Characterization of the Thermal Denaturation of Bence-Jones Proteins by Ultracentrifugation at Elevated Temperatures*

KENNETH E. NEET† and FRANK W. PUTNAM‡

From the Department of Biochemistry, College of Medicine, University of Florida, Gainesville, Florida 32603

SUMMARY

The unusual thermosolubility properties of Bence-Jones proteins have been investigated by the technique of ultracentrifugation at elevated temperature. Study of several purified Bence-Jones proteins has allowed description of the molecular changes that occur upon heating. Although the proteins participate in a common phenomenon, individual differences in their ultracentrifugal properties at high temperatures were observed. In certain cases an initial polymerization, followed by a depolymerization to an unfolded molecule, occurred as the temperature was raised. Sulfhydryl-binding reagents had little effect on the high temperature dissolution phenomenon is attributed to alterations in the noncovalent bonding and tertiary structure of the Bence-Jones molecule, occurred as the temperature was raised. Sulphydryl-binding reagents had little effect on the high temperature dissolution phenomenon is attributed to alterations in the noncovalent bonding and tertiary structure of the Bence-Jones protein.

The unusual thermal solubility of Bence-Jones proteins, which precipitate upon heating at 50-60° and redissolve at 100°, is both of clinical significance and theoretical interest. This property may be related to the role of light chains in antibody specificity since Bence-Jones proteins are monomeric or dimeric of the light chains of myeloma globulins (1). Previous work has established the optimum conditions for precipitation of Bence-Jones proteins (2), some of the molecular requirements for precipitation (3, 4), and the presence of a thermal transition observable by fluorescence measurements (5, 6). Recently, Gally and Edelman (7) have been able to obtain information about the molecular changes during heating by depolarization of fluorescence measurements.

Studies in this laboratory (8) have essentially excluded changes in covalent bonding as a contributing factor in the thermosolubility properties of Bence-Jones proteins. Therefore, the unique thermal properties of Bence-Jones proteins have been attributed to alterations in the tertiary and quaternary structure of the molecules. The present communication describes the investigation of these molecular changes by the method of ultracentrifugation at high temperatures. This method has previously been shown to be feasible for the study of aggregation reactions during protein denaturation (9).

MATERIALS AND METHODS

The purification and characterization of most of the 15 individual Bence-Jones proteins used in this investigation have been described (2, 3). All of the purified proteins appeared to be immunologically homogeneous, and most moved as a single band during starch gel electrophoresis in urea containing mercaptoethanol.

Ultracentrifugation experiments were performed over a range of temperatures from 20° to 100° in a Spinco model E-HT analytical ultracentrifuge equipped with schlieren phase plate optics. The model E-HT high temperature system enabled the temperature to be regulated within ±0.1° up to 100°. Sedimentation velocity experiments and calculations were performed as previously described (9). The Trautman modification (10, 11) of the Archibald approach to equilibrium method (12) was chosen as most feasible for molecular weight determination at high temperature.

It was assumed that the viscosity increment and density increment for the various salts did not vary with temperature. The solvent viscosity and density corrections become quite large at the highest temperatures used; therefore, the accuracy of the computed values is lowered. The partial specific volume, β, was assumed to be 0.73 at all of the temperatures for Bence-Jones proteins. Such assumptions appear to be justified at least for

* Supported by Research Grant CA-0983 of the National Cancer Institute, National Institutes of Health, United States Public Health Service. Taken from a dissertation presented to the Graduate School of the University of Florida by K. E. Neet in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† National Science Foundation Cooperative Graduate Fellow, 1964-1965. Present address, Department of Biochemistry, University of California, Berkeley, California.
‡ Present address, Division of Biological Sciences, Indiana University, Bloomington, Indiana. Reprint requests should be sent to this address.

1 Donations of additional samples from Dr. E. Osserman, Frances Delafield Hospital, Columbia University, New York; Dr. R. L. Engle, Jr., New York Hospital, New York; and Dr. B. Jirgensons, M. D. Anderson Hospital, Houston, are gratefully acknowledged.
2 If β were not constant as assumed, it would be reflected in a change in the calculated 20,°. Thus, an erroneous interpretation might occur, but the fact of some molecular transition at the temperature of study should remain valid. Such inconsistency in interpretation does not occur for the formation of polymers since the increased 20,° of the polymer would be generally much larger than could be accounted for by a change in β.
serum albumin (9). Actual measurement of the viscosity, density, or partial specific volume is impractical over the range of temperatures studied (20–100°C), thus necessitating these approximations.

RESULTS AND DISCUSSION

Variation in Thermal Properties of Bence-Jones Proteins—Marked individual differences were found in the ultracentrifugal properties at high temperatures of the various Bence-Jones proteins studied, even though all of the proteins gave the standard heat test. Variations in solubility, size of polymers formed, pH dependence, and critical temperature were observed despite the fact that all of the proteins participated in a common phenomenon. However, no correlation has been found between these thermal properties and other chemical and physicochemical characteristics such as amino acid composition, electrophoretic mobility, amount of dissociable dimer or polymer, and amino-terminal residues.

When heated at neutral pH, 12 of 15 Bence-Jones proteins precipitated. Ultracentrifugal analysis suggested the appearance of a more slowly sedimenting component at the temperature of precipitation. Although the validity of such an observation is difficult to assess, the result is compatible with the thermal transition suggested by fluorescence (5–7) and ultraviolet absorbance measurements.

One Bence-Jones protein, Hal (antigenic type K), formed large, unstable polymers (30 to 50 S) when heated above 48°C. The sedimentation coefficient of the polymer peak appeared to decrease as 100°C was approached.

The polymerization and depolymerization of Bence-Jones proteins on heating differs, of course, from the monomer-dimer equilibrium at room temperature (3), where different Bence-Jones proteins may exist as the monomer (mol wt about 22,500), the dissociable dimer, or the stable dimer.

Soluble Polymers of Bence-Jones Protein Oh—One Bence-Jones protein, Oh (antigenic type L), formed relatively stable, soluble polymers upon heating. This behavior was studied in detail in order to obtain information about the molecular changes that occur at high temperatures. When Oh was heated at pH 6.8, polymers formed. The sedimentation patterns of Fig. 1 show this effect at several temperatures between 51°C and 90°C. At 50°C a more rapidly sedimenting peak appears in addition to the slow peak originally present. As the temperature of analysis is increased to 65°C, more polymer is present, as is also true in the case of serum albumin (9). However, as the temperature is raised further, the relative concentration of the polymeric fraction of Oh decreases until at 90°C the protein boundary has reverted to a single, slowly sedimenting peak (Fig. 1). The sequence of photographs in Fig. 1 appears to depict quantitatively the changes implicit in the well known thermosolubility behavior of Bence-Jones proteins; that is, the polymerization and depolymerization phenomenon observed in the ultracentrifuge is completely analogous to the precipitation and redissolution phenomenon observed visually. Study of the behavior of the Oh monomer-polymer system should thus lead to a better understanding of the mechanism of the thermal dissolution of Bence-Jones proteins.

Analysis of the schlieren patterns of Fig. 1 yielded the sedimentation coefficients and relative polymer concentrations shown in Fig. 2. Values are given for results obtained after heating for 20 min or for 2 hours. From the curve showing the relative percentage of polymer, it may be seen that the maximum amount of polymer is formed between 65°C and 70°C, as was observed qualitatively in Fig. 1. The sedimentation coefficient of the fast peak tends to increase and decrease in concert with the polymer concentration, but no exact correlation between the size of $s_{20,w}$ and the amount of polymer could be made. The temperature also directly affects the $s_{20,w}$ of the polymer as evidenced by the small dip in the curve at 71°C. Furthermore, the slow change over 2 hours (circles versus squares) indicates that a rapid equilibration is not occurring; in such a case, the degree of polymerization would not be expected to be completely determined by the concentration of polymer.

The number of dimeric units of 3.5 S and 45,000 molecular weight that comprise the polymer may be estimated. Four to six such units of $s_{20,w}$ equal to 3.5 S could account for a polymer of 8 to 11 S. The $s_{20,w}$ value is much smaller than that of other Bence-Jones proteins.

All of the Bence-Jones proteins tested gave the proper heat test at pH 5, the pH specified (2, 3). Even at pH 7.5 the phenomenon of precipitation and dissolution upon heating was often present, although, in general, the critical temperatures for precipitation and dissolution were elevated. These observations validate study of the thermosolubility properties at neutral pH.

K. E. Neet, unpublished observations.
Bence-Jones proteins or even that of albumin; this probably accounts in part for its relative solubility.

At temperatures greater than those at which polymers are first observed, changes are also apparent in the sedimentation coefficient of the slow peak. A gradual decrease in $s_{20,w}$ of the slowly sedimenting peak commences around 65° (Fig. 2) and continues up to 100° where the $s_{20,w}$ levels off at about 2.5 S. The lowering of the $s_{20,w}$ of the slow peak occurs as the relative concentration of the polymer decreases, suggesting that the transition to 2.5 S material at 100° is responsible for the thermosolubility properties of Bence-Jones proteins. That is, the shift in equilibrium to favor the 2.5 S form at 100° accounts for the depolymerization of the other species present in solution. Characteristics of the protein molecules in the temperature range of 90–100° will be brought out in the discussion of other experiments.

To ascertain the generality of the observation on Oh, another Bence-Jones protein with similar properties (Pa, antigenic type L) was also studied. Although limited in scope, the studies with Pa justified the conclusions about the mechanism of the heat denaturation of Bence-Jones proteins made from the extensive studies with Oh. The main features, polymerization, depolymerization, and $s_{20,w}$ decrease above 90°, were present when Pa was heated, and were similar to those described for Oh. In addition, a Type K Bence-Jones protein, Lo, also polymerized similarly in the same temperature range.

The kinetics of the reactions occurring when Oh was heated at pH 6.8 was investigated with the results presented in Fig. 3. The upper, dotted curves of Fig. 3 show the slow increase in polymer concentration with time at 71° and at 79°. The shape of the curves suggests the usual asymptotic approach to an equilibrium condition which in this case is nearly reached in 5 hours.

The $s_{20,w}$ values of the polymer remain surprisingly constant over the 5-hour period at both temperatures studied even though the amount of polymer has increased 5-fold at 71°. The $s_{20,w}$ of the slow peak decreases gradually over the 5-hour period of heating to values of around 2.5 S at 90°, as well as at 71° and 79°.

The concentration dependence of the $s_{20,w}$ of the heated protein was studied at several temperatures (Fig. 4). The $s_{20,w}$ of the fast peak of both Oh and Pa increased as the initial concentration of protein was increased. At the same time, the relative proportion of polymer increased linearly (4 to 5% polymer per mg per ml, data not shown). Both facts are indicative of an interacting system in which monomers (or stable dimers) are associating to form polymers. The kinetic data suggested a
FIG. 4. Concentration dependence of the sedimentation coefficients of Bence-Jones proteins Oh and Pa (BJP-Oh and BJP-Pa).

Solid lines and open circles: Oh at 71°C and pH 6.8 heated for 2 hours. Dotted lines and filled circles: Pa at 70°C and pH 7.5. Dashed line and filled squares: Oh at 91.5°C and pH 7.5.

The pH range for the formation of soluble polymers of Oh is quite narrow. At pH 6.5 precipitation occurred, polymers were observed at pH 6.8 (Fig. 1), and at pH 7.5 essentially no aggregation was observable. At the latter pH neither precipitation nor polymerization occurred; instead, there was a slow decrease in $s_{20,w}$ to values around 2.5 S above 90°C in a manner similar to the transition seen at pH 0.8 (Fig. 2).

Molecular Weight of Bence-Jones Proteins at High Temperatures—Transition to a molecular species possessing a lowered $s_{20,w}$ appears to be a general mechanism for the thermosolubility of Bence-Jones proteins. Thus, a decreased $s_{20,w}$ above 90°C was observed with Oh at pH 6.8 and pH 7.5, with Hal after heating for 2 hours at pH 6.0, and with Hn at pH 6.0 after precipitation had occurred (Table I).

The alternatives of dissociation into subunits of about 23,000 molecular weight or of unfolding may be considered in order to explain the decrease in $s_{20,w}$. In Fig. 4 the $s_{20,w}$ of Oh at 91.5°C is seen to be nearly invariant as the protein concentration is changed. This suggests that dissociation does not occur. A more direct test of dissociation is the determination of the molecular weights of the Bence-Jones proteins at high temperatures (Table I). Oh has not dissociated at either 89°C or 94°C, and the calculated molecular weights are in good agreement with those determined in guanidine (8). Hal and Hn did dissociate to some extent, which accords with other evidence (8) that both preparations are composed of both dissociable and stable dimers. It appears that a certain fraction of the dimer form will dissociate into subunits at very high temperatures, whereas another fraction is stable in the dimer form. In any event, dissociation into monomeric subunits is not a requirement for the thermosolubility of Bence-Jones proteins although it may occur. The interchain disulfide bonds are seemingly sufficient to maintain the integrity of the 45,000 molecular weight form. The decreased $s_{20,w}$ thus suggests a conformational change in the molecule although a more sensitive method to determine such a change would be desirable.

Several experiments with Oh showed the interconversion of the species present at different temperatures and the facile shift of the equilibrium. When Oh was heated at 70–71°C to form polymer and then ultracentrifuged at 90°C, a single slow peak was observed.

Table I

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Oh</th>
<th>Hal</th>
<th>Hn</th>
<th>Pa</th>
</tr>
</thead>
<tbody>
<tr>
<td>$s_{20,w}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100°C</td>
<td>2.57e</td>
<td>1.70e</td>
<td>2.17h</td>
<td>9.73e</td>
</tr>
<tr>
<td>94°C</td>
<td>2.57e</td>
<td>1.70e</td>
<td>2.17h</td>
<td>9.73e</td>
</tr>
<tr>
<td>89°C</td>
<td>3.42e</td>
<td>3.40e</td>
<td>3.72e</td>
<td>3.45e</td>
</tr>
<tr>
<td>20°C</td>
<td>3.42e</td>
<td>3.40e</td>
<td>3.72e</td>
<td>3.45e</td>
</tr>
<tr>
<td>Molecular weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100°C</td>
<td>42,100y</td>
<td>31,000y</td>
<td>25,400y</td>
<td>24,400y</td>
</tr>
<tr>
<td>94°C</td>
<td>39,300y</td>
<td>25,400y</td>
<td>24,400y</td>
<td>24,400y</td>
</tr>
<tr>
<td>89°C</td>
<td>39,300y</td>
<td>25,400y</td>
<td>24,400y</td>
<td>24,400y</td>
</tr>
<tr>
<td>20°C</td>
<td>47,500y</td>
<td>25,400y</td>
<td>24,400y</td>
<td>24,400y</td>
</tr>
</tbody>
</table>

In dissociating solvents

<table>
<thead>
<tr>
<th>20°C</th>
<th>Oh</th>
<th>Hal</th>
<th>Hn</th>
<th>Pa</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.12e</td>
<td>1.57f</td>
<td>2.22f</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Sodium phosphate, 0.05 ionic strength, pH 8.0, 2 hours at 100°C.
* Sodium phosphate, 0.1 ionic strength, pH 6.0.
* Sodium phosphate, 0.1 ionic strength, pH 7.5.
* Sodium phosphate, 0.05 ionic strength, plus 0.05 M NaCl plus 2 M urea.
* Guanidine hydrochloride, 6 M.
/ Urea, 6 M.
observed with an $s_{20, w}$ similar to that obtained by heating directly at 90°. Thus, the polymer may form at one temperature and then depolymerize at a higher temperature. Polymer also formed on cooling to 70° after heating the solution at 100°. Indeed, when a solution of Oh was heated at either 100° or at 50° and then brought to 70°, the protein polymerized to the same degree and with similar kinetics. However, the polymerization reaction was shown to be irreversible upon cooling from 70° to 20° or even only to 50°. Thus, at least one reaction leading to polymers is irreversible with the obvious choice being the polymerization step itself.

Forces Involved in Thermal Interactions of Bence-Jones Proteins—Bence-Jones proteins were heated in the presence of various reagents to study the forces involved in the thermally induced reactions. Ethanol, which is considered to be a probe for hydrophobic interactions, lowered both the initial temperature of precipitation and the temperature of redissolution by 20° or more. This suggests that hydrophobic forces contribute to the stabilization of the tertiary structure of Bence-Jones proteins. When these forces are disrupted, aggregation occurs more readily. Support for this conclusion is also found in the dependence of the $s_{20, w}$ of Oh on ionic strength at 71°. The polymer size increases as the ionic strength is raised, and at high salt concentrations precipitation occurs. Increasing ionic strength reduces the electrokinetic potential between charged proteins and probably favors the “burying” of hydrophobic regions inside the aggregate.

Urea is known to have strong solubilizing effects on proteins; for example, it inhibits the heat precipitation of Bence-Jones proteins (2) and lowers their transition temperature (6). Concentrations of urea above 1.5 M prevented the thermal precipitation of Bence-Jones protein Hn, thus allowing ultracentrifugal analysis above 90°. As might be expected, the $s_{20, w}$ decreased as the urea concentration was increased at a given temperature.

Dodecyl sulfate, which is known to protect proteins against heat denaturation, completely inhibited the thermal precipitation of Bence-Jones proteins at pH 7.5. The effect of dodecyl sulfate on the sedimentation behavior was quite complex, difficult to interpret, and dependent on the concentration of the detergent. A comparatively large molar ratio of dodecyl sulfate was required (20 moles of dodecyl sulfate per 22,000 molecular weight unit). Interacting systems were produced at lower molar ratios of the detergent. In contrast, cetylpyridinium chloride, a cationic detergent, decreased the temperature of initial precipitation of Bence-Jones proteins. Thus, the charge on the protein plays an important role in the polymerization process; the same conclusion was reached from ultracentrifugal study of the pH dependence of the aggregation process.

Studies with specific blocking reagents have indicated that free sulfhydryl groups are not required for the thermal interactions of Bence-Jones proteins. Although Bence-Jones proteins do not have reactive sulfhydryl groups at room temperature (8), sulfhydryl groups might be formed by cleavage of disulfide bonds at high temperature. Hence, the effect of p-chloromercuribenzoate and iodoacetamide on the sedimentation of various Bence-Jones proteins was studied at several temperatures. Only slight changes occurred in the degree of polymerization of Hal or Pa at 61–70° in the presence of these sulfhydryl-binding reagents. No polymer peak was formed by Pa or Oh at 90–94° in either the presence or absence of p-chloromercuribenzoate; in fact, at these temperatures, the $s_{20, w}$ of both proteins decreased significantly in the presence of 2.27 × 10⁻³ m p-chloromercuribenzoate. Although this suggests the cleavage of disulfide bonds at 90–100° with binding of the liberated —SH groups by p-chloromercuribenzoate, free sulfhydryl groups were not detected by quantitative titration after cooling of solutions of these proteins after heating in the absence of p-chloromercuribenzoate (8).

The participation of disulfide bonds in the thermal reactions of Bence-Jones proteins is difficult to study because of the possibility of temporary cleavage and reformation of such bonds. In one experiment, a completely reduced and alkylated Bence-Jones protein⁷ was tested for thermosolubility. Although the preparation was insoluble at room temperature at pH 5, it dissolved at 100–110° and precipitated upon cooling below 90°. This indicates that intact disulfide bridges are unnecessary for the solubility of Bence-Jones proteins at 100°, and they probably are not needed for precipitation. Thus, the data presented here suggest that disulfide bonds do not participate in the characteristic thermal behavior of Bence-Jones proteins although a mechanism involving the rearrangement of disulfide bridges would be consistent with much of the data. However, if this were to occur, a much lower $s_{20, w}$ and a cleavage of interchain disulfide bonds would be expected; neither of these occurs.

From these experiments it would appear that nonequivalent bonds play an important role in the thermal transitions of Bence-Jones proteins, since ethanol, urea, detergents, and salts all have a profound influence on the reactions at high temperatures. Apolar bonding may be the driving force, but polar bonds probably make a contribution also. Sulfhydryl and disulfide groups appear to be of a lesser importance in the heat denaturation process of Bence-Jones proteins.

In conclusion, the discovery of a Bence Jones protein, Oh, which remains soluble up to 100° at neutral pH has allowed an ultracentrifugal investigation of its heat denaturation. Studies on other, similar Bence-Jones proteins and on less soluble specimens under various conditions have yielded additional data about this process. Polymerization occurs above 50°, followed by depolymerization to a conformationally altered molecule as the temperature approaches 100°. Shifts in the equilibrium between these forms may account for the thermosolubility of Bence-Jones proteins. Changes in nonequivalent bonding and tertiary structure appear to be the important processes.

REFERENCES
3 This sample was kindly prepared by Dr. K. Titani, of this department. Amino acid analysis showed the quantitative reduction and carboxymethylation of all of the cystine or cysteine residues.
4 The evidence for a molecule of Oh protein of different conformation at high temperatures rests on the similarity in molecular weight at 20° and 94° (Table 1) and on the decrease in $s_{20, w}$ from about 3.5 S to about 2.5 S. Since the latter change is small and the correction factors large, it should be emphasized that other data support the conclusion that the difference in $s_{20, w}$ is real; for example, the time dependence of $s_{20, w}$ (Fig. 3) where the correction factors are constant and $s_{20, w}$ goes from about 3.4 S to about 2.6 S with time. Furthermore, in previous experiments on the ultracentrifugation of serum albumin with use of the same assumptions and correction factors, $s_{20, w}$ was constant from 20° to about 68° (8).
Characterization of the Thermal Denaturation of Bence-Jones Proteins by Ultracentrifugation at Elevated Temperatures

Kenneth E. Neet and Frank W. Putnam


Access the most updated version of this article at http://www.jbc.org/content/241/10/2320

Alerts:
  - When this article is cited
  - When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/241/10/2320.full.html#ref-list-1