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Structural effects of cross-linking reagents on triple-helix reformation of intramolecularly cross-linked collagen

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Intramolecular cross-links were introduced into collagen molecules with six kinds of cross-linking reagents at a dilute concentration of protein where intermolecular cross-links rarely occurred. Cross-linked collagen molecules regenerated the triple-helical structure from the heat-denatured conformation. The cross-links consisting of an aliphatic hydrocarbon chain were more effective in the regeneration of the collagen structure than the cross-links containing one or two aromatic rings.

(Keywords: collagen; cross-link; helix content)

Introduction

Collagen is the most abundant protein in higher animals, and its function has been considered to maintain the body skeleton. Collagen is usually employed as the carrier of drugs[1,2] or the material for constructing artificial organs[3,4] because the collagen molecule is non-toxic toward an organism and has a high mechanical strength. The triple-helical structure of the collagen molecule has shown specific interactions with certain biomolecules such as $\alpha_2\beta_1$ integrin, a collagen receptor on the cell surface. A collagen molecule is transformed into gelatin by heat denaturation above body temperature. If the triple-helical structure of the collagen molecule is destroyed by heat, the properties of the polypeptides change entirely in spite of having the same chemical composition. Therefore, introduction of chemical cross-links into the collagen molecules has been performed in order to stabilize the structure of the protein[5,6]. Although a large number of investigations on cross-linked collagens have been carried out on a macroscopic level by many research groups[1,2,7], very few reports have focused on intramolecular cross-links. Veis and Drake[8] prepared collagen treated with formaldehyde in a dilute solution and examined the physical properties of the intramolecularly cross-linked collagen. In the present study, the effects of cross-links on the stability and interchange of the triple-helical structure of collagen were examined by the treatment of the protein with six kinds of bifunctional cross-linking reagents. The triple-helical content at various temperatures was evaluated using circular dichroism (c.d.) spectroscopy.

Experimental

Materials. Collagen (solubilized collagen with alkali, SCL) that was solubilized from steer hide with an alkaline solution was obtained from Nippi, Inc.

The bifunctional cross-linking reagents used are as follows. Dimethyl suberimidate dihydrochloride (DMS), 1,5-difluoro-2,4-dinitrobenzene (FNB), and 4,4′-difluoro-3,3′-dinitro-diphenylsulfone (FNP) were purchased from Wako Pure Chemical Industries, Ltd. Toluylene-2,4-diisocyanate (TDC) and *m*-xylylene diisocyanate (XDC) were obtained from Tokyo Kasei Kogyo Co., Ltd. Dimethyl adipimidate dihydrochloride (DMA) was purchased from Nacalai Tesque, Inc. All the bifunctional reagents were of the highest purity available and used without further purification. The structural formulas of the cross-linking reagents are illustrated in *Scheme 1*.

Preparation of cross-linked collagens. As a general procedure, the preparation of the cross-linked collagens was carried out according to the following process. First, SCL was dissolved in an alkaline solution. A bifunctional cross-linking reagent was continuously and uniformly added to the SCL solution at 25°C with gentle stirring in a flask. After the addition of the cross-linking reagent to the SCL solution had been completed, the mixture was continuously stirred in the dark at 25°C overnight. The cross-linking reaction was terminated by acidifying the alkaline solution of the reaction mixture with 1 M sulfuric acid or 1 M acetic acid. The reaction mixture was then dialysed against five changes of 20 litres of 5 mM acetic acid solution. The dialysate was lyophilized and stored below −20°C. The cross-linking reactions were performed as follows.

FNB modification: addition of 0.67 ml of 0.2% methanolic FNB solution to 300 ml of 1% $NaHCO_3$ solution containing 1.06 g (dry weight) of SCL.

FNP modification: addition of 1.2 ml of 0.14% FNP in acetone to 150 ml of 0.1 M Na_2HPO_4 containing 0.50 g (dry weight) of SCL.

TDC or XDC modification: addition of 1 μl of liquid TDC or liquid XDC into 250 ml of borate buffer, pH 9.5, containing 0.50 g (dry weight) of SCL.

DMA or DMS modification: the slow addition of 1.5 mg of powdered DMA or powdered DMS to 250 ml of 0.2% SCL solution, pH 10.

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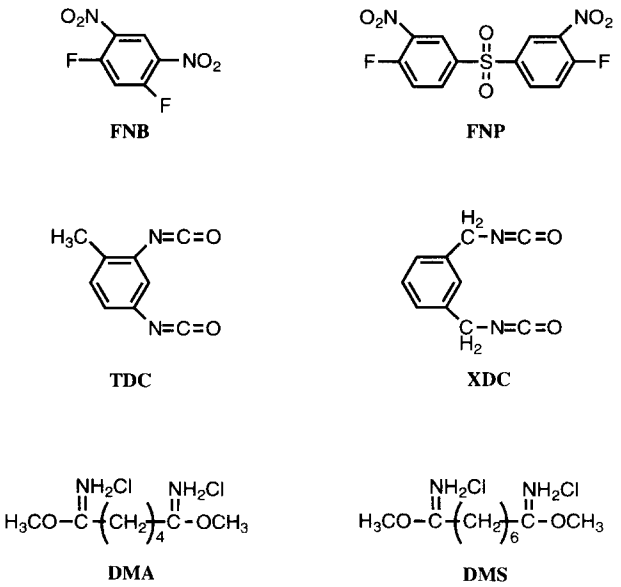

Scheme 1 Structural formulas of bifunctional cross-linking reagents employed in the present study

Gel permeation chromatography (g.p.c.). G.p.c. was carried out on two 7.5 × 300 mm columns (Shodex Protein KW-804, Showa Denko Co., Ltd, Japan) arranged in tandem. Each cross-linked collagen was dissolved in 0.1 M phosphate buffer, pH 6.9, and the concentration was adjusted to 0.5%. The sample solution was then heated in hot water at 100°C for 3 min. Insoluble particles were removed by filtration with 0.45 μm of membrane filter. Twenty microlitres of the sample were injected into a liquid chromatograph and eluted with 0.1 M phosphate buffer, pH 6.9. The flow rate of the developing solution was 1.0 ml min^{-1}, and the detection of polypeptides was carried out by ultraviolet absorption at 230 nm.

C.d. measurements. C.d. spectra were obtained using a Jasco J-600 spectropolarimeter. The temperature of the cross-linked collagen solution was controlled using a Taitec EZL-80 constant temperature circulator equipped with a Taitec PU-9 thermocontrol unit. The temperature change in the solution was performed by programming the pattern of the temperature change using the Taitec PU-9. Cross-linked collagen dissolved in 20 mM acetic acid was used for c.d. measurements. The concentration of the cross-linked collagen was adjusted to 0.01% based on the microbiuret method[9]. The molecular ellipticity was obtained as a mean residue weight of 91.2 (ref. 10).

Results and discussion

We used the six kinds of bifunctional chemicals shown in *Scheme 1* as the cross-linking reagents on collagen to examine the effects of the molecular structure of the reagents on the properties of the cross-linked collagens. Although there are three types of active groups in the six cross-linking reagents, the difference among the active groups is negligible in this study because each active group combines with amino groups of the protein; these cross-linking reagents are known to be introduced mainly between the ε-amino groups of the lysine residues of the protein molecule[11]. In addition, the active groups of the reagents probably also bind to the δ-amino groups of the ornithine residues, because part of the arginine residue in collagen is transformed to ornithine during the isolation of collagen with alkali[12]. Quantification of the cross-linking reagent introduced into collagen molecules was monitored by spectroscopic measurements of the treated collagen after removing the unbound reagents. FNB[13] and FNP[14] exhibit the absorption characteristics of light at or just below a short wavelength region of visible light due to the aromatic residues. The absorption spectra of the FNB- or FNP-treated collagen solution showed an absorbance wavelength above 250 nm (data not shown), indicating that these reagents are bound covalently to the collagen molecule, since collagen prior to the reaction has no absorption above 250 nm and the spectrum above 250 nm is consistent with that of the cross-linking reagent alone. We determined the average number of the FNB or FNP cross-links in a collagen molecule from the molar extinction coefficient of each reagent in the near-ultraviolet or short visible region[13,14] (*Table 1*). The amount of cross-linking reagents bound to the collagen molecule other than FNB and FNP was, in turn, estimated by quantifying the number of free amino groups in the cross-linked collagen using the spectrophotometric method with trinitrobenzenesulfonic acid[15], which stoichiometrically reacts with the amino groups of protein. The average numbers of cross-linking reagents bound per collagen molecule are presented in *Table 1*.

G.p.c. was performed to examine whether the cross-linking resulted in an increase in the polypeptide sizes. The percentage of each chain component of the heat-denatured collagens with and without the cross-links is

Table 1 Degree of cross-links in a collagen molecule and composition ratio of α, β and γ chains in cross-linked collagens

Cross-linking reagent on collagen	Number of reagent bound per collagen molecule	Ratio of α, β and γ chains (%)		
		α chain	β chain	γ chain
SCL[a]	0.0	67.4	25.3	7.3
FNB	2.1	61.1	27.9	11.0
FNP	2.1	57.6	28.5	13.9
TDC	2.3	61.3	26.8	11.9
XDC	2.7	60.8	26.5	12.7
DMA	1.9	56.1	27.6	16.3
DMS	2.6	55.6	28.7	15.7

[a] Unmodified collagen

summarized in *Table 1*. It is expected that the amount of the β- or γ-fraction of the cross-linked collagen molecule increases markedly if cross-links are frequently formed between two α chains or between the α and β chains. However, as shown in *Table 1*, this was not the case. These results suggest that a large part of the cross-linking reagent binds to the collagen molecule with one of the active groups, while another active group remains free or is inactivated by its reaction with water. The introduction of cross-links between the amino groups of the same α chain of collagen is also considered to be another reason for the small increases in the β- and γ-fractions.

In addition to the α fraction, SCL collagen contained the β- and γ-fractions that were probably produced through a non-enzymatic reaction[16]. If the artificial cross-links are introduced intermolecularly, the high molecular weight fractions above the γ chain should be observed on the chromatogram as a result of the α–γ,. β–β, β–γ and γ–γ conjugations. However, no polypeptides were recovered in the g.p.c. fraction corresponding to molecular weights above the γ chain of collagen. This result suggests that the cross-linking reaction mainly occurred intramolecularly. We then calculated the maximum proportion of the intermolecular cross-linking assuming that the increase in the quantity of the β- and γ-fractions of the cross-linked collagen was due to the formation of the intermolecular cross-links, and the numbers of the reagents contained in the α chain and in the β chain were one-third and two-thirds of the reagent bound per collagen molecule, respectively. For example, the proportion of intermolecular cross-links for the TDC-modified collagen was 11.2%. Even the highest content value of the intermolecular cross-links was 20.0% for the DMS-modified collagen. We concluded, therefore, that the cross-links were mainly intramolecular.

The c.d. spectrum of native collagen shows the characteristic spectrum pattern that has a maximum at 221 nm. As for the molecular ellipticity for the collagen triple helix, Na[17] reported that the molecular ellipticity at 221 nm of the acid-soluble calf-skin type I collagen in acidic solution, pH 4.0, at 20°C was about 6000 deg cm^2 dmol^{-1}, while Jenness *et al.*[18] reported $\sim$ 10 000 deg cm^2 dmol^{-1} for the acid-soluble calf-skin collagen in phosphate buffer, pH 3.5, at 15°C. Since the molecular ellipticity at 221 nm of the collagen isolated from steer hide with an alkaline solution (SCL), which is used in our present experiments, was approximately 8500 deg cm^2 dmol^{-1} in acidic solution at 20°C, the SCL probably maintains the triple-helical structure.

We then examined the effect of various cross-linking reagents on the collagen triple-helix reformation from the heat-denatured state by monitoring the ellipticities. The unmodified or cross-linked collagen solution (0.01% w/v) was first heated at 60°C for 15 min for heat denaturation of the collagen, and then the solution was kept at 4°C for 24 h in a refrigerator for the regeneration of the triple-helical structure of the collagen. We assumed that intact collagen consists of a 100% helix conformation, while gelatin consists of a 100% random coil conformation and that the molecular ellipticity of a collagen sample under various conditions depends on the content of the triple helix of collagen. The helical content (h.c.) recovered was estimated using the same method as described by Hayashi *et al.*[19], and the h.c. value of regenerated collagens was calculated using the following equation:

$$\text{h.c.} = 100([\theta] - [\theta]_g)/([\theta]_c - [\theta]_g)$$

where $[\theta]$ is the observed molecular ellipticity at 221 nm, and $[\theta]_c$ and $[\theta]_g$ are the molecular ellipticity of collagen and gelatin at 221 nm, respectively. The helix contents of the regenerated collagens with and without cross-links are indicated in *Table 2*. The h.c. value of SCL treated with cross-linking reagents is larger than that of SCL without reagent. Gelatinized polypeptides would have to come together as the first step before regeneration of the triple helix. When gelatin polypeptides are cross-linked with each other, the reagents might facilitate the assembly of the three polypeptides of gelatin because of the vicinal presence of the polypeptides with the cross-links. The h.c. values of the regenerated collagens treated with DMA or DMS are about 8% more than the others. In spite of the small difference in the number of the reagents bound per collagen molecule, the content ratio of the α chain in the DMA- or DMS-treated collagen seems to decrease slightly compared with the other cross-linked collagens (*Table 1*). Since DMA and DMS do not have a benzene ring in the molecule among the cross-linking reagents used in our present study, the cross-linking reagents containing an aliphatic backbone may be effectively incorporated into two chains constituting a collagen molecule, resulting in the slightly higher proportion of the β- and γ-fractions. The slight difference in the amount of cross-links may subsequently promote the formation of the triple-helical structure. On the other hand, one or two bulky benzene rings in the reagents other than DMA and DMS may inhibit the regeneration of the triple-helical structure through steric hindrance.

Table 2 Helix content (h.c.) and helix-to-coil transition temperature (T_m) of cross-linked collagens

Cross-linking reagent on collagen	h.c. (%)[b]	T_m (°C)
SCL[a]	11.9	34.3
FNB	18.7	34.1
FNP	19.7	34.5
TDC	17.1	34.6
XDC	17.7	34.4
DMA	27.7	34.7
DMS	29.2	35.0

[a] Unmodified collagen
[b] Helix content of regenerated collagens

The helix-to-coil transition of collagen by increasing the temperature at a rate of 15°C h^{-1} within the temperature range of 20 to 50°C was monitored by circular dichroism, and the denaturation temperature (T_m) of various cross-linked collagens was obtained by taking the midpoint of the ellipticity of the sample at 221 nm. Both the ellipticity values of unmodified collagen and of modified collagens decreased sharply between 30 and 40°C, and their T_m values are given in *Table 2*. The T_m of the acid-soluble collagen in acidic solution is about 38°C (ref. 17), whereas that of SCL is 34.3°C. A lower helix-to-coil transition temperature of SCL may be due to the change in the amino acid residues such as glutamine, asparagine and arginine to glutamic acid, aspartic acid and ornithine, respectively, during the

treatment with alkali[12]. The T_m values of the modified collagens were indistinguishable from that of SCL. This can be explained by the low content of cross-links in the collagen molecule because the T_m of SCL that contained a large number of aliphatic cross-links was as high as 41°C (data not shown).

Conclusions

We artificially prepared cross-linked collagens using six kinds of bifunctional cross-linking reagents under dilute protein concentration conditions, and examined several properties of the modified collagens in the solution. The helix-to-coil transition temperature of the collagens did not appreciably increase by cross-linking in comparison with that of native collagen. However, heat-denatured collagens cross-linked with the bifunctional reagents, particularly having an aliphatic backbone skeleton, showed a high regeneration ratio of the triple helix in comparison with the denatured collagen without cross-linking. The effect of the cross-linking reagent chain length on the properties of collagen cross-linked with the aliphatic reagent would be intriguing to examine by adjusting the distance between the two active groups of the reagent.

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