Configuration between Re-Formed Collagen Triple Helices and Artificially Introduced Cross-Links in Gelatin Gels

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ABSTRACT: A collagen cross-linked with a pyrene derivative was prepared, and the gel structure of the heat-denatured collagen (gelatin) containing the chemical cross-links was examined using the fluorescence depolarization method. The fluorescence anisotropy of the cross-linked collagen decreased with an increase in temperature, suggesting a helix-to-coil transition. The denaturation temperature of the collagen was determined by the fluorescence anisotropy. The Perrin−Weber equation was applied to the analysis of the denaturation and regeneration of the collagen triple helix. The quantity of the triple helix regenerated from random coils was estimated from the Perrin−Weber plot. The helix content values in the regenerated cross-linked collagen obtained by the fluorescence anisotropy were different from those obtained by the circular dichroism. The models of the gelatin gel containing chemical cross-links were considered from the helix content data obtained from the different methods. Cross-links promoted the regeneration of the collagen triple helix. However, the triple helix was re-formed by avoiding the cross-link at the higher degree of cross-linking.

Introduction

The heat transformation of collagen to gelatin is interpreted as a disintegration of the collagen triple helical structure into random coils. This is accompanied by a change in physical properties such as viscosity, sedimentation, diffusion, light scattering, and optical activity.[1−3] The overall helix-to-coil transition process has been observed by circular dichroism (CD).[4,5]

Gelatin (heat-denatured collagen) chains associate and partly regenerate the triple helix upon cooling. We have demonstrated using CD that the extent of the triple helix regeneration of the heat-denatured collagen increased by the introduction of chemical cross-links into the collagen molecules.[5−7] However, Hermel and co-workers[8] have reported that gelatin chains containing many natural cross-links produced *in vivo* did not regenerate the triple helix at 10 °C for 2 days. Cross-links may inhibit the regeneration of the collagen triple-helical structure. Oikawa and Nakanishi[9] have also reported that the artificial cross-links introduced between gelatin chains depressed the regeneration of the triple-helical segments; they obtained this result using dynamic light scattering. A further investigation at the molecular level would be necessary to explain the difference between our results and others.

Fluorescence depolarization is a powerful tool to detect phase transitions of polymer gels in a microvolume[10−12] and local molecular motions in linear polymers.[13−15] Hence, it is expected that information can be obtained about the microenvironment around the cross-links when a fluorescent cross-linking reagent is used in the experiments. In order to examine the structure of the gelatin gel containing chemical cross-links, a collagen cross-linked with a pyrene derivative was prepared.[7] In the present study, we examined the change in the microenvironment around the cross-links in the fluorescent collagen using the fluorescence depolarization method and detected the helix-to-coil transition of collagen at the molecular level. The gelatin gel structure was also estimated by comparing the results of the fluorescence anisotropy with those of the CD.

Experimental Section

Materials. Solubilized collagen with lime (SCL),[16] which was isolated from steer hide using an alkaline solution, was employed in this study. The cross-linking reagent, 1-acetoxypyrene-3,6,8-trisulfonyl chloride (APTS), was purchased from Lambda. A model compound of APTS, 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS), was obtained from Wako Pure Chemical Industries, Ltd.

Sample Preparation. The detailed procedure for the preparation of the collagen cross-linked with APTS (Py-SCL) was previously described.[7] The quantity of the pyrenyl compounds bound to SCL was determined using a molar extinction coefficient of HPTS ($\epsilon_{460\ nm} = 2.4 \times 10^4$).[17] The degree of cross-linking was expressed as the percentage that is the molar ratio of the binding cross-linker against all amino groups in SCL. Py-SCL solutions were prepared using 0.01 M sodium hydroxide. The Py-SCL concentration for spectroscopic measurements was adjusted to 0.01 wt % based on the microbiuret method.[18] As reference, the SCL solutions mixed with HPTS were prepared using 0.01 M sodium hydroxide. The concentrations of the SCL were determined by the microbiuret method,[18] and the absorbance of the HPTS dissolved in these solutions was adjusted to 0.1 at 460 nm.

Fluorescence Anisotropy and Fluorescence Decay Measurements. Fluorescence anisotropy measurements were made using a Jasco FP-770F spectrofluorometer equipped with a Jasco ADP-300 fluorescence depolarization attachment. The fluorescence anisotropy ratio of Py-SCL was obtained at 520 nm with the excitation wavelength at 450 nm. An alkaline HPTS solution was used as the blank for the correction of the instrumental factor. The temperature was controlled by circulating water through the metallic sample holder from an Advantec LCH-4 constant temperature circulator. First, the fluorescence anisotropy ratio of a Py-SCL solution was observed at a lower temperature. The temperature of the sample was stepwise increased to 50 °C, and the fluorescence aniso-

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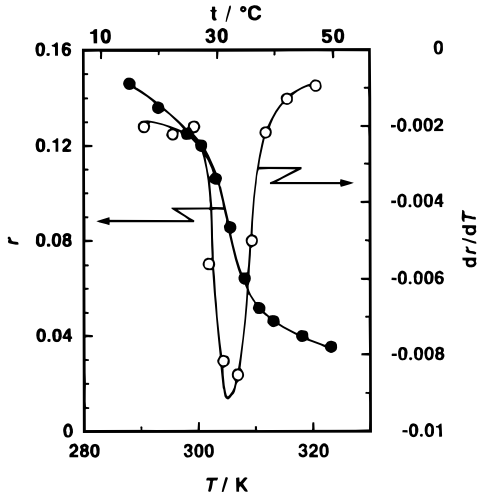

Figure 1. Fluorescence anisotropy ratio r of Py-SCL (●) and its differential coefficient (○).

tropy ratio at various temperatures was obtained. The Py-SCL solution heated to 50 °C was then cooled at 4 °C for 17 h. The fluorescence anisotropy ratio of the cooled sample was measured again according to the same procedure as the previous measurement. A series of fluorescence anisotropy measurements was repeated two times for every Py-SCL sample.

Further analysis of the fluorescence anisotropy was performed according to the Perrin−Weber equation[19]

$$1/r = (k_B\tau/\eta v r_0)T + 1/r_0 \quad (1)$$

where r is the fluorescence anisotropy ratio, k_B is the Boltzmann constant, τ is the fluorescence lifetime, η is the local viscosity around a fluorescent probe, v is the rotational volume of a fluorescent molecule, r_0 is the fluorescence anisotropy ratio at infinite viscosity, and T is the absolute temperature. The r_0 value of the 1-acetoxypyrene-3,6,8-trisulfonamide derivative, which is a monomer model compound of Py-SCL and was prepared by mixing APTS with glycine, was 0.375 in glycerol/water mixture.

Fluorescence decay curves were obtained on a Horiba NAES-1100 photon counting apparatus equipped with Toshiba UV-33 and Y-50 filters and with a Taitec EZL-80 constant temperature circulator. The decay data were analyzed using the Marquardt method.[20]

CD Measurements. The denaturation temperature and helix content of a Py-SCL solution were determined using a Jasco J-600 spectropolarimeter with a 10-mm path length water-jacketed cell.[4,5] Temperature of the sample was controlled by circulating water through the water-jacketed cell from a Taitec EZL-80 circulator equipped with a Taitec PU-9 thermoprogramming unit. The ellipticities at 221 nm for the sample were continuously observed from 20 to 50 °C with a temperature heating rate of 0.25 deg/min. Denaturation temperature of the sample was determined as the temperature at which the first derivative peak of the transition curve was indicated.[4]

Results and Discussion

Helix-to-Coil Transition of Py-SCL. As a typical result, the fluorescence anisotropy ratio of the Py-SCL, the degree of cross-linking of which was 0.85%, as a function of the temperature is shown in Figure 1. The value of the fluorescence anisotropy decreased with an increase in temperature. This change probably corresponds to the helix-to-coil transition of collagen. In order to determine the denaturation temperature (T_m), the r *vs* T curve was differentiated with T. The dr/dT *vs* T curve showed a manifest peak. The T_ms of Py-SCLs obtained from the fluorescence method are summarized in Table 1 together with the T_ms from the CD method. Both T_m values of a Py-SCL are very close, indicating that the fluorescence anisotropy can be utilized for the detection of the transition from the collagen triple helix to random coils as well as the CD method. The fluorescence method has the advantages of higher sensitivity *vs* the CD method and would offer information on the local environment around the fluorescent molecules.

Table 1. Comparison of Denaturation Temperatures Obtained from the Fluorescence Anisotropy Ratio (T_m^F) or CD (T_m^C)

proportion of modified amino group (%)	T_m^F (°C)	T_m^C (°C)
0[a]		34.3
0.85	32.7	32.5
2.0	32.0	32.3
2.9	33.6	33.0
6.9	31.7	32.0
22.3	31.6	32.4
25.0	33.0	32.6
82.1	33.0	32.9

[a] Unmodified SCL.

In order to further examine the helix-to-coil transition of the Py-SCL, the obtained fluorescence anisotropy ratio was analyzed using the Perrin−Weber equation.[19] In principle, the reciprocal of the fluorescence anisotropy ratio is proportional to the temperature when both the fluorescence lifetime and viscosity are constant (see eq 1). The fluorescence decay of the Py-SCL was measured at temperatures ranging from 20 to 50 °C. The fluorescence decay curves of the pyrene derivative cross-linking collagens were well fitted with a single-exponential curve having a lifetime of 5.38 ± 0.05 ns within the experimental temperature range. Therefore, the coefficient for the absolute temperature in eq 1 depends on the viscosity around the pyrenyl cross-linker in a system.

When the reciprocal of the fluorescence anisotropy ratio of the Py-SCL plots *vs* the absolute temperature (the modified Perrin−Weber plot[19]), the slope of the obtained straight line may markedly change with an increase in the temperature because the viscosity of collagen is significantly different from that of gelatin. As a typical result, the modified Perrin−Weber plot of the Py-SCL, the degree of cross-linking of which was 82.1%, is shown in Figure 2 (closed circle). The fluorescence anisotropy at temperatures below T_m probably reflects the mobility of the APTS on collagen, and that at temperatures above T_m would reflect the mobility of the APTS on gelatin.

On the other hand, the modified Perrin−Weber plot of the HPTS-mixed SCL solution, whose SCL concentration was 0.01 wt %, was linear between 15 and 50 °C. The 1/r values of the HPTS dissolved in the SCL solution were 88.6 at 15°C and 318 at 50 °C. The reciprocal of the fluorescence anisotropy values of the fixed APTS was smaller than that of the free HPTS at the same temperature. The mobility of the APTS molecule would be strongly suppressed by binding the APTS with the SCL. A single straight line was observed on a modified Perrin−Weber plot of the SCL solution mixed with HPTS even when the SCL concentration was 6 wt %. However, two straight lines, which were probably based on the collagen and gelatin states, were found on the modified Perrin−Weber plot of the HPTS dissolved in the SCL solution whose SCL concentration was 7 wt % (data not shown). Influence of the change

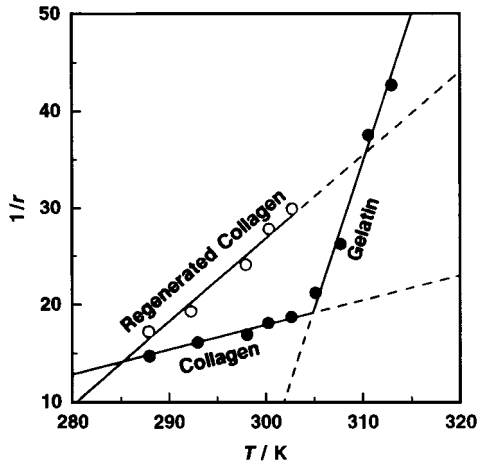

Figure 2. Modified Perrin−Weber plot of the Py-SCL of which the starting conditions of the measurements were before heat denaturation (●) or after regeneration of the triple helix by cooling the heat-denatured collagen (○).

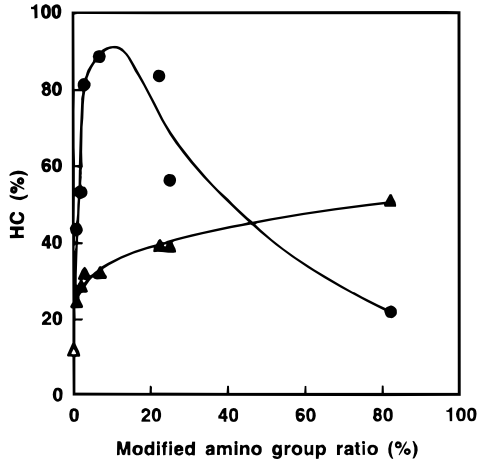

Figure 3. Helix content in re-formed Py-SCL determined by the fluorescence (●) or CD (▲) method. The open triangle is un-cross-linked SCL.

in the viscosity of a SCL solution system on the fluorescence anisotropy measurements may appear within the SCL concentration above 7 wt %, but may not appear below 6 wt %. Consequently, the fluorescence anisotropy in Figure 2 certainly reflects the mobility of the pyrenyl cross-linkage combined with SCL side chains.

Regeneration of Collagen Triple Helix. The helix content (HC) in the regenerated collagen may be obtained from the fluorescence anisotropy data because the mobility of the fluorescent cross-link, namely η in eq 1, depends on the quantity of the collagen helix formed around the cross-link. The reciprocal of the slope value of the modified Perrin−Weber plot would be proportional to HC. The HC of Py-SCL before heat denaturation was determined using the CD,[7] and the HC of the heat-denatured Py-SCL was considered to be 0%. As a typical result, the modified Perrin−Weber plot of the regenerated Py-SCL, whose degree of cross-linking was 82.1%, is represented in Figure 2 (open circle). The slope values of the standard lines, which correspond to the collagen and gelatin states, were 0.266 (94.0% HC[7]) and 2.85 (0% HC), respectively. The slope value of the regenerated Py-SCL was 0.875. Accordingly, the HC in the regenerated Py-SCL was 21.7%.

Figure 3 shows plots of the HC in the regenerated Py-SCL *vs* the proportion of the closed amino group on the SCL side chain. The HC obtained by the CD monotonously increased with an increase in the degree of cross-linking. In contrast to the result from the CD, the HC determined by our new approach showed a peak. The HC obtained by the CD method certainly indicates the average helix content in a system. On the other hand, the HC obtained from the fluorescence anisotropy would be the quantity of the collagen helix re-formed near the fluorescent cross-link because the fluorescence anisotropy only reflects the situation in the microregion around the fluorescent cross-link. A small amount of the cross-link probably promotes the regeneration of the collagen triple helix because a larger quantity of the triple helix, more than the average quantity, was regenerated around the chemical cross-link. On the contrary, the cross-link would inhibit the regeneration of the triple helix around the cross-link when the degree of cross-linking is above 80%, because the HC determined from the fluorescence anisotropy was smaller than the HC obtained from the CD. A decrease in the extent of the triple helix re-formation around the chemical cross-link may be caused by aggregation among the pyrenyl cross-links based on hydrophobic interaction because the random aggregation probably attracts the gelatin chains containing the aromatic cross-link with incorrect configuration.

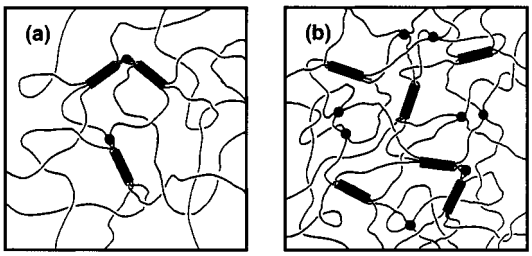

Figure 4. Schematic illustrations of gelatin gels containing (a) a small amount of or (b) a large amount of the chemical cross-links. Solid rectangles and circles represent the re-formed collagen triple-helical segments and chemical cross-links, respectively.

In conclusion, schematic illustrations of the cross-linked gelatin gels are displayed in Figure 4. The amount of the triple-helical segments, which have a similar size,[9] increased with an increase in the degree of cross-linking. However, the triple helices were re-formed by avoiding the cross-link under the higher cross-link content conditions. These models may mimic the gel structure of the gelatin isolated from animal bodies because a natural cross-linkage produced *in vivo* has aromatic ring(s).[21,22] The difference between our previous reports[5−7] and other ones[8,9] may be consistently explained by the present results as the difference in the degree of cross-linking or in the monitor scale detected by different methods.

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