, was complete within experimental uncertainty for measurements below about 40° at all pressures. At higher temperatures, slow irreversible changes in absorbance were observed, and are apparently similar to those encountered by Brandts (1964). At atmospheric pressure, where equilibrium occurs quickly, this creates no problem. At higher pressure, however, the transition occurs more slowly and above approximately 45° and 3500 atm it is impossible to distinguish reversible from irreversible changes; and a direct determination of the extinction coefficient of the reversibly denatured state is not practical. For the few measurements where this presents difficulties, the extinction coefficient has been extrapolated from data obtained at lower pressures and temperatures.

Solutions were prepared from salt-free crystalline chymo-

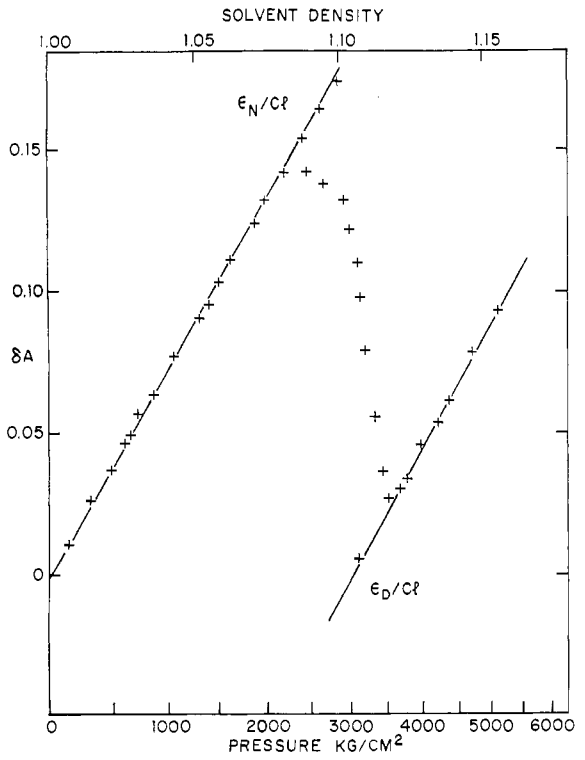

FIGURE 1: Absorbance difference, δA as a function of pressure at 8.5°, 292 nm. The independent axis is linear with respect to density of water with values shown on the superior abscissa.

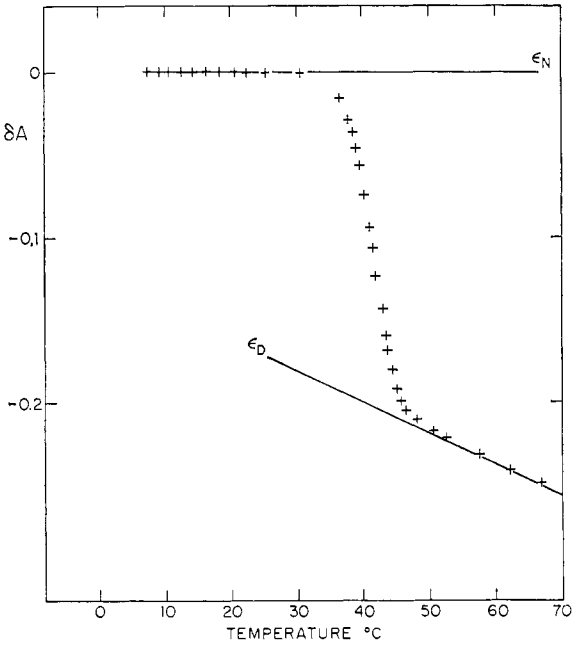

FIGURE 2: Absorbance difference, δA as a function of temperature at atmospheric pressure.

trypsinogen obtained from Mann Research Laboratories and the pH was adjusted with HCl with no added salt. Concentration ranged from about 0.03 to 0.05% depending on the sample holder employed.

Results

Thermodynamic information was obtained in the usual way. Assuming that two distinguishable states are involved, corresponding to the native and denatured species, and that Beer's law is applicable, the observed absorbance difference, δA, may be written as

$$\delta A = cl\rho[\epsilon_N X_N + \epsilon_D X_D] - A_0$$

where c is the *molal* concentration of protein, l is the path length, ρ is the solution density, A_0 is the reference absorbance, and ϵ_N, X_N, ϵ_D, and X_D are the extinction coefficients and species fraction of the native and denatured states, respectively. Other nonrelevant effects which might be expected to contribute to the variation of δA with the intensive parameters P and T are (1) variation of light intensity arising from strain birefringence in the cell windows in conjunction with any reflection polarization of the light beam that arises in the monochromator, (2) variation of the normal reflection coefficient of the two window-sample interfaces caused by changes in refractive index of the sample, (3) variations of solvent polarizability, and (4) changes of the path length, l, that accompany pressure deformation of the sample holder and cell windows. Although the experimental characteristics of these effects have not been examined in detail, they are expected to account for only a small part of the observed absorbance changes and further, expected to vary smoothly with temperature and pressure. The principal limitation imposed by these nonrelevant optical effects regards the physical meaning that can be ascribed to the derived extinction coefficients. These effects are not important, however, in the determination of the fraction denatured as long as it is possible to adequately determine the relative contributions of the denatured and native species to the observed absorbance throughout the transition region.

Representative experiments are depicted in Figures 1 and 2. In Figure 1, observations collected in an experiment made at 8.5° are shown as a function of increasing pressure where the independent axis has been made to be linear with respect to the density of water. Here direct determination of the behavior of the native and denatured states was possible as a result of the very slow transition rate. More often it was necessary to obtain the absorbance associated with the individual states within the transition region by extrapolation, as illustrated in Figure 2 which depicts an experiment conducted at atmospheric pressure. Such extrapolations were made from least-squares fittings of data outside the transition region. In most cases a linear relation between absorbance and temperature and/or density of water was sufficient to represent the experimental data within experimental error. For this purpose the polynomial representation of the specific volume of water derived by Vedam and Holton (1968) was particularly useful.

The fraction denatured, X_D, may be determined from the usual relation, $X_D = (\epsilon - \epsilon_N)/(\epsilon_D - \epsilon_N)$, where $\epsilon = (\delta A + A_0)/\rho cl$. The free-energy difference between the two states, ΔG, is calculated from $\Delta G = G_D - G_N = -RT \ln [X_D/(1 - X_D)]$.

Figure 3 depicts contours of constant X_D on the pressure–temperature plane where values of X_D have been interpolated from the various cuts such as those of Figures 1 and 2.

It is perhaps worthwhile to discuss the qualitative thermodynamic features of the transition that can be evoked from this diagram. Along the atmospheric pressure axis, the free-energy difference decreases with increasing temperature, indicating that there is a positive entropy contribution asso-

ciated with the transition. Noting that the $\Delta G = 0$ ($X_D = 0.5$) contour emerges from the atmospheric pressure axis at about 42° with a positive slope, it is evident from the Clausius–Clapeyron relation, *viz.*

$$\frac{dP}{dT_{\Delta G=0}} = \frac{S_D - S_N}{V_D - V_N} = \frac{\Delta S}{\Delta V}$$

that ΔV at atmospheric pressure also must be positive. Continuing to higher pressure (dT/dP) passes through zero at about 1500 atm and is negative at higher pressures. Since ΔS is evidently still positive at this point, the change in slope indicates ΔV has become negative, suggesting that the compressibility factor, $\Delta\beta = (\partial\Delta V/\partial P)_T$, is negative. At higher pressures the transition occurs at lower temperatures, and at about 3800 atm and 30° the contour becomes parallel to the temperature axis indicating a reversal in the sign of ΔS which apparently is negative at lower temperatures. This suggests that ΔC_p is positive for the transition, *i.e.*, the denatured state has a greater specific heat than the native state. One would expect that a continuation of this trend (ΔS, ΔV negative) would result in a reintersection of the $\Delta G = 0$ contour with the atmospheric pressure axis somewhere below 0° with (dP/dT) positive.

In order to assess these effects in detail it is necessary to include the coupling of ΔS and ΔV *via* the thermal expansivity factor $\Delta\alpha$. If we assume that $\Delta\alpha$, $\Delta\beta$, and ΔC_p are constant and further that the transition occurs with no net change in the number of particles, the free-energy difference between the two states may be easily obtained by integration of the relation, $d(\Delta G) = -\Delta S dT + \Delta V dP$, yielding eq 1,

$$\Delta G = \frac{\Delta\beta}{2}(P - P_0)^2 + \Delta\alpha(P - P_0)(T - T_0) - \Delta C_p\left[T\left(\ln\frac{T}{T_0} - 1\right) + T_0\right] + \Delta V_0(P - P_0) - \Delta S_0(T - T_0) + \Delta G_0 \quad (1)$$

where the various parameters are defined in eq 2. To be compatible with units of cal/mole for ΔG, the units of pressure must be cal/cm³ (cal/cm³ = 41.0 atm). Temperature is in °K; T_0 and P_0 represent reference temperature and pressure. In further discussion and analysis T_0 will be 273°K and P_0 will be 1 atm.

The only essential differences between eq 1 and those normally employed to analyze transition data (Brandts, 1964; Brandts *et al.*, 1970) are the presence of the contribution to free energy attributable to thermal expansivity and the logarithmic form of the specific heat contribution. Brandts (1964) analyzes the transition of chymotrypsinogen along the atmospheric pressure isobar employing a third-degree polynomial in temperature and obtains a temperature-dependent specific heat contribution. This perhaps requires more accuracy than is warranted by the data, however (Tanford, 1968; Brandts *et al.*, 1970).

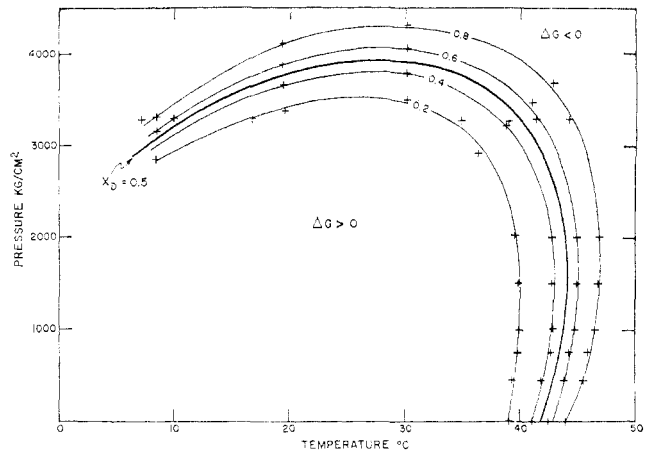

FIGURE 3: Contours of constant fraction denatured, X_D, on the pressure temperature plane. Values shown (+) have been interpolated from various data sets.

TABLE I: Thermodynamic Transition Parameters for Chymotrypsinogen (pH 2.07) and Ribonuclease (pH 2.0).[a]

	Chymotrypsinogen		Ribonuclease		
		σ		σ	Units
$\Delta\beta$	−1.24	0.13	−0.817	0.53	cm⁶/cal per mole
ΔV_0	−14.3	13.0	−48.6	17.0	cm³/mole
$\Delta\alpha$	1.32	0.24	0.252	0.28	cm³/mole per °K
ΔS_0	−227	41.0	12.5	32	cal/mole per °K
ΔC_p	3800	260	1700	250	cal/mole per °K
ΔG_0	2530	480	2510	570	cal/mole

[a] Standard temperature 0°, standard pressure 1 atm.

The functional form of eq 1 was computer fitted to the transition data employing a weighted, nonlinear iterative technique with the results listed in Table I. The statistical weight of each point, W_i, was calculated from the relation $W_i = 1/(\sigma_{G,i})^2$. The uncertainty associated with ΔG was determined from the following relation (Brandts *et al.*, 1970)

$$\sigma_{G,i} = \frac{2RT_i(\epsilon_D - \epsilon_N)\sigma_{\epsilon,i}}{(\epsilon_i - \epsilon_N)(\epsilon_D - \epsilon_i)} = 8RT_i\sigma_{\epsilon,i}' \cos h^2(\Delta G_i/2RT_i)$$

where $\sigma_{\epsilon,i}' = \sigma_{\epsilon,i}/(\epsilon_D - \epsilon_N)$ and where $\sigma_{\epsilon,i}'$ is a measure of the relative uncertainty in the extinction coefficient of the ith point. This relation assumes that the uncertainties in ϵ, ϵ_D, and ϵ_N are about the same and are much more significant sources of error than either pressure or temperature.

Fittings were also obtained for the pressure data of Brandts *et al.* (1970) characterizing the transition of ribonuclease

$$\Delta G = G_D - G_N \qquad \Delta S = S_D - S_N = -\left(\frac{\partial\Delta G}{\partial T}\right)_p \qquad \Delta V = V_D - V_N = \left(\frac{\partial\Delta G}{\partial P}\right)_T$$

$$\Delta G_0 = \Delta G(P_0, T_0) \qquad \Delta S_0 = \Delta S(P_0, T_0) \qquad \Delta V_0 = \Delta V(P_0, T_0) \quad (2)$$

$$\Delta C_p = T\left(\frac{\partial\Delta S}{\partial T}\right)_p \qquad \Delta\alpha = \left(\frac{\partial\Delta V}{\partial T}\right)_p = -\left(\frac{\partial\Delta S}{\partial P}\right)_T \qquad \Delta\beta = \left(\frac{\partial\Delta V}{\partial P}\right)_T$$

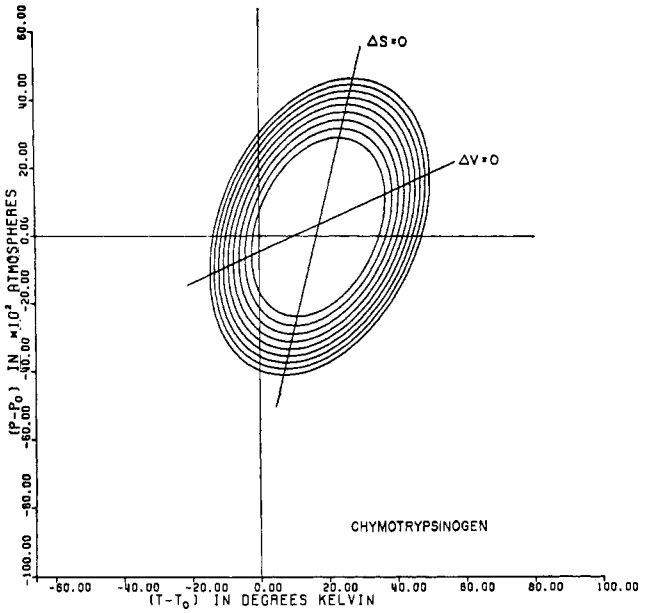

FIGURE 4: Contours of constant free-energy difference, ΔG, for chymotrypsinogen (pH 2.07). See text for details.

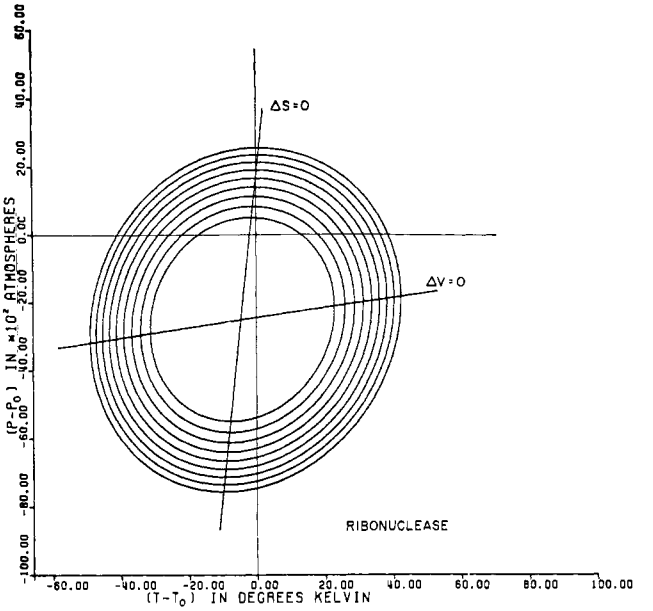

FIGURE 5: Contours of constant free-energy difference, ΔG, for ribonuclease (pH 2.0). Data from Brandts *et al.* (1970). See text for details.

at pH 2.0 and the relevant parameters also appear in Table I. For both data sets, convergence proceeded rapidly and was virtually independent of initial estimates of the adjustable parameters.

A primary difficulty in assessing the goodness of fit provided by eq 1 arises in evaluating $\sigma_{\epsilon,i}'$. The evaluation is particularly difficult in the present investigation for which the time course of experiments ranged from hours to weeks. Employing nominal estimates of 0.015 ranging to 0.03, constant for all $\sigma_{\epsilon,i}'$, the weighted sum of the square of the residuals, chi square, was observed to be in the vicinity of its most probable value (the number of points minus the number of adjustable parameters) for randomly distributed errors (Wolberg, 1968). Both the ribonuclease data of Brandts *et al.* and the chymotrypsinogen data provide a comparable fitting behavior.[1] The rapid convergence and the chi-square behavior indicate that the functional form of eq 1 is consistent with the uncertainty associated with the measurements. That is, the addition of terms of higher power in P or T with additional adjustable parameters would have statistical significance only if the measurement error could be decreased.

The relative uncertainties listed in Table I for the various parameters are somewhat greater for the ribonuclease data of Brandts *et al.* These uncertainties are not so much determined by experimental errors as by the limited information content intrinsic to their data set; unlike the transition of chymotrypsinogen, neither extremum corresponding to $\Delta S = 0$ or $\Delta V = 0$ is explicit to the ribonuclease data. Brandts *et al.* do not determine the thermal parameters ΔS and ΔC_p from their pressure data, but do so elsewhere from atmospheric pressure measurements (Brandts and Hunt, 1967). The present analysis reveals very good consistency between the separate investigations of Brandts and his coworkers. For example, Brandts and Hunt determine a value of about 2000 cal/mole per °K for ΔC_p at pH 2.10 as opposed to 1700 observed here at pH 2.0, a value of 207 eu for $\Delta S(30°)$ as opposed to 185 eu predicted in the present analysis, and ΔG_0 about equal to 3000 cal/mole as opposed to 2500 cal/mole found here. Further, both data sets lead to a prediction that the temperature of maximum stability will be between 0 and −5° at 1 atm, and cold denaturation will occur at a somewhat lower temperature. From their pressure studies Brandts *et al.* (1970) conclude $\Delta\alpha$ is positive, which is consistent with the present analysis, and that $\Delta V(27.5°) = -43.5$ cm^3/mole which compares well to −40 cm^3/mole estimated *via* eq 1. The agreement between the pressure denaturation results and atmospheric pressure parameters determined elsewhere for chymotrypsinogen is also reasonably good. Jackson and Brandts (1970) find a value of 3900 cal/mole per °K at pH 2.08 for ΔC_p obtained calormetrically. The data of Brandts (1964) predict a temperature of maximum stability (pH 2.0) at about 12° and a value of about 3200 cal/mole for ΔG_0. The present study yields 15° and 2500 cal/mole at pH 2.07.

Examination of the pressure denaturation of chymotrypsinogen at lower pH and at 19.5° revealed that the melting pressure decreases approximately linearly from about 3750 atm at pH 2.07 to about 3400 atm at pH 1.65, with ΔV at transition midpoint remaining approximately constant. The apparent compressibility becomes more negative as pH is lowered, however, and is qualitatively similar in this regard to ribonuclease (Brandts *et al.*, 1970). The two proteins differ, however, in the respect that the apparent maximum melting pressure for ribonuclease at sufficiently low pH (1.13) is close to atmospheric pressure and significant amounts of denatured protein exist at all temperatures, whereas this apparently is not the case for chymotrypsinogen (Brandts and Hunt, 1967; Brandts, 1964).

Figures 4 and 5 depict the contours of constant free energy on the PT plane for chymotrypsinogen and ribonuclease as determined by eq 1 employing the mean values of the parameters listed in Table I. To illustrate the behavior, the entire contours have been drawn, although the region where measurements have been obtained include only the

[1] The value of σ_ϵ given by Brandts *et al.* (1970) is interpreted here as $\sigma(\delta A)$.

first quadrant representing positive pressure and positive centigrade temperature. The free-energy difference, ΔG, is positive at the interior region of the ellipses and negative on the exterior with a 250-cal/mole separation between contours with the innermost contour at 2 kcal/mole and the outermost at -2 kcal/mole. The elliptical character of the contours reflects the quadratic nature of eq 1 in the vicinity of T_0 where

$$-\Delta C_p\left[T\left(\ln\frac{T}{T_0}-1\right)+T_0\right]\approx\frac{\Delta C_p}{2T_0}(T-T_0)^2$$

The contours will therefore be elliptical near T_0 whenever $(\Delta\alpha)^2 > \Delta C_p\Delta\beta/T_0$, a condition which is fulfilled by the opposite signs of ΔC_p and $\Delta\beta$ (Table I).

Since the left side of the inequality is positive or zero, and the right negative, we may rewrite the condition as $0 > (\Delta\alpha)^2T_0/\Delta C_p\Delta\beta$ or $\Delta C_{\Delta V}/\Delta C_p < 1$ since $\Delta C_{\Delta V}/\Delta C_p = 1 + (\Delta\alpha)^2T_0/\Delta C_p\Delta\beta$, where $\Delta C_{\Delta V}$ is defined as $T(\partial\Delta S/\partial T)_{\Delta V}$ and is the specific heat difference measured along a contour of constant transition volume. The condition for ellipticity in the present case therefore requires that $\Delta C_{\Delta V}$ be less than ΔC_p.

The equation for the loci of maximum stability ($\Delta S = 0$) and zero volume change for the transition are simply derived from eq 1 and are given by

$$(P-P_0)=\frac{\Delta C_p}{\Delta\alpha}\ln\frac{T}{T_0}+\frac{\Delta S_0}{\Delta\alpha};\ \Delta S=0$$

$$(P-P_0)=-\frac{\Delta\alpha}{\Delta\beta}(T-T_0)-\frac{\Delta V_0}{\Delta\beta};\ \Delta V=0$$

and are shown in Figures 4 and 5.

Discussion

The results described above suggest that the pressure–temperature transitions of chymotrypsinogen and ribonuclease have a relatively simple thermodynamic basis. While it is evident that a simple transition equation is capable of describing a great deal of experimental information, the transition parameters are directly related to instrumental operations and cannot be directly interpreted in terms of molecular models for the transition process. One immediate limitation is the use of ultraviolet difference spectra and consequently, the solvation of tryptophanyl and tyrosyl residues as the observable parameter. At atmospheric pressure the coincidence of the transition as observed by different types of measurements (Brandts and Lumry, 1964) including ultraviolet difference spectra would indicate that this is probably not an important restriction.

A question of greater concern is how accurately the thermodynamic transition surfaces represent those which would be elaborated by direct thermal and volume measurements. Some direct measurements are available for comparison but are confined to the atmospheric pressure axis. As previously indicated, the calorimetric determination of ΔC_p by Jackson and Brandts (1970) for the thermal transition of chymotrypsinogen agrees with the van't Hoff estimate listed in Table I within the experimental uncertainty of the analysis. Similarly, Brandts and Hunt (1967) have found that the direct determination of ΔC_p for the ribonuclease transition observed by Danforth *et al.* (1967) compares well to van't Hoff values obtained spectrophotometrically at atmospheric pressure. The value listed in Table I, deduced from Brandts *et al.* (1970) pressure data is somewhat lower but in reasonable accord with these estimates.

Unfortunately, there are very few direct measurements of transition volume. Perhaps the only direct measurement that is useful for comparison is the dilatometric measurement by Holcombe and Van Holde (1962) for thermal denaturation of ribonuclease at atmospheric pressure and pH 2.8. The direct measurement of volume is very difficult to perform, however. To obtain useful resolution Holcombe and Van Holde employed concentrations in excess of 1% and were not able to examine the transition for reversibility. They observed a value of about 15 cm^3/mole per °K for $\Delta\alpha$ which is many times larger than the values found here or by Brandts *et al.* (1970) to be characteristic of the ribonuclease transition. Further, Holcombe and Van Holde observe a volume decrease of about 240 cm^3/mole or about six times the van't Hoff value obtained at comparable temperature and pressure. Gill and Glogovsky have examined the pressure denaturation of ribonuclease using optical rotation and also observe that the apparent ΔV is several times smaller than the direct measurement, a situation which they suggest arises from the failure of the two-state assumption. Lumry *et al.* (1966) have discounted the importance of the disagreement because of uncertainties associated with the direct volume measurement, however.

Thus, while the calorimetric measurements indicate the van't Hoff analysis provides useful measure of the thermal character of the ribonuclease transition, it is possible there is significant disagreement between the measured and predicted transition volume at atmospheric pressure. The evidence is insufficient to resolve this difficulty and for the present, the thermodynamic significance of the elaborated transition surfaces must remain somewhat speculative.

Perhaps the most important observation associated with this investigation is the relatively simple equation of state that is necessary to order equilibrium properties associated with the interaction of pressure and temperature with the reversible transition of ribonuclease and chymotrypsinogen. Since the behavior of a number of proteins is consistent with the expectation of "cold" melting (Brandts, 1968), it is conceivable that the type of transition surface exhibited by ribonuclease and chymotrypsinogen is not restricted to these two proteins.

Acknowledgments

The author is indebted to Professor Gerald Holton for his interest and support of this work, and to Professor J. F. Brandts, University of Massachusetts, for making both advice and details of experimental work conducted in his laboratory freely available during the course of this investigation. Data analyses were conducted on the Harvard University Physics Department AEC-supported computer facility.

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Structural Properties of Hydrogenase from *Clostridium pasteurianum* W5*

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ABSTRACT: Hydrogenase from *Clostridium pasteurianum* has an adsorption maximum at 280 mμ and a broad absorption between 350 and 500 mμ with a molar extinction coefficient at 400 mμ of 8000. Hydrogenase as isolated exhibits an intense, temperature-sensitive electron spin resonance signal of the $g = 1.94$ type. The signal is lost on oxidation and can be restored almost completely by incubation under H_2 or addition of dithionite in the absence of ferredoxin. Sodium dodecyl sulfate treated hydrogenase dissociates into two subunits of identical size with a molecular weight of about 30,000. The behavior of the enzyme on polyacrylamide gels made 8.0 M in urea suggests that its two subunits also have the same isoelectric point. Hydrogenase incubated with 4.0 M urea retains its activity for several hours. When dialyzed against 4.0 M urea, it rapidly loses iron, "acid-labile" sulfide, and enzymatic activity. Amino acid analysis of hydrogenase shows the presence of four half-cystine residues and the absence of tryptophan. All the other usual amino acids are present. Titration of hydrogenase with two mercurials and 5,5′-dithiobis(2-nitrobenzoic acid) indicates the presence of 12 SH equiv. Amino acid analysis, titration with mercurials, and elemental analysis establish the presence of both cysteine sulfur and acid-labile sulfide in hydrogenase. Treatment of hydrogenase with *o*-phenanthroline removes two of its four iron atoms but its hydrogen evolution and hydrogen-uptake activities are not decreased. Sodium mersalyl treatment removes the iron and acid-labile sulfide from the protein with concomitant loss of its enzymatic activity. The finding of hydrogenase "iso enzymes" in crude or partially purified preparations of the enzyme appears to be an artifact.

Hydrogenase (H_2:ferredoxin oxidoreductase, EC 1.12.1.1) is involved in hydrogen metabolism and electron transport of a number of microorganisms (Gray and Gest, 1965), of certain plants (Renwick *et al.*, 1964), and probably of animals (Kuruta, 1962).

Hydrogenase from *Clostridium pasteurianum* has been purified to a state that shows one protein band and one coincident activity peak on analytical polyacrylamide gel electrophoresis (Nakos and Mortenson, 1971). These authors also found that hydrogenase from the same organism is an iron–sulfur protein containing 4.0 iron atoms and 4.0 "acid-labile" sulfide groups per molecule.

This paper describes some physical and chemical characteristics of the purified hydrogenase from *C. pasteurianum*. The theory that hydrogenase exists as iso enzymes (Ackrell *et al.*, 1966; Kidman *et al.*, 1969; Kleiner and Burris, 1970) is also investigated.

Materials and Methods

Chemicals. The chemicals used were obtained: from Fisher Scientific Co., *o*-phenathroline; from K & K Laboratories, disodium 1,2-dihydroxybenzene-3,5-disulfonate (Tiron); from Mann Research Laboratories, sodium *O*-(3-hydroxymercuri-2-methoxypropyl)carbamylphenoxyacetate (sodium mersalyl), methyl viologen, and Ultra Pure urea; from Pierce Chemical

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