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Influence of Temperature on the Intrinsic Viscosities of Proteins in Random Coil Conformation†

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ABSTRACT: Measurements have been made of the intrinsic viscosities of proteins consisting of one polypeptide chain, in 6 M guanidine hydrochloride plus β-mercaptoethanol at different temperatures in the range 25–55°. The molecular weight dependence and the values of Huggins constant and end-to-end distance were determined at 25° for the reduced denatured proteins including papain and were consistent with their linear random coil behavior. The behavior of reduced ovalbumin in 9 M urea was found to be similar. The dimensions of randomly coiled proteins generally decreased with increasing temperature. The unperturbed dimensions calculated from viscosity data also showed variation with temperature. The intrinsic viscosity–temperature profiles of random coil proteins were characterized with a minimum at 35° and a hump at 40°. Similar observations were made on randomly coiled ovalbumin in 9 M urea. However, such features were not noticed in the curves for cross-linked randomly coiled ovalbumin and lysozyme. Our results, therefore, suggest that proteins which are well-behaved, linear random coils in denaturing solvents at 25° show conformational anomalies at higher temperatures that are independent of amino acid composition, chain length, and the nature of the denaturing solvent.

Intrinsic viscosity measurements have been successfully used in the detection of conformational changes in proteins (Tanford, 1968). For native globular proteins intrinsic viscosity, $[\eta]$, is low, about 3–4 cm^3/g, regardless of their molecular weights. Moreover, it is independent of temperature and of the nature of the solvent, as long as the protein molecule retains its native conformation and the extent of its solvation is not markedly changed (Tanford, 1961). On the other hand, intrinsic viscosity for linear random coil proteins shows strong molecular weight dependence and this was successfully used by Tanford (1968) in the demonstration of the linear

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random coil behavior of proteins in 6 M guanidine hydrochloride (Gdn·HCl) plus β-mercaptoethanol (HSEtOH).[1]

In linear random coil proteins, free from long-range interactions, peptide backbone rotations are feasible around two single bonds defining the dihedral angles ϕ and ψ. Therefore, depending on the availability of ϕ–ψ space, factors such as increase in temperature, which facilitate backbone rotations, may reduce the dimension making $d[\eta]/dT$ negative. However, to our knowledge the temperature dependence of the intrinsic viscosity of randomly coiled proteins has not been systematically investigated so far. In this paper we report our results on the intrinsic viscosity of eight proteins, each containing one polypeptide chain, in 6 M Gdn·HCl–0.1 M HSEtOH at different temperatures, in the range 25–55°. Viscosity results demonstrating the random coil behavior of papain in 6 M Gdn·HCl containing the thiol compound are being reported here for the first time. Indeed, the data generally showed a decrease in intrinsic viscosity and end-to-end distance with an increase in temperature. A striking feature common to all the proteins was that, contrary to the expectation based on the above considerations, the dimension of randomly coiled proteins was significantly higher at 40° than that at 35°. Furthermore, an intrinsic viscosity–temperature profile for randomly coiled ovalbumin in 9 M urea–0.1 M HSEtOH was found to be similar. However, these characteristic features were absent in the viscosity–temperature profile of cross-linked randomly coiled ovalbumin and lysozyme in 6 M Gdn·HCl.

Experimental Section

Pancreatic ribonuclease (type II-A), bovine serum albumin (type F), egg-white lysozyme (grade IV), and β-mercaptoethanol were purchased from Sigma Chemical Co. A salt-free myoglobin (crystallized and lyophilized) preparation was from Mann Research Laboratory, Inc. Chymotrypsinogen A (3× crystallized) and pepsinogen were from Worthington Biochemical Corporation and Nutritional Biochemicals Corporation, respectively. The lyophilized preparation of papain was from V. P. Chest Institute, Delhi; on polyacrylamide gel electrophoresis it gave a single band (A. Rafi, unpublished results). The ovalbumin preparation isolated from egg white according to the method of Kekwick and Cannan (1936) was crystallized five times. The purified sample gave three peaks on a carboxymethylcellulose column (Rhodes *et al.*, 1958), but these were found to be indistinguishable by immunological criteria (Ansari, 1971). Reagent grade guanidine hydrochloride obtained from Nutritional Biochemicals Corporation was first washed with distilled acetone and then crystallized twice from ethanol and was finally recrystallized from water. Analytical reagent grade urea from BDH (India) was crystallized two times from hot ethanol. Ion exchange resins were from J. T. Baker Chemical Co. Carboxymethylcellulose was from Calbiochem, Los Angeles, Calif. Other chemicals were of analytical grade.

Preparation of Protein Solution. For all proteins, except lysozyme, a relatively concentrated aqueous solution was first prepared and dialyzed extensively in cold against several changes of water, each change after an interval of 30 min. The traces of insoluble material, if any, were removed by centrifugation. Isoionic protein solutions were obtained by passing dialyzed protein solution through a Dintzis column (1952), and their concentrations were determined with a precision better than 1% by taking isoionic protein solutions in weighing bottles and heating them in air at 105–107° to constant weight. Following Aune (1968), the lysozyme solution was prepared in 0.1 M KCl and its concentration determined by the dry-weight method. Appropriate amounts of a stock solution of isoionic protein and Gdn·HCl were taken by weight in a 5-ml volumetric flask and the volume was made up by the addition of required volumes of HSEtOH and solvent. Since aggregation of isoionic ovalbumin was noticed on standing for 3 or more days, its fresh solution was used in the preparation of solution. The final concentration of the thiol compound was the same, *viz.* 0.1 M for all proteins except for bovine serum albumin and ribonuclease where the concentration was 0.2 M. The pH of the resulting solution measured on an Elico pH meter Model LI-10 was near 5.0 for lysozyme and ovalbumin and 6.0–7.7 for the rest of the proteins. The solutions were generally kept for 24 hr to ensure complete reduction of the disulfide bonds. The time required for complete reduction of the disulfide bonds in lysozyme at the desired pH was 30 hr. Solutions of lysozyme and ovalbumin in 6 M Gdn·HCl and that of ovalbumin in 9 M urea–HSEtOH were prepared on a weight basis.

Viscosity Measurements. Calibrated Cannon-Fenske viscometers with a flow time of about 400 sec for distilled water at 25° were used. The time of fall of the solvent, t_0, and that of the protein solution, t, were recorded by a stop watch reading to 0.1 sec. The viscometer was dipped in a well-insulated glass tank filled with distilled water whose temperature was maintained within ±0.05° by circulating water from Neo Thermostat (Budapest, Hungary). Extreme care was taken to ensure that (i) all the solutions were free from dust, (ii) the viscometer was clean and dust free, and (iii) the position of the viscometer was vertical during measurements. Each protein solution was filtered through a Millipore filter (pore size 0.8 μ) and the filtered solution was taken in the viscometer. A minimum of eight independent measurements of the time of fall of each sample were made and all observations agreed within ±0.3 sec.

The reduced viscosity, η_{red}, was computed from the measured values of t, t_0, and protein concentration, c (in grams per cubic centimeter), by the help of expression 1 (Tanford,

$$\eta_{red} = (t - t_0)/t_0c + (1 - \bar{v}_2\rho_0)/\rho_0 \quad (1)$$

1955), where $\bar{v}_2$ is the partial specific volume of protein and ρ_0 is the density of the solvent, *i.e.*, 6 M Gdn·HCl–0.1 M HSEtOH, which was measured to be 1.1360 g/cm^3, which is comparable (about 0.9% lower) to that determined by Kawahara and Tanford (1966). The data on reduced viscosity were analyzed by the help of least squares using an IBM-1130 computer and were fitted to eq 2, where k is Huggins constant. The values

$$\eta_{red} = [\eta] + k[\eta]^2c \quad (2)$$

of k and $[\eta]$ were calculated from the intercept and the slope of the plot of reduced viscosity *vs.* c. The precision with which intrinsic viscosity could be measured, as shown by actual observations in triplicate, in these studies was better than 2%. It should be noted that protein denaturation and changes in temperature may influence $\bar{v}_2$ and ρ_0 but these variations were ignored in the calculation of intrinsic viscosity for the following reasons. First, the contribution of the term $(1 - \bar{v}_2\rho_0)/\rho_0$ of eq 1 was found to be 0.3–1% of the measured intrinsic viscosity which is experimentally insignificant. Second, $d\bar{v}_2/dt$

[1] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; HSEtOH, β-mercaptoethanol.

TABLE I: Intrinsic Viscosities of Proteins in 6 M Gdn·HCl–ESTtOH, at 25°.[a]

Protein	M	n	$\bar{v}_2$ (cm³/g)	$[\eta]$ (cm³/g)	Huggins Constant	$\langle L^2\rangle^{1/2}$ (Å)
Ribonuclease	13,700	124	0.728	16.1 (16.6)	0.35	102 (103)
Lysozyme	14,300	129	0.688	17.6 (17.1)	0.15	106 (105)
Myoglobin	17,200	153	0.730[e]	20.1 (20.9)	0.34	118 (120)
Papain	23,406[b]	212[b]	0.723[b]	24.5	0.40	140
Chymotrypsinogen	25,700	245	0.734[f]	26.3 (26.8)	0.52	148 (148)
Ovalbumin	43,000[d]	345[c]	0.748	31.0	0.31	183
Pepsinogen	40,000	376	0.730[g]	29.0 (31.5)	0.46	177 (179)
Bovine serum albumin	69,000	605	0.734	51.6 (52.2)	0.34	257 (258)

[a] The values of n and M and of $[\eta]$ in parentheses were taken from Tanford (1968) and those of $\bar{v}_2$ came from Tanford (1961), unless otherwise stated. The values of $\langle L^2\rangle^{1/2}$ in parentheses were taken from Kurata and Stockmayer (1963). [b] Mitchel *et al.*, 1970. [c] Weintraub and Schlamowitz, 1970. [d] Fish *et al.*, 1970. [e] Breslow and Gurd, 1962. [f] Lapanje and Skerjanc, 1971. [g] Williams and Rajagopalan, 1966.

is positive (Hunter, 1966), whereas $d\rho_0/dt$ was found to be negative. Finally, the change in $\bar{v}_2$ caused by denaturation (Lapanje and Skerjanc, 1971) is too low to be of any significance. Accordingly, the values of $\bar{v}_2$ listed in Table I for native proteins were used in the calculations.

Results

Viscosities at 25°. Intrinsic viscosities of proteins in 6 M Gdn·HCl–HSEtOH, at 25°, are given in Table I, where it can be seen that our values are generally in good agreement with those reported in the literature (Tanford, 1968) for randomly coiled proteins with disulfide bonds reduced. The least-squares analysis of the data gave a straight line between log $[\eta]M_0$ and log n which corresponds to the relation

$$[\eta]M_0 = 76.1n^{0.660} \quad (3)$$

where M_0 is the mean residue weight, M/n, n is the number of amino acid residues per polypeptide chain, and M is the molecular weight of the polypeptide. For linear random coil polymers in a nonideal solvent, the value of the exponent lies between 0.5 and 0.8 and the coefficient is a constant. The values of the exponent and the constant of eq 3 compare fairly well with those determined by Tanford (1968) for a larger number of randomly coiled proteins, showing that the proteins listed in Table I also adhere to Tanford's empirical relationship (1968). Furthermore, the values of Huggins constant shown in Table I are of the same order of magnitude as that reported for random coil polymers, which is 0.35 (Tanford, 1961). These facts strongly suggest that the proteins including papain behave as linear random coils in 6 M Gdn·HCl–HSEtOH. The intrinsic viscosity of ovalbumin in 6 M Gdn·HCl alone, where the protein would exist as a cross-linked random coil, was measured to be 27.0 cm³/g, which is 13% lower than that for ovalbumin in linear random coil conformation. The intrinsic viscosity of reduced ovalbumin in 9 M urea at 25° was determined to be 29.5 cm³/g which agrees with the value calculated from the empirical equations obtained for linear random coil proteins in concentrated urea solutions (Tanford, 1968; Lapanje, 1969).

The root-mean-square end-to-end distance, $\langle L^2\rangle^{1/2}$, for the randomly coiled proteins, was calculated by the help of eq 4 (Tanford, 1961; Kurata and Stockmayer, 1963), where

$$\langle L^2\rangle^{3/2} = nM_0[\eta]/\Phi \quad (4)$$

Φ is a universal constant having a value of about 2.1×10^{23} in cgs units for impenetrable random coil polymers in a good solvent such as a concentrated solution of Gdn·HCl. The least-squares analysis of the values of $\langle L^2\rangle^{1/2}$ calculated from viscosity data gives the equation: $\langle L^2\rangle = 111n - 4251$. As can be seen in Table I, our values are again in good agreement with those found by Tanford (1968). The real dimensions of randomly coiled proteins are obviously greater than the unperturbed dimensions, $\langle L^2\rangle_0^{1/2}$, in a thermodynamically ideal solvent. Randomly coiled proteins do not behave as ideal solutes in 6 M Gdn·HCl. One way to evaluate $\langle L^2\rangle_0^{1/2}$ from the observed dimension is to correct the latter for the thermodynamic nonideality of the randomly coiled proteins in 6 M Gdn·HCl–HSEtOH by the help of osomtic pressure data. Lapanje and Tanford (1967) used this approach for determining $\langle L^2\rangle_0^{1/2}$ for six proteins and obtained an empirical relation: $\langle L^2\rangle_0 = (60 \pm 10)n$. The unperturbed dimensions can be calculated from viscosity data alone using the procedure of Stockmayer and Fixman (1963) who, for randomly coiled polymers with negligible draining effects, found the relation

$$[\eta] = KM^{1/2} + 0.51\Phi BM \quad (5)$$

where K and B are constants independent of molecular weight, and the former is related to $\langle L^2\rangle_0^{1/2}$ through the expression

$$K = (\langle L^2\rangle_0/M)^{3/2} \quad (6)$$

The values of K were computed by the method of least squares from the intercept of the plot between $[\eta]M^{-1/2}$ and $M^{1/2}$. The least-squares analysis of the values of $\langle L^2\rangle_0$ determined by the help of eq 5 and 6 gives the relation

$$\langle K^2\rangle_0 = 77n \quad (7)$$

Effect of Temperature on Intrinsic Viscosity. The effect of temperature on the intrinsic viscosity of randomly coiled proteins in 6 M Gdn·HCl–HSEtOH was generally investigated in the temperature range 25–55° and the results are shown in Table II. No change in the measured value of time of fall, t,

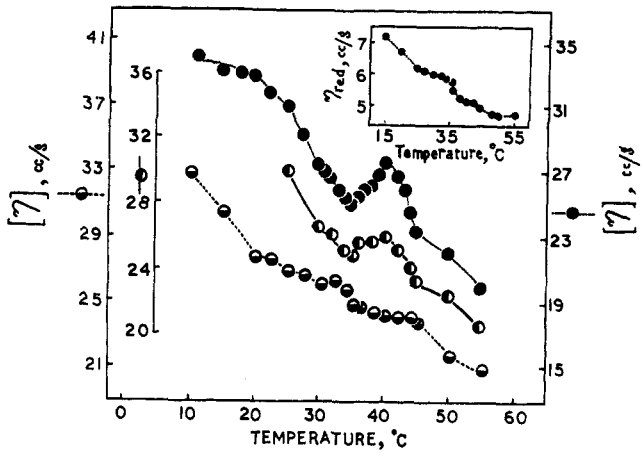

FIGURE 1: Temperature dependence of intrinsic viscosity of ovalbumin in 6 M Gdn·HCl–0.1 M HSEtOH (pH 5.1) (···●); 9 M urea–0.1 M HSEtOH (pH 2.0) (–◐); and 6 M Gdn·HCl (pH 6.8) (- -◑). The inset shows a reduced viscosity–temperature profile for lysozyme (0.39 g/100 ml) in 6 M Gdn·HCl (pH 5.0).

at 25° was observed either by heating from a lower temperature to 25° or by cooling from 55 to 25°. Typical intrinsic viscosity–temperature profiles of cross-linked as well as linear random coil ovalbumin are shown in Figure 1, whose inset depicts the variation of reduced viscosity of cross-linked randomly coiled lysozyme with temperature.

From Table II it is evident that there is appreciable decrease in intrinsic viscosity with a rise in temperature. The decrease in intrinsic viscosity with a rise in temperature from 25 to 35° for the first four proteins is about 40%, and is 33% for chymotrypsinogen, 28% for pepsinogen, 19% for ovalbumin, and 14% for bovine serum albumin. There was a definite increase in intrinsic viscosity as the temperature was raised further up to 40° and the extent of increase was way above the experimental uncertainty involved in the measurement of the intrinsic viscosity. At still higher temperatures, intrinsic viscosity invariably decreased with an increase in temperature. The increase was most pronounced (19%) for pepsinogen and was relatively lower (3–5%), but experimentally significant, for ovalbumin and myoglobin; it was 8–9% for the rest of the proteins. The Huggins constant exhibited no unusual behavior when passing through the anomalous region. Similar features of intrinsic viscosity–temperature plot were also observed for randomly coiled ovalbumin in 9 M urea–0.1 M HSEtOH (see Figure 1). However, the characteristic minimum at 35° and the distinct hump at 40°, observed for random coil proteins, are not present in the curve for cross-linked randomly coiled ovalbumin. There is a definite, but somewhat irregular, decrease in the viscosity of the cross-linked randomly coiled ovalbumin and lysozyme with an increase in temperature. The reduced viscosity of lysozyme decreased rather slowly.

TABLE II: Effect of Temperature on the Intrinsic Viscosity of Proteins, with Disulfide Bonds Broken, in 6 M Gdn·HCl.

Protein	Intrinsic Viscosity (cm^3/g) at					
	30°	35°	40°	45°	50°	55°
Ribonuclease	10.2	9.1	9.8	7.2	5.5	5.0
Lysozyme	11.2	9.9	10.8	6.9	5.0	4.8
Myoglobin	12.0	10.7	11.2	8.7	8.9	8.5
Papain	17.4	14.8	16.2	12.9	12.0	10.5
Chymotrypsinogen	25.6	17.5	19.0	15.9	13.8	12.0
Ovalbumin	27.4	25.2	27.5	23.4	22.1	19.9
Pepsinogen	26.2	20.8	24.7	19.5	17.0	14.1
Bovine serum ovalbumin	48.5	44.3	47.9	38.0	35.0	33.0

Discussion

The intrinsic viscosity results, summarized in Table I, amply demonstrated that the reduced proteins under study lose all the elements of their native conformation in 6 M Gdn·HCl and behave as linear random coils. Thus, the molecular weight dependence of their intrinsic viscosities described by eq 3, Huggins constants, and end-to-end distances are all consistent with the random coil behavior. The unperturbed dimensions computed from viscosity data alone, using the Stockmayer and Fixman (1963) procedure, fit eq 7 which is identical with the equation obtained by Tanford and coworkers (1968) using a similar procedure, and is comparable to the equation put forward by Lapanje and Tanford (1967) after correcting for the thermodynamic nonideality of the reduced proteins in 6 M Gdn·HCl. Furthermore, whereas the unperturbed dimensions reported here are smaller than those expected from the equation $\langle L^2\rangle_0 = 90n$, which was obtained (Miller *et al.*, 1967) from theoretical considerations for polypeptide chains containing 10% glycine (the glycine content of the proteins of Table I is 9% or lower), these are much larger than those expected on the basis of completely free rotations (Flory, 1960) around single bonds, *i.e.*, angles ϕ and ψ. The discrepancy between our results and those expected from the theoretical considerations of Flory and coworkers is, however, not serious. First, the values of the parameters used by Flory and coworkers in the theoretical calculations of $\langle L^2\rangle_0$ are not free from uncertainty. Second, precise theoretical estimates of the influence of possible contraction (due to the presence of proline) or expansion (due to valine, isoleucine, and threonine) of the randomly coiled polypeptide chain on the unperturbed dimension are not available.

Effect of Temperature. Ideally, backbone rotations, *i.e.*, ϕ and ψ, in a randomly coiled polypeptide chain should occur with the same ease as they do in simple model peptides. These rotations are, however, never entirely free and are severely restricted by short-range nonbonded interactions operating within the random coil. The net effect of restriction of the rotations is to expand the random coil, while facilitation of rotations is expected (Miller *et al.*, 1967; Flory, 1960; Brant and Flory, 1965; Brant *et al.*, 1967) to have an opposite effect on the dimension. The increase in temperature would facilitate backbone rotations which in turn may cause a decrease in the dimension and hence in the intrinsic viscosities of the randomly coiled proteins. As a result of appreciable conformational constraint imposed by intrachain disulfide bonds in cross-linked randomly coiled proteins, the intrinsic viscosities of the latter, besides being considerably lower than those of linear random coils, are expected to show smaller temperature dependence. Our results are in general agreement with these contentions. Among the proteins studied, the contraction of the linear random coil observed on increasing the temperature from 25 to 35° was most pronounced for ribonuclease and least for bovine serum albumin which suggests that the extent to which backbone rotations, ϕ and ψ, could be facilitated, thereby shortening the chain by increasing temperature, was maximum for ribonuclease and minimum for bovine serum albumin. This behavior could not be attributed

to the difference in the amino acid composition of the proteins, particularly to the relative amounts of glycine, proline, valine, isoleucine, and threonine in the two proteins. Furthermore, following the Stockmayer and Fixman (1963) procedure the values of intrinsic viscosity under θ condition, *i.e.*, $[\eta]_\theta$ for randomly coiled proteins, were calculated at different temperatures. To our surprise, $[\eta]_\theta$ was found to be dependent on temperature.

As expected, the temperature-induced decrease in the dimension, and hence in the viscosity, was found to be less marked for cross-linked randomly coiled proteins, *i.e.*, ovalbumin and lysozyme in 6 M Gdn·HCl (Figure 1). Thus, the decrease in intrinsic viscosity on heating from 25 to 35° was about 19% for linear randomly coiled ovalbumin as against an 8% decrease for the cross-linked ovalbumin. The decrease in the reduced viscosity of lysozyme in 6 M Gdn·HCl on heating appears to be even lower. It should be noted that O'Malley and Weaver (1972) recently observed a relatively lower decrease (about 10%) in the intrinsic viscosity of glucose oxidase from *Aspergillus niger* in 6 M Gdn·HCl–0.1 M HSEtOH on raising the temperature from 25 to 50°. The enzyme containing about 16% carbohydrate appears to behave as a branched chain random coil whose radius of gyration and intrinsic viscosity would be lower than that expected from its chain length according to eq 3. Thus, the branched chain randomly coiled glucose oxidase behaves more as a cross-linked random coil than as a linear randomly coiled polypeptide chain.

The intrinsic viscosity of randomly coiled proteins invariably decreased up to 35° and increased at 40° and finally decreased on heating beyond 40°. It seems that, on raising temperature, the backbone rotations in the randomly coiled proteins are facilitated up to 35° causing contraction of the random coil. However, at 40° the nonbonded repulsive forces tend to expand the coil. Further increases in temperature from 40 to 55° facilitate rotations to remove these repulsive forces and consequently the dimension decreased monotonically. The presence of a minimum at 35° and a hump at 40° in the intrinsic viscosity–temperature profile of the random coil proteins is the most interesting feature of this study. This feature seems to be independent of amino acid composition and the length of the polypeptide chain. As can be seen in Figure 1, these are not shared by cross-linked randomly coiled proteins. Preliminary results from this laboratory (A. Waheed, F. Ahmad, and A. Salahuddin, unpublished results) showed that ovomucoid which contains about 25% carbohydrate (Melamed, 1966) behaves as a branched chain random coil both in 6 M Gdn·HCl–HSEtOH and in 9 M urea–HSEtOH, and the intrinsic viscosity–temperature profile lacks the minimum at 35° and the hump at 40°. That the latter were indeed characteristic features of the randomly coiled proteins and were not due to any solvent effect exerted specifically by 6 M Gdn·HCl is evident from Figure 1, where it can be clearly seen that the curve for randomly coiled ovalbumin in 9 M urea is qualitatively identical with that obtained in 6 M Gdn·HCl.

If the viscosity data for sulfhydryl-reduced proteins in 6 M Gdn·HCl are analyzed at each temperature according to eq 5, a curious result is obtained. Below 35° and above 45° the data behave as expected and give meaningful K values. But in the anomalous temperature region, *i.e.*, where the intrinsic viscosity goes through a minimum and a maximum for each protein, the data extrapolate to give negative K values. This, of course, means that the theory of linear random coils is not applicable to the denatured proteins in this region. This cannot be due to aggregation and an erroneous extrapolation of the viscosity data as the Huggins constant is normal, and not that observed for aggregating systems (Priel and Silberberg, 1970). This strongly suggests that some type of intramolecular interaction, perhaps hydrophobic, must be significant in this temperature region.

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