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Influence of Pressure on the Reversible Unfolding of Ribonuclease and Poly-γ-benzyl-L-glutamate[1]

by S. J. Gill and Robert L. Glogovsky

Department of Chemistry, University of Colorado, Boulder, Colorado *(Received September 23, 1964)*

The effect of pressure on the thermal transitions of ribonuclease aqueous solution at pH 2.80 and of poly-γ-benzyl-L-glutamate (PBG) in dichloroacetic acid and 1,2-dichloroethane has been investigated to 1400 atm. by optical rotation measurements. An increase in pressure enhances unfolding for both ribonuclease and PBG. The pressure dependence on the extent of unfolding can be used to calculate the volume change for assumed basic steps of the transition process. For ribonuclease the volume change as calculated for a single-step reaction mechanism was found to be sufficiently smaller than that given by direct determinations of Holcomb and Van Holde, so that the reaction mechanism must consist of more than one reaction step. For the PBG transition an application of the helix-coil transition theory of Zimm, Doty, and [illegible] with the measured pressure dependence upon the extent of transition, showed that the volume change of the process is at the experimental limit of direct determinations. The shift in the transition temperature with pressure for PBG was used to estimate a heat capacity change at constant pressure of approximately 140 cal./mole-deg. for the helix to random coil transition.

Introduction

Thermally induced transitions of macromolecular structures have been found for a variety of polymer materials.[2] The reversible transitions of ribonuclease[3-6] and synthetic polypeptides[7-10] have been the subjects of experimental and theoretical investigation. One of the primary purposes of such studies has been to gain insight into the mechanism of the transition process. In the case of ribonuclease, the strategy has been to interpret the extent of reaction as a function of temperature by assumed simple reaction mechanisms. The heat of the assumed reaction may then be found from the dependence of the extent of reaction upon transition.[11] Until recently,[12] direct calorimetric meas-

(1) This work was supported by the National Science Foundation under Grant GP732.

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urements for the process were unavailable for comparative purposes. In analogous reasoning the temperature dependence on polypeptide helix coil transition has been used to test helix coil transition theories,[13,14] and from such studies at various chain lengths theoretical parameters for the PBG transition have been established. Recently, direct calorimetric measurements have provided an even more direct evaluation of the theoretical parameters.[15,16]

Analogous information should also be revealed from studies of the effect of pressure on the transition along with a determination of the volume change of the transition. There may be situations where this latter approach is experimentally more feasible than the determination of thermal transitions along with calorimetric measurements. In this paper we report results obtained for two known reversible transitions, ribonuclease and PBG.

Experimental

The ribonuclease A used was Lot RAF 6069, lyophilized and phosphate-free, and was obtained from Worthington Biochemical Corp., Freehold, N. J. The material was used without further purification, and calculations were based on the weight of the vacuum-desiccated material.

The buffer solutions used were 0.01 *M* in potassium acid phthalate and 0.15 *M* in KCl. The solution conforms to that used by Holcomb and Van Holde.[6] In preparation of the ribonuclease solution, the buffer solution was pipetted into a weighed flask containing the ribonuclease; the flask was then tightly stoppered. The pH of the solution was adjusted to 2.80 using a Beckman Zeromatic pH meter. The solution was refrigerated until used. The high pressure optical rotation apparatus has been previously described.[17]

The PBG used was Lot G51. It was obtained from Pilot Chemicals Inc., Watertown, Mass., and was specified to have a molecular weight of 275,000. The material was used without further purification, and calculations were based on the weight of the desiccated material.

The solvent for the PBG was 76 vol. % Eastman practical dichloroacetic acid (DCA) and 24 vol. % Eastman reagent 1,2-dichloroethane (EDC). Both components of the solvent were redistilled prior to use. The solvent mixture was tightly capped and refrigerated until needed.

Theory

The basic reaction process for these polymer transitions can be written in terms of the extreme conditions as

$$\text{folded} \rightleftharpoons \text{unfolded} \qquad (1)$$

where, if the process involves more than one step, there will be a series of intermediate states and equations within eq. 1.

In the case of a complex molecule such as ribonuclease, it is not possible to predict logically the nature of intermediate steps, if any. It becomes convenient either to assume a single-step reaction process of eq. 1 or, as Tanford has done,[18] to suppose that reaction 1 consists of r equivalent and independent steps. This latter situation reduces to the one-step case when $r = 1$. The appropriate result which expresses the pressure dependence upon the fraction of folding f is then given by

$$\left(\frac{\partial f}{\partial P}\right)_T = \frac{f(1-f)\Delta V^\circ}{rRT} \qquad (2)$$

where ΔV° is the standard state volume change per mole of polymer taken under the conditions of infinite dilution, at temperature T and pressure P. A dilatometric determination of the volume change of transition, even at finite concentrations, should be nearly equivalent to ΔV°. With the assumptions contained in eq. 2 it should then be possible to determine $\Delta V^\circ/r$ from pressure measurements, and, when this value is compared to a dilatometric determination of ΔV°, an estimate of r can be made. If a multiple-step mechanism is indicated from the experimental findings, it is quite probable that cooperative effects exist between different portions of the molecule. The assumption of independent steps then becomes invalid. A simple mechanism of successive steps which must follow in a definite order and which are characterized by the same equilibrium constants can be analyzed, but it seems the presentation of such an analysis would be superficial until some more definite facts about the ribonuclease transition are available.

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In the case of PBG the helix coil transition theory describes the appropriate mechanism for the transition process. For our purposes it is sufficient to extend the results of Applequist,[8] who made use of the model of Zimm and Bragg[14] for long chains, to describe the pressure dependence on the fraction f of hydrogen-bonded carbonyls at the midpoint of the transition

$$\left(\frac{\partial f}{\partial P}\right)_{T,f=1/2} = + \frac{\Delta V_m^\circ}{4RT_r\sqrt{\sigma}} \qquad (3)$$

where ΔV_m° is the standard state volume increase upon disruption of 1 mole of hydrogen-bonded carbonyl groups, T_r is the temperature at the center of the transition, and σ is the parameter which represents the equilibrium for the formation of an interruption in a sequence of bonds by a process which maintains a constant number of bonds. An equation analogous to eq. 3 is given by Applequist for the temperature dependence as

$$\left(\frac{\partial f}{\partial T}\right)_{P,f=1/2} = \frac{-\Delta H_m^\circ}{4RT_r^2\sqrt{\sigma}} \qquad (4)$$

where we define ΔH_m° as the standard state enthalpy of transition for disruption of 1 mole of hydrogen-bonded carbonyl groups. For internal consistency the Δ terms in eq. 3 and 4 have been defined according to the reaction expressed by eq. 1. Applequist uses the reverse equation and describes the enthalpy for the reaction of formation of hydrogen bonds. The two equations may be combined to yield an equation which has the familiar form of the Clapeyron equation

$$\left(\frac{\partial P}{\partial T}\right)_f = \frac{\Delta H_m^\circ}{T\Delta V_m^\circ} \qquad (5)$$

Equation 5 actually applies for any constant helical content fraction f, but if $f = 1/2$ then $T = T_r$.

Results and Discussion

Some preliminary attempts were made to induce the entire transitions of ribonuclease and PBG isothermally by application of pressure, but the entire transition could not be covered for either case within our range of pressures to 1500 atm. Consequently, reversible thermal transitions were examined at various fixed pressures. We shall describe the results as they apply to each material in the next two sections. In the following results it is helpful to remember that an increase in temperature promotes unfolding for ribonuclease but folding for PBG.

Ribonuclease A. A 5.491 wt.–vol. % solution of ribonuclease A was studied at 589 mμ. Studies on less concentrated solutions of ribonuclease yielded similar results but with less precision.

Reversible thermal transitions were observed at atmospheric pressure, 680, 1020, and 1360 atm. Transition temperatures (where $f = 1/2$) were found to be 46.0, 44.4, 43.5, and 40.7° for the above pressures, respectively.

The effect of pressure on the thermal denaturations is seen in Figure 1 which illustrates the change in observed rotation with temperatures at various pressures. The filled symbols represent values obtained for decreasing temperatures and substantiate the reversibility of the transition. At low temperatures a significant increase in the optical rotation was found with increase of pressure. This effect is possibly due to solvent effects. The effect of pressure on the unfolded form from 1 to 681 atm. may be due to small apparatus changes or to denaturation. These latter two effects are insignificant if relative properties are examined as we shall do in studying the fraction of denaturation as a function of temperature and pressure. Figure 2 shows the effect of temperature on undenatured fraction at various pressures. The undenatured fraction, f, is assumed to be determined by the fractional change of the optical rotation upon temperature.

The results of the effect of pressure on the undenatured fraction at various temperatures are shown in Figure 3. The slopes at the center of the transition, $(\partial f/\partial P)_{f=1/2}$, have values of -3.5×10^{-4} and -3.0×10^{-4}/atm. for 42.5 and 45.0°, respectively. On the basis of an independent r-step denaturation mechanism, eq. 2 yields values of -36 and -31 ml./mole for 42.5

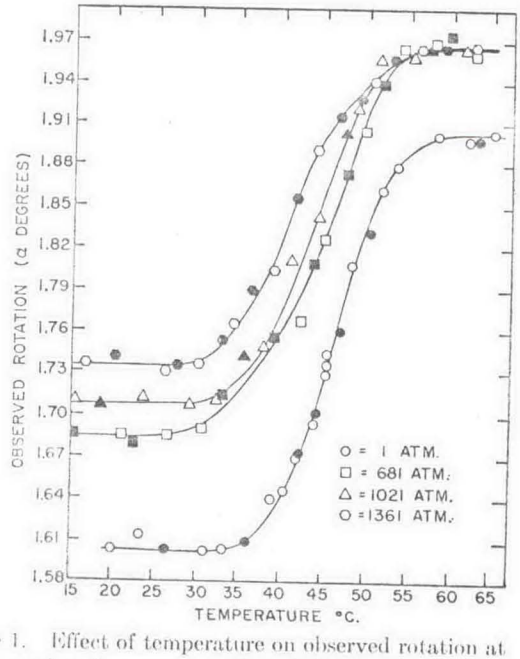

Figure 1. Effect of temperature on observed rotation at 589 mμ and various pressures for a 5.491 wt.–vol. %, pH 2.80 buffer solution of ribonuclease A.

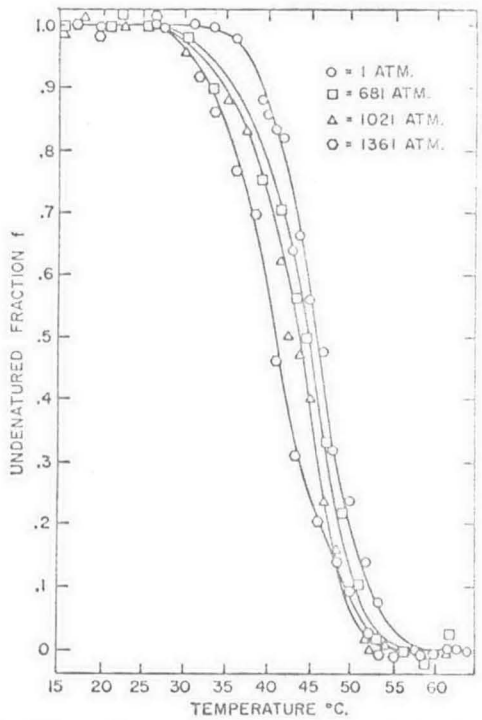

Figure 2. Effect of temperature on undenatured fraction (f) at various pressures for ribonuclease A.

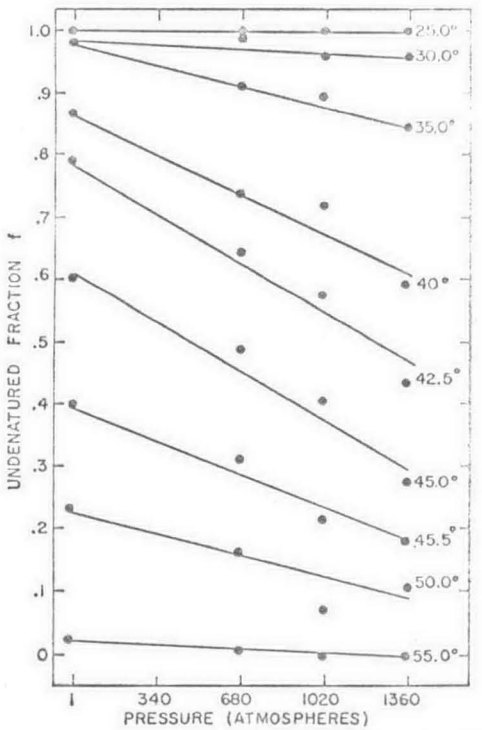

Figure 3. Effect of pressure on undenatured fraction (f) at various temperatures for ribonuclease A.

and 45.0°, respectively, for $\Delta V°/r$. It is also known from direct measurements that the folded configuration (low-temperature form) has greater volume than the unfolded configuration.[6] The observation (Figure 2) that the amount of folded form decreases with increasing pressure is in agreement with this finding.

The volume changes calculated from the pressure measurements are much lower than those observed by Holcomb and Van Holde,[6] who found a value of −240 ml./mole at 45.0° by density measurements using twin dilatometers. This immediately suggests a multiple-step process. However, before drawing any more definite conclusions, the possible errors in the values of these two volume changes should be noted.

Although the dilatometric method yields excellent results, a linear extrapolation of the slopes for the high- and low-temperature determinations must necessarily be made to evaluate the volume change occurring for the denaturation process. The determination of the slopes and the necessary extrapolations could affect the evaluation of the volume change by as much as 35%. A similar order of magnitude of error may also exist in the value derived from the pressure measurements since the effect was so small.

Thus, we shall take for the dilatometric volume change −240 ± 100 ml./mole, and for the pressure-calculated value, −30 ± 10 ml./mole. The ratio of these volume changes yields a value for r, the number of independent steps of the mechanism, of somewhere between 3 and 17. The range of values is rather unsatisfactory, but, on the basis of independent equal reaction steps, the number of regions with which the folding to unfolding process occurs is between 3 and 17. Tanford[18] has analyzed data which indicate that three regions seem to unfold independently. Scott and Scheraga have interpreted optical density changes by a two-step mechanism. It seems highly probable that cooperative effects would be present in this transition, and the safest conclusion is one which states that more than one reaction step is involved.

Poly-γ-benzyl-L-glutamate. Reversible thermal transitions as observed for a 4.33 wt. % solution of PBG at 1, 681, and 1021 atm. are shown in Figure 4 which illustrates the change of observed rotation for the thermal transition at various pressures. Figure 5 shows the effect of temperature on the fraction of folding at the three pressures. The fraction, f, is assumed to be determined by fractional change of the optical rotation upon transition. Open and filled symbols on Figure 4 represent data obtained for increasing and decreasing temperatures, respectively, and illustrate the reversibility of the transition.

Dilatometric measurement was attempted to evaluate the volume change accompanying the transition, but it was found that the change was too small to be

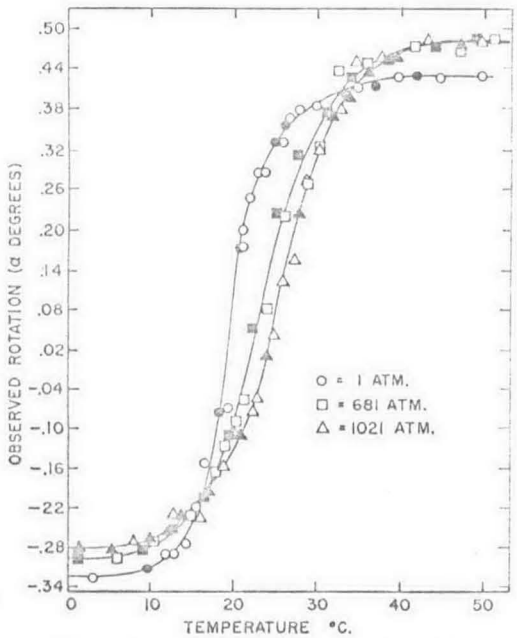

Figure 4. Effect of temperature on observed rotation at 589 mμ and various pressures for a 4.330 wt. % solution of PBG in a 76:24 vol. % solvent of DCA and EDC, respectively.

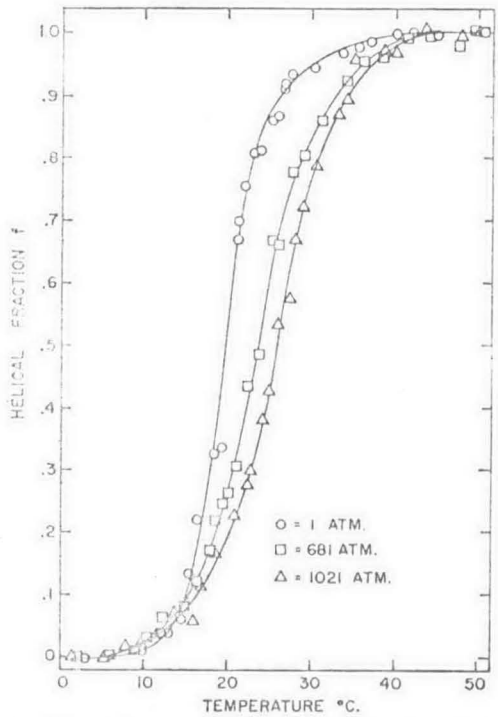

Figure 5. Effect of temperature on helical fraction (f) of PBG at various pressures.

adequately determined without resorting to special methods.

The effect of pressure on fraction f noted in Figure 5 yields an average value of -4.6×10^{-4}/atm. for $(\partial f/\partial P)_{T,f=1/2}$ with T approximately 296°K. Using Zimm and Bragg's value for σ (2×10^{-4}), the ΔV_m° is found to be -0.6 ml./mole of monomer or -0.003 ml./g., which substantiates the difficulty encountered in the attempt of the direct determination. It should be noted that ΔV_m° is negative which means that the folded configuration has a slightly larger partial molar volume than does the unfolded form.

If Zimm's value of σ is used with the experimental evaluation of $(\partial f/\partial T)_{P,f=1/2}$ at the center of the transition, then ΔH_m° may be calculated for the three pressure determinations using eq. 4. Table I summarizes the results. Values of ΔH_m° are seen to increase with increasing pressure and transition temperature. Since ΔV_m° is very small, we shall assume the variation of ΔH_m° is governed by the change in temperature and ΔC_p. This yields a value of approximately 140 cal./mole-deg. This finding might be explained by noting that the helical configuration has less degrees of freedom than the coiled form.

As may be noted in Table I, the transition temperature was found to increase by the effect of increasing pressure and yields a value of 5.6×10^{-3} deg./atm. for $(\partial T/\partial P)_{f=1/2}$. By eq. 5 the positive sign of this result shows that both ΔH_m° and ΔV_m° must have the same sign. Since ΔV_m° has been shown to be negative, ΔH_m° must also be negative. This is in agreement with the known negative value of ΔH_m°.[15,16]

Table I

Transition temp., °C.	Press., atm.	$(\partial f/\partial T)_{f=1/2} \times 10^2$	$\Delta H_m^\circ \pm 20\%$, cal./mole
20.2	1	3	−1400
24.1	680	5	−800
25.9	1020	6	−600

Although it has been possible to determine the effect of pressure on the helix–coil transition of PBG and thereby calculate an expected change of ΔV_m°, the smallness of the calculated ΔV_m° makes a direct determination highly unfavorable. It therefore appears that a direct evaluation of the σ-parameter for this transition will have to depend upon the direct measurement of the enthalpy change in conjunction with the effect of temperature on the transition.[15,16]