Transactions of the Materials Research Society of Japan 25 [1] 1-4 (2000)

Multiple Phase Behaviors of Cross-linked Biopolymer Gels

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A multiple phase transition was observed in gels made by covalently cross-linking proteins in either native or denatured state. The observation of multiple phase behaviors of cross-linked proteins and the reversibility of the swelling degree upon phase transition indicate that proteins are at free energy minimum and thus in thermodynamic phases. One of the observed phases may correspond to the native stable state.

Key Words: Protein, Polypeptide, Polymer Gel, Volume Phase Transition, Multiple Phases

1. INTRODUCTION

PROTEIN has a unique and stable structure. To have the structure is essential in creating functions such as the accurate molecular recognition and high efficient catalysis[1]. Extensive studies showed that denaturation and renaturation of proteins are reversible, which indicates that the structure of a natural protein should be determined thermodynamically. The uniqueness and reproducibility of a conformation mean that protein must be at its free energy minimum separated by free energy barriers from other possible conformations. By thermodynamic definition the protein conformation should be a phase. Recent theoretical analyses suggest that protein should have multiple free energy minima in the conformational space[2].

No observation has been made, however, on the existence of multiple phases and phase transition in protein molecules. Circular dichroism, optical rotation, and light scattering studies show that configuration of protein changes continuously in response to the environment changes[3-4]. We report here that gels made by cross-linking denatured or native albumin and polypeptide have multiple phases and undergo discontinuous volume transitions among the phases as pH, temperature, or urea, a denaturant, are varied. Cross-linking allows macroscopic observation of the delicate change in protein volume.

2. EXPERIMENTAL

Gels were made by cross-linking proteins in the denatured or native states. Bovine albumin (Sigma) denatured by 1.0 mol urea aqueous solution (20 wt%) was cross-linked using ethylene glycol diglycidyl ether (20 wt% based on bovine albumin) at 70 °C in glass micropipettes of inner diameter 140 μm. After gelation was completed, the gel was taken out from micropipette and was immersed in a large amount of water to wash away residual chemicals. Water was repeatedly changed until the diameter of the gel reached equilibrium value. The gel was placed in a glass cell whose temperature was controlled within 0.1 °C. It was continuously flushed with aqueous solution whose pH was varied using HCl for pH < 7, and NaOH for pH ≥ 7. In order to minimize the effect of carbon dioxide the experiments were carried out under the nitrogen atmosphere. The gel diameter was measured under a microscope.

The cross-linked bovine albumin (20 wt%) in the native state was prepared using glutardehyde (20 wt% base on bovine albumin) in water at 23°C.

The cross-linking of gelatin (General Foods Co., 10 wt%) was carried out by ethylene glycol diglycidyl ether (20 wt% based on gelatin) at 60 °C[5].

3. RESULTS AND DISCUSSION

In Figure 1-a, equilibrium swelling degree of the albumin gel cross-linked in denatured state is plotted as a function of pH at 23°C. There were six distinct volume phases in the gel at higher pH. Each is denoted by its diameter normalized by the original diameter: $d/d_0 = 0.91$, 1.09, 2.02, 2.77, 3.33, and 5.13. At neutral pH the gel was shrunken (phase 0.91), but not in the most compact

phase. As pH was raised it swelled discontinuously to phase 2.77 at pH 7.05. As pH was lowered from pH 7.05, the gel collapsed to phase 1.09 at pH 6.33 and further reduction of pH caused the gel collapse back to phase 0.91 continuously. If, instead, pH was increased, the gel swelled discontinuously to phase 2.77 again at pH 7.01. If pH was increased from pH 6.33, the gel swelled discontinuously to phase 3.33 at pH 7.92. As pH was lowered from pH 7.92, the gel collapsed into phase 2.02 at pH 6.62 and by further reduction of pH, the gel collapsed into phase 1.09. Instead, if pH was increased from pH 6.62, the gel swelled discontinuously from phase 2.02 to 2.77 (not phase 3.33) at pH 6.99. If, instead, pH was increased to 12.0, the gel diameter changed continuously, and when pH was lowered again, it swelled continuously to phase 5.13. If pH was lowered further, the gel collapsed to phase 0.91 at pH 6.06. This cycle was repeated with excellent reproducibility. Three phases 0.91, 1.66, and 1.68 were found at lower pH.

Another gel was made by cross-linking bovine albumin in the native state by glutardehyde at 23°C. The swelling degree of the gel was determined as a function of pH (Figure 1-b) and urea concentration (Figure 1-c). The gel showed five phases of diameter d/d_0 = 0.93, 1.03, 1.10, 1.17, and 1.38.

Multiple phases have recently been found in copolymer gels consisting of cationic and anionic groups that form inter-polymer hydrogen bonding[6]. As temperature or pH is varied, the gel changes its volume discontinuously among many phases distinguished by different volumes. The number of phases and the transition thresholds depend on the ratio of the cationic and anionic monomers in the polymer network. Each phase can be reached by following a different way of changing pH. Multiple phases have also been observed in a gel made of homogeneous polymers where polymers are interacting through hydrogen bonding and hydrophobic interactions[7]. The sufficient criterion for a polymer system to have multiple phases is that it has a certain combination of at least two of the fundamental attractive interactions of biology, that is, hydrogen bonding, hydrophobic interactions, van der Waals interactions, and electrostatic interactions. Proteins have all these interactions within themselves. Thus one may expect that protein should have multiple phases and one of them may correspond to the native stable state.

Cross-linked gelatin gel was prepared and the swelling curves were determined as a function of pH. Figure 2a shows a swelling behavior of cross-linked gelatin gel obtained as pH was varied at 25 °C. Five phases were observed in this gel. Figure 2b shows the temperature dependence of the swelling curve of Cross-linked gelatin gel in pure water. Three distinct phases were observed in the gel. The gel underwent discontinuous transition between some of the phases confirmed in the pH experiment.

Coexistence of multiple phases is possible if they each correspond to a minimum in free energy. However, the lowest minimum is the stable equilibrium state and the others represent metastable phases. As the environment varies the minima can cross, leading to a discontinuous phase transition. The fact that observed transitions always involve a discrete set of swelling degrees and none of the intermediate values, suggests that these swelling degrees correspond to separate free energy minima. The different stimuli create the different minima crossings, which leads to the discontinuous phase transitions between different phases.

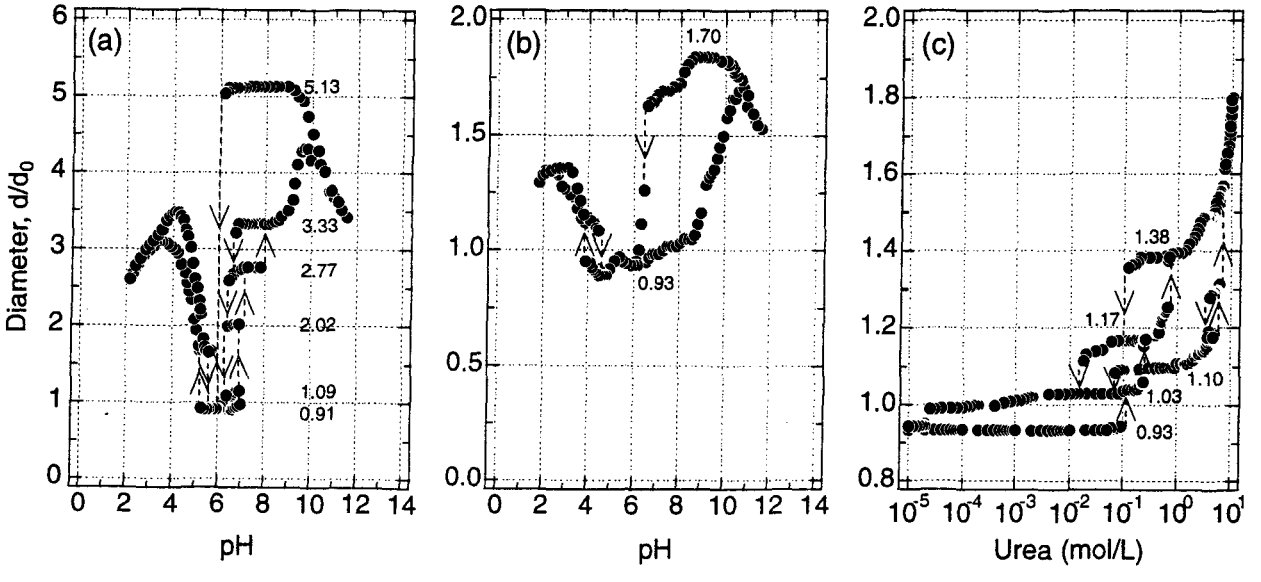

Figure 1. Equilibrium diameter of the gel as a function of pH / urea concentration made by cross-linking denatured albumin denatured by 1.0 M urea (a), and by cross-linking native albumin (b), (c). Numbers in the phase diagrams indicate the equilibrium swelling degrees, d/d_0.

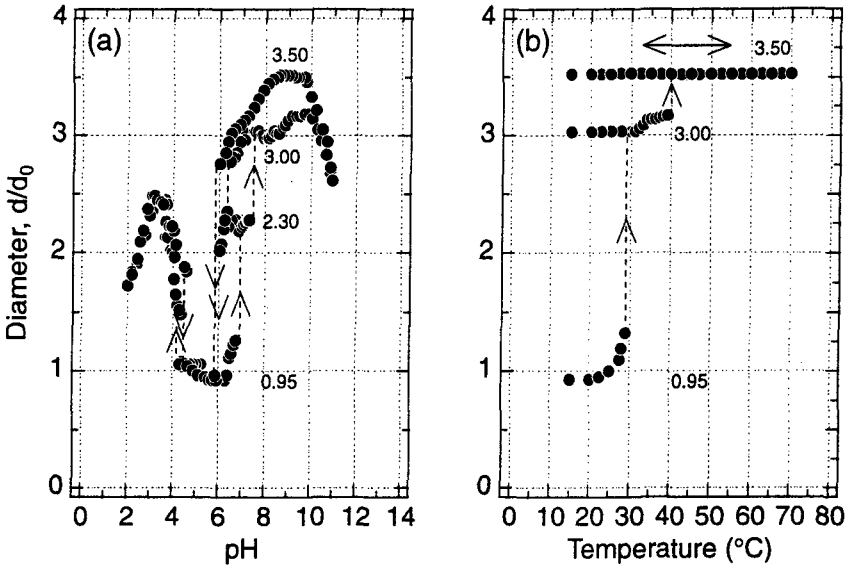

Figure 2. Equilibrium swelling degree, d/d_0, of cross-linked gelatin gel as a function of pH at 25 °C (a) and as a function of temperature in pure water (b). Numbers in the phase diagrams indicate the equilibrium swelling degrees, d/d_0.

Reversibility of denaturation and renaturation of proteins indicates that the structure of a natural protein should be determined thermodynamically. The conditions for a polymer system to have multiple phases is that it has a certain combination of at least two of the fundamental attractive interactions, that is, hydrogen bonding; hydrophobic interactions; van der Waals interactions; and electrostatic interactions[5,6]. Proteins have all these interactions within themselves. Therefor the observation of multiple phase behaviors of cross-linked proteins indicates that proteins are in thermodynamic phases and one of them may correspond to the native stable state. It will be of interest to study the microscopic structure at each phase of these proteins using neutron scattering, NMR, and other techniques.

4. BIOLOGICAL IMPLICATION OF MULTIPLE PHASES OF GELS

Proteins are virtually unique in being linear macromolecules with a nonrepetitive covalent structure and in being able to adopt a relatively fixed three-dimensional structure, or conformation. The covalent structure in determined by the structures of the 20 different amino acids and by the order in which they are linked together, using the generic information, into a polypeptide chain. The conformation of a protein is specified by the rotations about all the single bonds of its covalent structure.

How and why a protein adopts a specific native conformation? Any molecule can adopt different conformations, but it is a special problem with proteins because of the vast number of conformation is possible. With an average of m equally probable conformations per amino acid residue and n residues in the polypeptide chain, total number of possible conformations of the polypeptide chain will be m^n. Not all such conformations will be possible, of course, because atoms would overlap in space in some of them, but the numbers are so large that even if only a small fraction of these conformations are permitted, there will still be a very large number. For example, an average protein contains about 400 residues, and each residue on average might be capable of existing in ten distinctly different conformations, which would suggest that 10^{400} conformation are possible. Even with relatively small protein of only 100 residues, and a very conservative estimate of only two conformations per residue, the number is 10^{30} total possible conformations.

What determines which of these many conformations is adopted by a protein as its "native" conformation? The ultimate determinant of the native conformation is the amino acid sequence, but each amino acid sequence (and there are many more amino acid sequences possible than there are conformations, 20^n for n residues) does not produce a different conformation. What are the rules governing the relationship between amino acid sequence and three-dimensional structure? Given the amino acid sequence, can the native three-dimensional structure be predicted?

The finding of multiple phases of gels may have opened the door to the solutions of these difficulties. It has shown that an ordinary polymer can have a finite number of stable phases, a possible solution to the first problem, and that the necessary condition for a polymer to have such phases is a combination of at least two of the fundamental biological interactions in a certain proportion. It may not be necessary that a polymer has a specific sequence, nor may it have to be a polypeptide. This may provide a solution to the second problem. Recent theoretical works by Shakhnovich and Gutin[7]

clearly showed that majority of copolymers can have unique structure if they have randomness larger than a threshold. Since no unique structure are found in these gels the above mentioned discussion is a speculation.

5. CONCLUSION

A multiple phase transition was observed in gels made by covalently cross-linking proteins in either native or denatured state. The observation of multiple phase behaviors of cross-linked proteins and the reversibility of the swelling degree upon phase transition indicate that proteins are at free energy minimum and thus in thermodynamic phases. One of the observed phases may correspond to the native stable state.

ACKOWLEDGEMENT

This work has been supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture (No.11740382 for MA).

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