Studies of Completely Deuteriated Proteins.* II. Thermal Denaturation in D_2O

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Thermal denaturation studies of a fully deuteriated protein and its hydrogen analog in buffered D_2O demonstrate that the deuterio protein always denatures at a lower temperature than its hydrogen analog. Cross comparisons of thermal denaturation in D_2O and H_2O do not give a simple easily interpreted picture. All results are, however, consistent with the suggestion that internal rotation is an important factor in the determination of protein structure.

In previous publications (Berns *et al.*, 1963; Berns, 1963) it has been demonstrated that the algal chromoprotein phycocyanin, isolated from *Plectonema calothricoides* grown in 99.8% D_2O, is quite likely identical in primary, secondary, and tertiary structure with phycocyanin isolated from this alga grown in water. The thermal denaturation of the deuterio- and protio-proteins[1] in water was followed by fluorescence (Berns *et al.*, 1963). The interesting fact uncovered in these studies was that independent of the *p*H and ionic strengths used, the deuterio-protein always denatured at a lower temperature than the protio-protein. Further studies were undertaken to investigate whether the thermal denaturation behavior of the deuterio- and hydrogen protein would be similar in D_2O solutions. These studies were also of interest since the work of Hermans and Scheraga (1959) and of Maybury and Katz (1956) indicate an increase in the configurational stability of some proteins in D_2O over that found in H_2O.

EXPERIMENTAL

The thermal denaturation of the deuterio- and hydrogen proteins was investigated by examining the fluorescent intensity with a Brice-Phoenix light-scattering photometer adapted for this purpose (Berns *et al.*, 1963). The temperature profiles of fluorescence were obtained in a manner analogous to that previously reported (Berns *et al.*, 1963).

The deuterio- and protio-phycocyanins were isolated and purified as previously described (Berns *et al.*, 1963). The denaturation experiments were carried out in 0.1 μ potassium phosphate buffer of the same composition as that used in the H_2O thermal denaturation experiments. The protein-buffered solution was made up to 15.00 ml in H_2O and lyophilized. The lyophilized material was taken up in several milliliters of D_2O, allowed to stand overnight at 4°, and lyophilized once again. This lyophilized material was then taken up in 15.00 ml of D_2O. This procedure was used for the deuterio-protein and protio-protein. The same procedure was followed with a sample of deuterio- and protio-protein except that H_2O was added to the lyophilized protein. The temperature-fluorescence profiles of these control protein solutions measured before and after lyophilization were identical. The observed *p*D of the phosphate-buffered D_2O-protein solutions measured on a Radiometer TTT 1a *p*H meter was 6.90.[2]

Sodium acetate buffer, $\mu = 0.1$, of identical composition to that used in the thermal denaturation studies in H_2O was prepared in D_2O with anhydrous reagent grade sodium acetate (J. T. Baker), and Fisher reagent grade glacial acetic acid. The solution was still greater than 99.6 mole % D_2O. The D_2O used throughout these studies was 99.8 mole % obtained from Bio-Rad Laboratories, Richmond, California. One ml of deuterio- and protio-protein solution was dialyzed into about 20 ml of D_2O-acetate-buffered solutions for 3 days at 4° with at least four changes of dialysate so that the final protein solution was greater than 99.6 mole % in D_2O. This solution was diluted with D_2O-acetate buffer before use. The deuterio and protio solutions in D_2O-acetate buffer were prepared simultaneously from the same buffer solution. The measured *p*D of the protein solutions was 4.85.

RESULTS

The behavior of the deuterio and protio solutions in the D_2O-buffered solutions was quite similar to that observed in the H_2O-buffered solutions. In the phosphate buffer, the thermal denaturation was accompanied by an irreversible loss in fluorescence and a change in absorption spectrum analogous to that observed in the H_2O thermal denaturation (Berns *et al.*, 1963). There was very little if any observable turbidity. In the acetate-buffered solutions, the thermal denaturation was characterized by the colloidal appearance of the solutions within a degree or two of the transition temperature with the major portion of the protein eventually precipitating as higher temperatures were reached. The supernatant solution in these cases was quite similar to the phosphate solutions in attenuation of fluorescence and alteration of absorption spectrum.

The results of the thermal denaturation experiments are shown in Figures 1 and 2. In each of the buffers used the deuterio-protein denatures at a lower temperature than the protio-protein. In acetate the deuterio transition begins at about 50° with a mid-point at approximately 54°, and the protio transition at about 55° with the mid-point at about 60°. In phosphate buffer the deuterio transition begins at

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[1] The term "deuterio-protein" refers to the protein with deuterium substituted for hydrogen in all normally non-exchangeable positions. "Protio-protein" is the normal hydrogen-containing protein.

[2] The *p*D of all buffers was measured on the Radiometer TTT 1a using the normal H_2O Beckman standard buffers at *p*H 4.00 and 7.00 to calibrate the *p*H meter. Therefore in order to arrive at the "true" *p*D one must add 0.4 to the observed *p*D (Glasoe and Long, 1960; Mikkelson and Nielson, 1960).

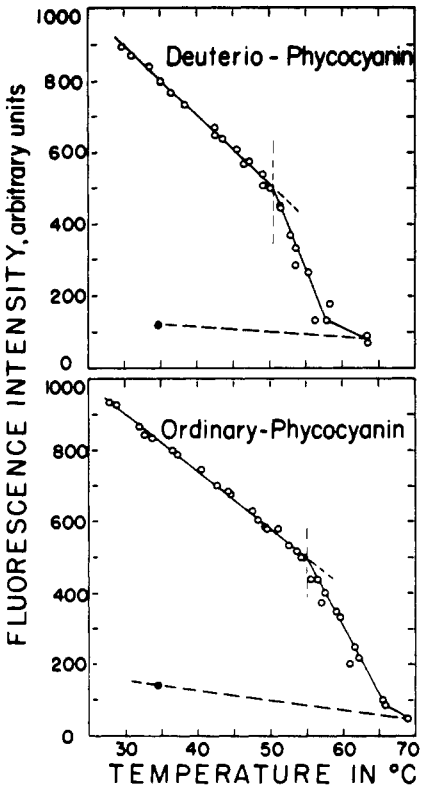

FIG. 1.—Thermal denaturation of phycocyanins in D_2O-acetate buffer: ● descending temperature.

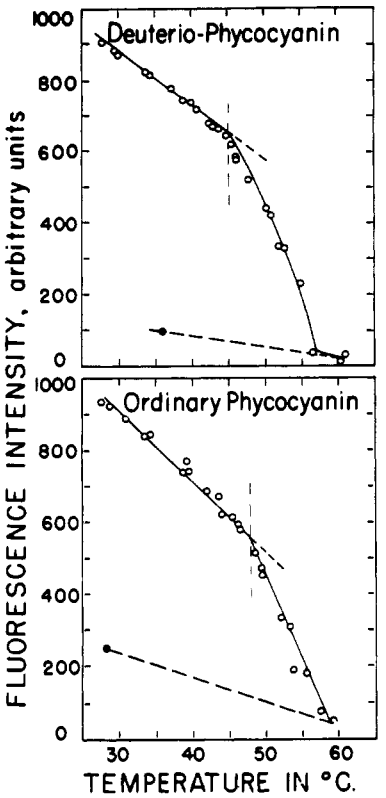

FIG. 2.—Thermal denaturation of phycocyanins in D_2O-phosphate buffer: ● descending temperature.

approximately 45° with the mid-point at about 51°, and the protio transition at about 48° with the mid-point close to 54°.

DISCUSSION

The observation that the deuterio-protein in D_2O-buffered solutions denatures at a lower temperature than its protio analog in the same buffer is in agreement with the behavior reported for these proteins in H_2O buffered solutions. To have a true and simple comparison of the behavior of deuterio- and protio-proteins, it is best to examine and compare them in the same solvent, namely, both in D_2O or both in H_2O, since there is a significant difference in physical properties of D_2O and H_2O (Kirshenbaum, 1951). The cross comparison of the deuterio-protein in D_2O and H_2O (Table I) is, however, instructive from the viewpoint of past experiments.

It is observed here that the deuterio-protein in acetate buffer denatures in H_2O at 44° and in D_2O at about 50°. The protio-phycocyanin in acetate in H_2O denatures at 48° and in D_2O at 55°. Both results would be in essential agreement with those of Hermans and Scheraga (1959), with ribonuclease in D_2O and H_2O, and of Maybury and Katz (1956) with ovalbumin. These investigators found that in D_2O apparently the configurational stability is greater than that in H_2O. If the results are examined in phosphate buffer, essentially the reverse is found. The protio-protein in phosphate in D_2O denatures at about 48° and in H_2O at about 51°. The deuterio-protein in phosphate in D_2O denatures at 45° and in H_2O also at about 45°. Since there is at least one other change accompanying the loss in fluorescence, namely, a large attenuation of the absorption at 620 mμ, it would seem reasonable to assume that a dramatic structural change is occurring, although admittedly not necessarily the same sort as occurs in ribonuclease or ovalbumin.

In a recent study, Lavorel and Moniot (1962) reported the effect of temperature on the absorption spectrum and fluorescence of C-phycocyanin isolated from *Aphanocapsa thermalis*. Their results are in essential agreement with the observations of the present investigator. A linear reversible decrease in fluorescence is observed with increasing temperature and an eventual irreversible change in fluorescence occurs with a subsequent change in slope of the fluorescence attenuation versus temperature curve at higher temperatures. An irreversible loss of 615 mμ absorption also accompanies the irreversible change. Lavorel and Moniot (1962) assign the irreversible change to thermal denaturation of the protein, although the observed properties are clearly identified with the chromophore, a phycobilin, which is an open tetrapyrrole ring (Ó hEocha, 1962). The chromophore is a sensitive indicator of the changes that may occur on thermal denaturation of the protein. The covalent chromophore

TABLE I

TEMPERATURES AT WHICH DENATURATION IS INITIALLY NOTED

Phycocyanin	Buffer System	Temperature
Deuterio-	Acetate-H_2O	44°[a]
Protio-		48°[a]
Deuterio-	Acetate-D_2O	50°[b]
Protio-		55°[b]
Deuterio-	Phosphate-H_2O	45°[a]
Protio-		51°[a]
Deuterio-	Phosphate-D_2O	45°[b]
Protio-		48°[b]

[a] Berns *et al.* (1963). [b] This work.

linkage and labile hydrogen bonds are thought of as forcing the chromophore to take a rigid coplanar configuration and perturbation of this structure is indicative of changes in protein structure and is observed by changes in fluorescence and absorption. Ó hEocha and Ó Carra (1961) reported that concentrated urea treatment of phycoerythrin (a related biliprotein) exhibited an attenuation and shift of absorption and loss of fluorescence which closely approximate the results of thermal denaturation. Recent unreported similar studies in our laboratory with phycocyanin indicate analogous results.

While it is not possible without additional studies to conclude specifically what structural changes are occurring in the phycocyanin and in this fashion draw a direct analogy with the ovalbumin and ribonuclease work, it is fairly certain from the results reported here that the relative configurational stability of a protein in D_2O over that in H_2O cannot be used simply as an indication of the importance of hydrogen bonding in stabilizing the α helix. It is clear, judging from these data, that these results seem to be too complex to interpret simply in terms of the importance of hydrogen bonding in the α helix. It is also important to note that under comparable conditions the deuterio-protein is always apparently less stable than the protio-protein.

When the behavior of the deuterio- and protio-proteins is considered in H_2O and D_2O separately, it is quite consistent with the proposal that internal rotation is one of the determining factors in protein structure.[3] In H_2O and D_2O in the same buffer systems, the deuterio-protein always denatures at a lower temperature than the protio-protein, an observation that would be difficult to explain without considering a factor other than hydrogen bonding which also contributes to the determination of protein configuration.

It is necessary to consider the possibility of differences in the ionization state of the protein induced by the presence of deuterium at nonexchangeable positions. In a previous study (Berns *et al.*, 1963), whether ionic equilibria are significantly different for deuterio- and protio–amino acids and peptides was studied by ion-exchange chromatography of mixtures of deuterio- and protio-glycine and deuterio- and protio-phycocyanin hydrolysates. Within the same solvent system it was concluded that the ionic equilibria are quite probably the same for protio- and deuterio–amino acids as far as ion-exchange behavior is concerned. Certainly the ionization behavior from D_2O to H_2O differs; however there will be no attempt at this time to discuss in detail the observed differences in D_2O and H_2O, but instead only the behavior within a single solvent in a specific buffer.

In the light of the previously mentioned experimental evidence (Berns, 1963; Berns *et al.*, 1963), it appears that there is no difference in primary, secondary, and tertiary structure of the deuterio- and protio-phycocyanin from *P. calothricoides*. In aqueous solution experimental evidence has indicated (Berns *et al.*, 1963) that the deuterio-protein has approximately 20% hydrogen, which would be accounted for by exchange of hydrogens bonded to oxygen atoms and amino hydrogens. Therefore, in aqueous solution deuterium is very likely present in the protein only at carbon deuterium positions which normally account for 80% of the hydrogen in proteins. In D_2O solutions the deuterio- and protio-proteins would still differ in isotopic substitution by the presence of deuterium in the nonexchangeable positions on the carbon atoms. The present evidence does not indicate that this difference in thermal denaturation is in any way directly associated with possible differences in hydrogen bonding or hydrophobic areas. There has been, however, some suggestion that stable configurations of polypeptide chains are explicable in terms of hindered rotation about single bonds and the effect of secondary force, from within and without; e.g., intramolecular and intermolecular hydrogen bonds, which tend to compensate for the excess energy arising from the internal rotation potential (Mizushima, 1954). Indeed, quite recently Tanford (1962), in proposing a model that attempts to explain the stability of globular conformation of proteins on the basis of hydrophobic interactions, mentions that at least in the discussion of thermal denaturation of proteins in water, the existence of potential energy barriers to rotation may be a contributing factor to the inability of his model to explain thermal denaturation.

It has been demonstrated by Aranow *et al.* (1958) that in long-chain hydrocarbons the high entropy of fusion may be attributed to the onset at melting of the molecule to undergo hindered rotation about each carbon-to-carbon bond. While this system is not truly analogous to globular proteins, it does seem to have some bearing on the question of what changes occur when thermal transitions take place and lends credibility to the assumption that hindered rotation probably occurs when the macromolecule is in the unfolded conformation.

The observed difference of temperature of thermal denaturation in the case of the deuterio- and hydrogen protein in the same solvent and buffer may be explained in terms of a simple difference in moments of inertia of the C-D vs. the C-H. It should be possible to calculate at least semiquantitatively what sort of temperature difference might be necessary to achieve the same conformational state in the two isotopically different proteins. At this time no attempt will be made to put forth a detailed model. For a protein to undergo thermal denaturation, a certain critical free energy must be reached. It is possible to propose that the hindered rotation contribution must at this point overcome the stabilizing influence of the other structural forces, e.g., hydrogen bonds. This suggestion, however, while being plausible, has no ultimate bearing on the development. The free energy of the macromolecule in the aqueous media may in theory be calculated by an evaluation of the total partition function for the protein molecule. For the liquid phase the Gibbs free energy F and the maximum work function are almost identical, therefore

$$F = -kT \ln Q$$

where Q is the total partition coefficient for the protein. The total partition function can then be separated into its internal and translational contributions. In the present problem we are only concerned with changes involving the internal states and, therefore, need only consider $Q_{int.}$. Furthermore, the energies involved would not permit excitation to higher electronic or vibrational levels. This leaves the rotational partition function as the major factor to consider in explaining the observed difference in thermal denaturation. The difference in temperature at which the thermal denaturation is observed in the two isotopic species may then be directly related to energy level differences in the rotational partition coefficient. In the deuterio-protein species the rotational energy levels will be more closely spaced than in the hydrogen protein and the heat capac-

[3] A semiquantitative development of the theoretical difference in rotational energy levels in the deuterio- and protio-protein and consequent heat capacity difference that would account for the observed thermal denaturation results will be presented in a subsequent publication.

ity for the deuterio-protein will be correspondingly larger. This will permit hindered rotation to take place at a lower temperature in the deuterio-protein than in the hydrogen protein, thus a lower denaturation temperature in deuterio-protein than hydrogen protein. To substantiate this it would be best to attempt a calculation of the temperature difference that would be expected as a result of the differences in moment of inertia.

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ADDED IN PROOF

It should be noted that although the thermal denaturation as observed in these studies is irreversible, Lavorel and Moniot (1962) have observed that with extremely fast heating and cooling the process is apparently reversible. The transition temperature is taken as the onset of the denaturation. Therefore, it is suggested that at the transition temperature we have a reversible process. This would allow the data to be treated by conventional thermodynamic analysis.

REFERENCES

Aranow, R. H., Witten, L., and Andrews, D. H. (1958), *J. Phys. Chem. 62*, 812.
Berns, D. S. (1963), *J. Am. Chem. Soc. 85*, 1676.
Berns, D. S., Crespi, H. L., and Katz, J. J. (1963), *J. Am. Chem. Soc. 85*, 8.
Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.
Hermans, J., and Scheraga, H. A. (1959), *Biochim. Biophys. Acta 36*, 534.
Kirshenbaum, I. (1951), Physical Properties and Analysis of Heavy Water, New York, McGraw-Hill, p. 1.
Lavorel, J., and Moniot, C. (1962), *J. Chim. Phys. 59*, 1007.
Maybury, R. H., and Katz, J. J. (1956), *Nature 177*, 629.
Mikkelsen, K., and Nielson, S. O. (1960), *J. Phys. Chem. 64*, 632.
Mizushima, S.-I. (1954), *Advan. Protein Chem. 9*, 299.
Ó hEocha, C. (1962) *in* Physiology and Biochemistry of Algae, Lewin, R. A., ed., New York, Academic, p. 421.
Ó hEocha, C., and Ó Carra, P. (1961), *J. Am. Chem. Soc. 83*, 1091.
Tanford, C. (1962), *J. Am. Chem. Soc. 84*, 4240.

The Relationship of Structure to the Thermal Solubility Characteristics of a Bence-Jones Protein*

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A Bence-Jones protein characterized as type A and having a molecular weight of 48,000 was degraded on heating to 100° to a main polypeptide chain and to other unidentified polypeptides of lower molecular weight. Similar degradation products were found after exposure of the Bence-Jones protein to 2 M urea. The main polypeptide chain, isolated by CM-cellulose and DEAE-cellulose chromatography, had a molecular weight of 22,500 and was soluble and stable between 0° and 100°. The peculiar thermal characteristics of the Bence-Jones proteins are explained on the basis that the protein forms an insoluble aggregate between 50° and 70°, which dissolves on heating to 100° owing to deaggregation and degradation to polypeptide chains. On cooling, the various polypeptide chains recombine to form a product resembling the starting material, which may reaggregate at temperatures ranging between 70° and 50°.

Two unusual proteins are elaborated by persons suffering from multiple myeloma. One, the myeloma protein, appears in the serum often in large quantity, and has a molecular weight of approximately 160,000. Although it shares at least one antigenic determinant with the portion of 7 S gamma globulin concerned with antibody function (Olins and Edelman, 1962; Migita and Putnam, 1963), no immunologic activity has as yet been ascribed to it. The other protein occurs mainly in the urine and has a molecular weight of 40,000–60,000. This protein was first described by Henry Bence Jones (1847) and has aroused much curiosity because of its unique thermal characteristics. The Bence-Jones protein precipitates between 45° and 60°, redissolves on heating to 100°, and reprecipitates on subsequent cooling. The nature of the thermal changes have not been explained to date. The relationship of the serum protein to the urinary protein, on the other hand, has been explained recently. As these proteins were found to share polypeptide chains, it has been suggested (Edelman and Gally, 1962) that "Bence-Jones proteins appear to be polypeptide chains . . . that have not been incorporated into myeloma proteins."

One of the difficulties encountered in working with these proteins is the fact that they are not identical in different individuals. As the result of evidence provided by Putnam (1957), it is now commonly accepted that each human with the disease multiple myeloma elaborates individually specific myeloma and Bence-Jones proteins.

Working with a well-characterized Bence-Jones protein from one subject offered an opportunity for intensive study of its structural peculiarities. One of the components of this protein, prepared either by heating to 100° or by urea treatment, was a heat-stable polypeptide chain. At *p*H 5.0 this polypeptide chain remained soluble over a wide temperature range. When associated with lower molecular weight polypeptides also derived from this protein it lost its solubility between 50° and 70°, but not at higher temperatures. This finding suggested that reversible association be-

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