Crosslinking Structure of Keratin. V. Number and Type of Crosslinks in Microstructures of Untreated and Potassium Cyanide Treated Human Hair

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SYNOPSIS

The pattern of the crosslinks in the microstructures of hair was investigated by analyzing the reaction of hair with aqueous KCN by means of chemical and physical methods. It was found that the disulfide (SS) bonds in hair were selectively converted to monosulfide (S) crosslinks by the treatment with 0.08*M* aqueous KCN solution. The KCN-treated hairs swollen with an 11*M* LiBr solution containing *N*-ethylmaleimide showed rubberlike elasticity in a solution composed of equal volumes of 8*M* LiBr and diethylene glycol monobutyl ether. Stress–strain relations of the swollen hairs were analyzed by applying a rubber elasticity theory. It was demonstrated that: the conversion reactions of SS to S links occur between the low-sulfur (LS) proteins at the initial step of the reaction; SS bond scission between the high-sulfur (HS) proteins commences at a faster rate through the conversion reactions of intermolecular SS bonds to intramolecular S bonds; and the SS bonds within the matrix proteins are less reactive than the intermolecular bonds in the LS proteins. The number and the type of crosslinks in microstructures of the intact hair were also determined. The percentage ratios of the different crosslinks in LS proteins were 27.0% intermolecular SS, 39.0% intermolecular *X*, and 34.0% intramolecular SS + *X* links, where *X* are the crosslinks other than SS links; the values in the HS proteins were 11.9% intermolecular SS and 88.1% intramolecular SS links. The percentages of the number of crosslinks in LS and HS proteins were 13.8 and 86.2% of the total number of crosslinks of hair, 627 μmol/g, respectively.

INTRODUCTION

Keratin fiber contains cellular components of its core cortex and external cuticle. The cortex constitutes the largest amount of the fiber and accounts for many of its important physical properties, such as elasticity, pliability, and durability. The major component of the cortex is closely packed, cylindrical macrofibrils. The macrofibril is a complicated, crosslinked system comprising crystalline microfibrils of low-sulfur (LS) proteins and a globular matrix of high-sulfur (HS) proteins.

A number of different types of covalent crosslinks occur in native keratin. As a major amount of crosslinks, the disulfide (SS) bonds originating from cystine (Cys) residues are involved. Minor amounts of lanthionine (Lan), as a transformation product from Cys residues,[1,2] and isopeptide crosslinks[3–7] are more or less incorporated. These crosslinks occur in the protein in two different ways: as an intermolecular crosslink between two adjacent protein chains and as an intramolecular loop within a single peptide chain. One of the most important crosslinks for the mechanical properties of keratin are the SS crosslinks. The SS bonding in the filamentous regions of

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the microfibril was analyzed from the sequential and conformational structure of the proteins.[8] As suggested by Zahn,[9] the crosslinking structure of the SS links in the keratin macrofibril has remained as an interesting, unresolved problem.

Chemical treatments of wool fabrics and hair fibers in aqueous media are an important aspect of the textile and cosmetic industries. The majority of chemical modifications in these areas are mainly concerned with the chemical reactions of the SS bonds. Improvement of the chemical and physical properties of fibers is, therefore, closely related to the reactivities of the SS bonds with their modifiers. However, there has been a lack of information for the differences in chemical reactivity among the SS bonds located in different microstructures of the keratin.

In a previous article, the bonding pattern of SS crosslinks in a variety of keratins was extensively studied.[10] The stress–strain properties of the swollen keratin fibers were measured in a mixed solution of 8*M* lithium bromide aqueous solution and diethylene glycol mono-*n*-butyl ether (BC), and the network structures of swollen keratins were analyzed on the basis of a two-phase model by applying a rubber elasticity theory.[10,11] It was shown that: the number of intermolecular crosslinks in the LS proteins is about 65–75% of the total content of SS bonds in the LS proteins; the SS bonding between the matrix and the microfibril proteins is considerably lower; and some intermolecular SS crosslinks occur between the globular matrix proteins.

Potassium cyanide (KCN) is a typical self-crosslinking agent attacking the SS bonds to form monosulfide (S) crosslinks without incorporation of the other bridge residues. Such a selective conversion reaction of the SS bonds to the stable S bonds against the reducing agents has been widely used for characterization of the crosslinks in keratins.[12–15] For the investigation of the crosslinked structure of keratin, further advantages of this reaction are as follows: hydrolytic cleavage of peptide bonds is negligible,[15] which is responsible for the exact interpretation of the stress–strain data; and the amount of the α crystallite present in an aqueous KCN-treated fiber is approximately similar to the amount before the treatment,[16] which gives obvious evidence that the α-helical conformation in LS proteins remains intact during the reaction of the formation of new crosslinks.

The purpose of the present study is to elucidate the reactivities of SS bonds with cyanide ions, to determine the number of intermolecular crosslinks of newly formed S bonds, to demonstrate the location and type of crosslinkages in aqueous KCN-treated fibers, and to prompt further discussion on the location of SS crosslinks in microstructures of the intact hair keratin.

EXPERIMENTAL

Materials

Human hairs (18-year-old Japanese female) were purified by Soxhlet extraction with acetone for 24 h, washing with ethanol at room temperature for 24 h, washing with cold distilled water, and then air drying. Potassium cyanide, and tri-*n*-butyl phosphine (TBP) used as a reducing agent of disulfides were special reagent grade. *N*-Ethylmaleimide (NEMI) used as a blocking agent of free thiol groups was special reagent grade. BC was obtained by distillation as reported previously.[11]

Determination of Disulfide and Sulfhydryl Contents

SS and sulfhydryl (SH) contents in reduced and unreduced hair samples were analyzed by a polarographic method using methyl mercury iodide.[17]

Preparation of KCN-Treated Human Hair (KCNH)

The hairs (0.1 g) were treated with a 0.08*M* KCN solution (10 mL) at 50, 70, and 90°C for 0.25, 0.5, 1, 2, 3, 5, and 10 h, and thoroughly washed with distilled water and then air dried. The KCNH fibers thus obtained were subjected to amino acid analysis.

Preparations of Reduced Hair (RH), and Completely Reduced and NEMI-Blocked KCNH (RKCNH) Fibers

The hairs and KCNH fibers (0.05 g) were reduced with a 2% TBP solution containing 1-propanol (5 mL) and borate–phosphate buffer adjusted at pH 9.6 (5 mL) for 24 h at 25°C, washed three times with the same buffer containing 1-propanol (10 mL), and then treated with a 10^{-2} *M* NEMI solution composed of 1-propanol (5 mL) and the same buffer (5 mL) for 24 h at 25°C to block free thiol groups. These reduction and blocking cycles were carried out three times for the preparations of RKCNH.

Amino Acid Analysis

The KCNH and untreated human hairs (5 mg each) were hydrolyzed with 6*M* HCl (2 mL) for 24 h at

110°C in deaerated conditions, dried under reduced pressure, and then diluted with 0.02*M* HCl (2 mL) for the amino acid analysis. The amino acid analyzer used was a Hitachi automatic high-speed amino acid analyzer (type L-8500).

Preparation of Swollen Fibers

The hair and chemically modified fibers (20 mg) were treated with an 11*M* LiBr aqueous solution containing 10^{-2} *M* NEMI (2 mL) at 90°C for 1 h and were subsequently immersed in a mixed solution composed of equal volumes of 8*M* LiBr aqueous solution and BC at room temperature. The swollen fibers thus obtained were subjected to mechanical tests.

Mechanical Tests for Swollen Fibers

Stress–strain relations for the swollen fibers were obtained in the mixed solution at a constant temperature of 70°C. The hair samples were elongated at a constant extension ratio, λ, and allowed to relax for a time until an equilibrium force, F, was obtained. The F and λ relationship was constructed as described in a previous article.[11]

Determination of Structural Parameters of Swollen Keratins

On the basis of a two-phase model, the crosslinked structure of hair keratin was analyzed. It was assumed that the swollen keratin is composed of a continuous, lightly crosslinked rubbery phase and densely crosslinked microdomains, which act as reinforcing filler particles in rubber. This assumption corresponds to the structure of swollen keratin fiber including the globular HS proteins dispersed in the swollen network of LS proteins. Elastic forces originating from a simple extension of the swollen fiber with such a nonuniform structure are shown by eq. (1).[18]

$$F = G(\sqrt{n}/3)\{L^{-1}(\alpha/\sqrt{n}) - \alpha^{-3/2}L^{-1}(1/\sqrt{\alpha n})\} \quad (1)$$

where F is the equilibrium force, α is the extension ratio of rubbery chain, n is the number of segments in network chain, $L^{-1}(X)$ is the inverse Langevin function, and G is the shear modulus of the swollen sample and is represented as eq. (2).[11,19]

$$G = (\rho RT/M_c)\{(v_2 - \phi_d)/(1 - \phi_d)\}^{1/3}(1 - 2M_c/M)\gamma \quad (2)$$

where ρ is the density of unswollen sample, M_c is the number average molecular weight between crosslinks in the rubber region, M is the number average molecular weight of the primary molecule, R is the gas constant, T is the absolute temperature, v_2 is the volume fraction of polymer in the swollen sample, ϕ_d is the volume fraction of domains in the swollen sample, and γ is the filler effect of domains in the rubber network. Here γ is a function of κ and ϕ_d, where κ is the shape factor as the length : breadth ratio for a rodlike filler and can be expressed by eq. (3),[20]

$$\gamma = 1 + a\kappa\phi_d + b\kappa^2\phi_d^2 \quad (3)$$

The extension ratio of the swollen sample, λ, can be related by the extension ratios of the rubber region, α, as eq. (4).[11]

$$\alpha = (\lambda - \phi_d)/(1 - \phi_d) \quad (4)$$

To evaluate the values of ϕ_d, M_c or ρ/M_c, and κ, it was attempted to fit the equation to the experimental data with adjustable parameters, using a damping Gauss method of nonlinear least squares. Experimental force-extension data used for the fitting was the range of extensions to the inflection point observed at a higher extension of the stress–strain curve. The origin of the stress–strain curve was determined by extrapolating the linear region of the plots of F versus $(\lambda - \lambda^{-2})$ to the latter axis. These procedures were carried out by computer-aided programs.

With regard to the application of eq. (1) for the keratin system, the following assumptions were made[11]:

1. the densities of unswollen keratin samples used in this experiment are 1.30 g/mL;
2. the number average molecular weight of the primary molecule of the keratin chain in the rubbery network, (M) is 50,000;
3. the molecular weight of the segment for the keratin chain comprising LS proteins, M_c/n, is a constant with the value of 1250;
4. the filler particles are near spherical as $1 \leq \kappa \leq 2$; and
5. the values of two constants a and b in eq. (3) are equal to the values given by Guth[20] for the spherical shape, namely, $a = 2.5$ and $b = 14.1$.

The four parameters ϕ_d, M_c, κ, and n in eq. (1) were reduced to the three parameters ϕ_d, M_c, and κ

by introducing the above assumption 3. The fitting to the experimental data for eq. (1) was performed by repeating the cycles on the two adjustable parameters, ϕ_d and M_c, under the condition that κ was fixed at constant values in the range from 1 to 2 with 0.1 intervals. With the deviation (Δx) of the curve fitted at a fixed κ from the experimental force at constant extensions, the sum of $(\Delta x)^2$ was made in the whole extension range.

The relationship between S [$= \sum (\Delta x)^2$] and κ was constructed from the value of $\kappa = 1$ to 2 with 0.1 intervals. The curve of S plotted against κ showed a leveling-off region but no recognizable minimum within the above range. Here, assumption was made that the most probable value of κ is the nearest value to unity at the leveling-off region. The nearest value to unity at the leveling-off region, κi, was empirically defined by eq. (5),

$$(S_{\kappa i+0.1}/S_{\kappa i}) - (S_{\kappa i}/S_{\kappa i-0.1}) \fallingdotseq 0.05 \qquad (5)$$

where $S_{\kappa i}$, $S_{\kappa i+0.1}$, and $S_{\kappa i-0.1}$ are the values of S at κi, $\kappa i + 0.1$, and $\kappa i - 0.1$, respectively. The κi was determined with the aid of a computer as the most probable value of the shape factor of domains in the swollen network. The values of the other parameters M_c and ϕ_d can therefore be obtained as the values adjusted by the fitting at the κi.

RESULTS

Rate of Formation of Lan Links

Figures 1 and 2 show the variation of amino acids cystine (Cys) and Lan contents with the time of treatment at different temperatures. The rate of formation of Lan markedly increases with an increase of the temperature. The content of Cys + Lan is approximately constant at different times, suggesting a quantitative conversion of Cys to Lan residues during the course of treatment. Similar results were reported for Lincoln wool fibers.[15] The analytical data of Cys tend to scatter more than those of Lan. According to a good reproducibility of the analysis of Lan content, rate of formation of Lan crosslinks was treated kinetically.

Kinetic Analysis

The reaction of the SS bonds with cyanide ions proceeds via substitution mechanism of S_N2 and is expressed by eq. (6).[21-24]

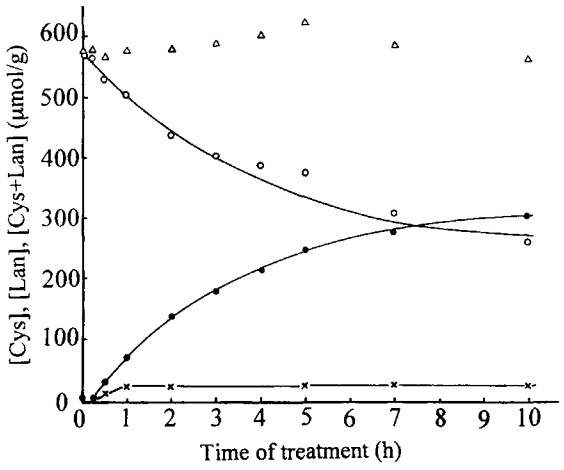
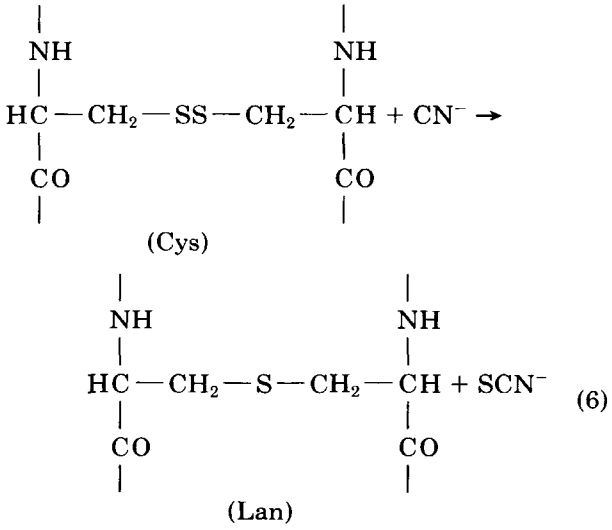


Figure 1 Changes of cystine (Cys) and lanthionine (Lan) contents with time of treatment with aqueous KCN at 50°C: (○) [Cys], (●) [Lan], (△) [Cys] + [Lan]. The Lan content of the untreated hair is 7.9 μmol/g. The number of intermolecular monosulfide (S) crosslinks in low-sulfur (LS) protein, $[S]_{inter}$ in micromoles per gram of whole hair protein (×) is also shown.



(6)

Assuming a pseudo-first-order reaction, eq. (7) is obtained.

$$\ln\{[\mathrm{Cys}]_{t=0}/[\mathrm{Cys}]_t\} = kt \qquad (7)$$

where k is the rate constant, $[\mathrm{Cys}]_t$ is the concentration of Cys residues at time t, and $[\mathrm{Cys}]_{t=0}$ is the concentration of Cys at $t = 0$, namely, the initial concentration of the reactive Cys groups associated with the reaction itself in the human hair keratin.

From the results shown in Figures 1 and 2, it can be assumed that the Cys residues are quantitatively converted into Lan residues. Then, eq. (7) is rewritten by eq. (8).

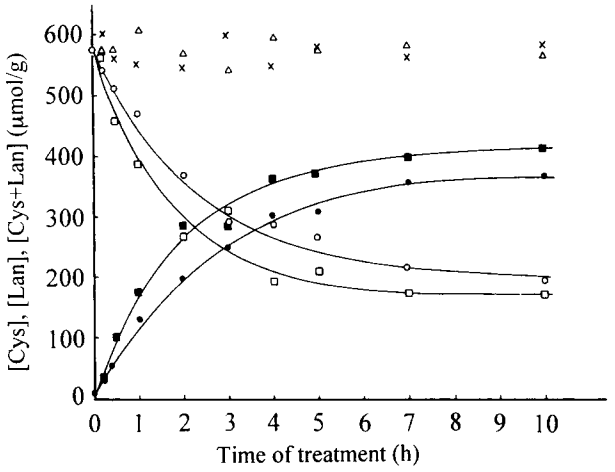


Figure 2 Changes of cystine (Cys) and lanthionine (Lan) contents with time of treatment with aqueous KCN at different temperatures (°C). [Cys]: (○) 60, (□) 70. [Lan]: (●) 60, (■) 70. [Cys] + [Lan]: (△) 60, (×) 70.

$$\ln\{[\text{Lan}]_{t=\alpha}/([\text{Lan}]_{t=\alpha} - [\text{Lan}]_t)\} = kt \quad (8)$$

where $[\text{Lan}]_{t=\alpha}$ is the concentration of Lan residues that will be formed at $t = \infty$ and $[\text{Lan}]_t$ is the concentration of Lan at time t. The untreated hair contains 7.9 μmol/g for Lan as shown in Figure 1. As the concentration of Lan, the values subtracted from this amount of Lan, independent of the reaction from analytical values, were taken for kinetic consideration. The value of $[\text{Lan}]_{t=\alpha}$ was evaluated by using a method of equal time intervals.[15,25,26]

Figure 3 shows the plots for the relation represented by eq. (8). The curves consist of two different slopes, and the difference between the two slopes tends to be larger at higher reaction temperature. It is noted that an induction period exists for every reaction. This suggests that the multiple layers of human hair cuticle act as a barrier for diffusing the reagent into the fibers; and, therefore, the initial reaction steps are diffusion controlled. From the slopes of the linear part of the initial region and toward the end of reaction, the rate constants for faster reacting groups (k_f) and slower reacting groups (k_s) were determined, respectively. The Lan content of faster reacting groups, which equals the content of faster reactive Cys, $[\text{Cys}]_f$, can be estimated by the equation: $[\text{Cys}]_f = [\text{Lan}]_t/[1\text{-exp}(-k_f t)]$. The content of slower reactive Cys, $[\text{Cys}]_s$, equals the value of $[\text{Cys}]_{t=\alpha} - [\text{Cys}]_f$, where $[\text{Cys}]_{t=\alpha}$ is the amount of Cys reacted at $t = \infty$, which equals the value of $[\text{Lan}]_{t=\alpha}$.

The magnitudes of k_f value at 50, 60, and 70°C were, respectively, 5.4×10^{-3}, 8.1×10^{-3}, and 12.7×10^{-3} min^{-1}. The corresponding k_s values were a similar order of magnitude of k_f values, that is, 4.0×10^{-3}, 4.9×10^{-3}, and 6.0×10^{-3} min^{-1}. The k_f values obtained for the human hair was one order less than the values obtained for wool fibers.[15] This may be ascribed to the reaction involving a diffusion-controlled mechanism for human hair as described in the preceding discussion.

Kinetic results are shown in Table I. The major part of reactive Cys occurs in slower reacting groups. The reactive Cys content increases with an increase of the reaction temperature. Nonreactive Cys content, $[\text{Cys}]_{\text{nonreactive}}$, reached 43.1, 33.2, and 27.9% of total Cys residues at the treatment temperatures of 50, 60, and 70°C, respectively. Reactivity of Cys residues was probably due to the difference in accessibility of CN^- ions in the fiber. However, no information about the location of Cys residues reacted with CN^- ions can be obtained from these kinetic results.

Determination of Structural Parameters

Figure 4 shows the relationships between equilibrium stress, F, and strain, λ, for the swollen human hair and the KCN-treated hairs obtained by varying the time of reaction at 50°C. The initial modulus decreases with increase of the time of treatment. According to eq. (2), the modulus depends on the crosslink density in the LS protein (ρ/M_c), the volume fraction of microdomains comprising the HS

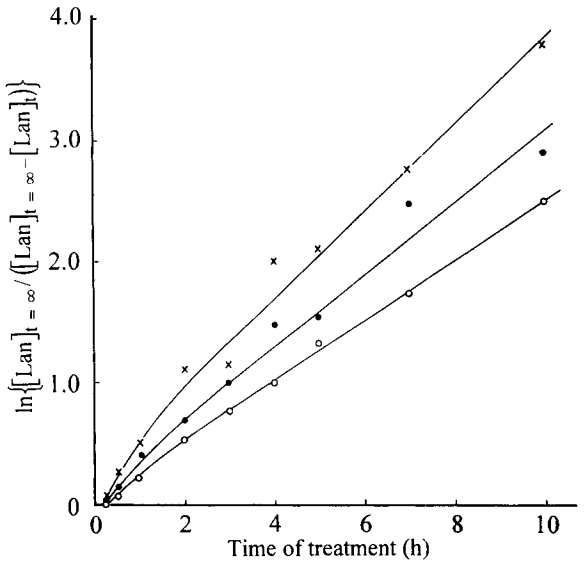


Figure 3 Relationships of $\ln\{[\text{Lan}]_{t=\alpha}/([\text{Lan}]_{t=\alpha} - [\text{Lan}]_t)\}$ versus time of treatment with aqueous KCN at different temperatures (°C): (○) 50, (●) 60, (×) 70.

Table I Reacting and Nonreacting Cys Contents in Human Hair Treated with 0.08*M* Aqueous KCN at Different Temperatures

Content of Cys with Different Reactivities	Treatment Temperatures (°C)					
	50		60		70	
	μmol/g	%	μmol/g	%	μmol/g	%
$[Cys]_{total}$	573	100	573	100	573	100
$[Cys]_{t=\infty}$	326	56.9	383	66.8	413	72.1
$[Cys]_f$	1	0.2	3	0.5	13	2.3
$[Cys]_s$	325	56.7	380	66.3	400	69.8
$[Cys]_{nonreactive}$	247	43.1	190	33.2	160	27.9

matrix in the swollen fiber (ϕ_d), and the shape factor of the microdomains that act as filler particles (κ). The changes in the magnitude of these structural parameters may represent the structural changes resulting from the chemical treatments.

Figure 5 shows the plots of S [$=\sum (\Delta x)^2$] against κ values varied with 0.1 intervals in the range from 1 to 2. The values of S sharply decrease initially with an increase of κ, and then level off. Similar curves were also obtained for all the cases examined. No minimum can be observed for S within the range of $1 < \kappa < 2$ which is a range in magnitude for the case of the near-spherical filler particles. We may assume, therefore, that the most probable value of κ is the nearest value to unity at the leveling-off region defined as κ_i in eq. (5). The solid lines fitted to the experimental data are also shown in Figure 4. Good fit can be realized in the whole extension range.

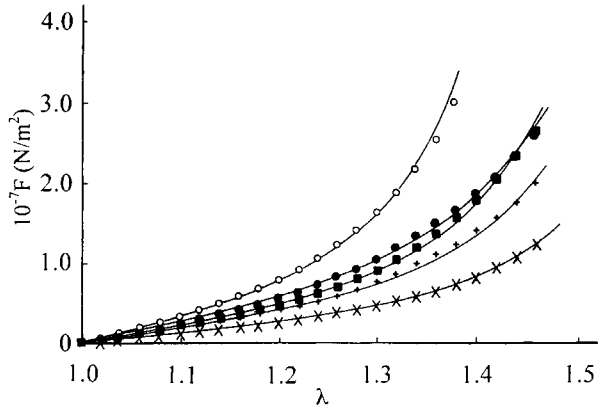


Figure 4 Relationships between equilibrium stress, F, and strain, λ, for the swollen human hair and swollen hairs treated with aqueous KCN at different times (h): (○) untreated hair; treated hair: (●) 0.25, (■) 0.5, (+) 1, (×) 10. The solid lines fit to experimental data by eq. (1).

Structural Parameters for KCNH Hairs

Figure 6 shows the relationships of the shear modulus, G, versus time of treatment at 50 and 70°C. The values of G sharply decrease within 2 h at any temperature and then slightly decrease to around 5 $\times 10^6$ N/m^2 after 10 h, which corresponds to about 40% of the value for the untreated hair. This suggests that considerable changes of the network structure occur during the treatments.

Figure 7 shows the relationships of ρ/M_c versus time of treatment. Although the plots are somewhat scattered within 2 h, it is of interest that at 50°C the values of ρ/M_c are approximately constant, in-

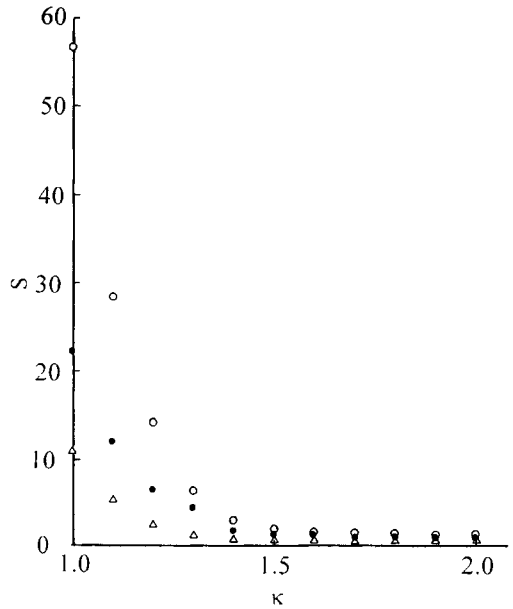


Figure 5 Plots of S against κ values varied with 0.1 intervals in the range from 1 to 2: (○) untreated hair, (●) hair treated with aqueous KCN for 1 h, (△) hair treated for 5 h.

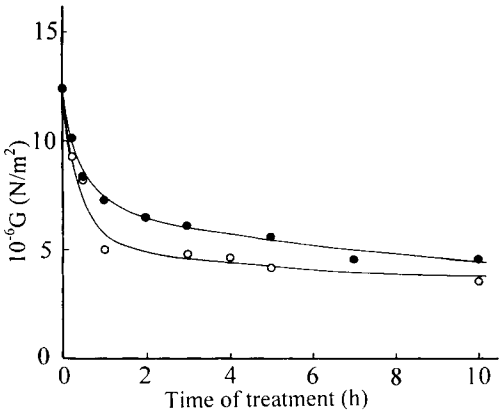


Figure 6 Relationships of shear modulus, G, versus time of treatment with aqueous KCN at (●) 50°C and (○) 70°C.

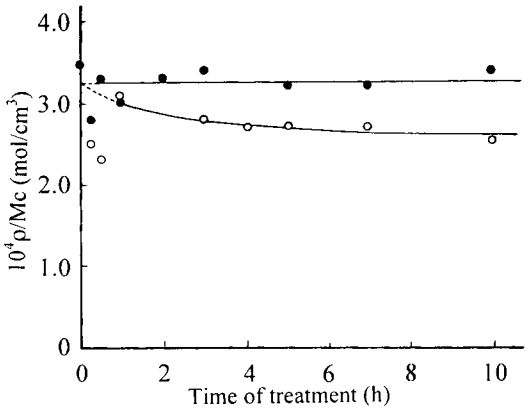


Figure 7 Relationships of ρ/M_c versus time of treatment with aqueous KCN at (●) 50°C and (○) 70°C.

dependent of the time of treatment, namely, 3.3×10^{-4} mol/mL, which is slightly less than the corresponding value of the untreated hair (3.42×10^{-4}). Under the treatment condition at 70°C, the crosslink densities tend to decrease as the time of treatment increases.

Figure 8 shows the relationships of ϕ_d and κ versus time of treatment at two different temperatures. The values of ϕ_d sharply decrease in the initial stage of treatment and then tend to decrease slowly up to about 67% of that value obtained for the untreated hair. The values of κ also decrease from ca. 1.7 to 1.5 at the leveling-off region. Such decreasing tendencies of both parameters suggest that at the initial stage of reaction, deaggregation of the globular matrix proteins is enhanced, and the elliptical shape of the matrix is changed into more spherical. This clearly shows that the sites of the formation of S crosslinks are more or less different from the original sites of the SS bonds located in the matrix proteins. These structural changes are responsible for the decrease of filler effect on fiber stretching, and as a result, this may lead to a decrease in the shear modulus of the modified hair. It is further noted that no significant difference is observed between the two different temperatures for both ϕ_d and κ versus the time curves. This implies that the reaction of the SS bonds with CN^- ions within the matricular components in hair may proceed in a similar reaction rate, even when the treatment temperature differs.

From the results shown in Figures 7 and 8, the difference between the curves observed for G versus time relationships at 50 and 70°C (Fig. 6) is ascribed to the difference between the values of ρ/M_c, namely, the crosslink densities in the LS proteins of the hair treated at different temperatures. For the hair treated at 50°C, the values are approximately constant independent of the time of treatment. This implies that in LS proteins, the conversion reactions of SS bonds into S bonds proceed at the same sites as those of the initial SS links. For the case of 70°C, a different crosslinking mechanism such as the formation of intramolecular S bonds from intermolecular SS bonds is clearly involved. To avoid the uncertainty from the complexity of the crosslinking reactions at higher temperature, detailed consideration about the location of SS and S links will be made for the KCNH fibers prepared at 50°C.

Structural Parameters for TBP-Reduced Hairs and Wools

Table II shows the structural parameters for the untreated and the reduced hairs and wools. After three cycles of reduction treatments with TBP, the SS content drops to only 1.3 and 1.8 μmol/g for hair and wool, respectively. The shear modulus of the

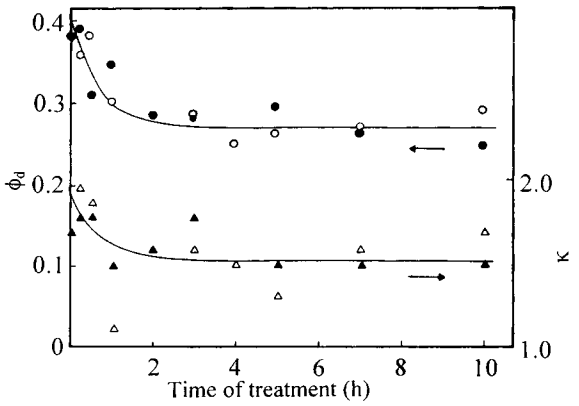


Figure 8 Relationships of ϕ_d and κ versus time of treatment with aqueous KCN at different temperatures (°C). ϕ_d: (●) 50, (○) 70. κ: (▲) 50, (△) 70.

Table II Results Obtained for Unreduced and Reduced Hairs and Wools

Samples	TBP-Reduction Cycles	SS and SH Contents (μmol/g) SS	SH	10^{-5} G (N/m^2)	v_2	10^4 ρ/M_c[a] (mol/mL)	M_c (g/mol)	κ	ϕ_d	ϕ'_d	$10^6/2M_c$[b] (μmol/g)
Human hair											
Untreated	—	581	12	124.0	0.703	3.42	3800	1.7	0.397	0.565[c]	132[d]
Reduced	1	123	7	6.15	0.220	0.86	15200	1.0	0.004	0.018	32.9[e]
Reduced	2	56	10	6.18	0.218	0.90	14400	1.0	0.002	0.009	34.7[e]
Reduced	3	1.3	3	5.61	0.193	0.88	14800	1.0	0.002	0.010	33.8[e]
Lincoln wool											
Untreated	—	409	25	49.1	0.600	3.50	3700	1.7	0.216	0.360	135[d]
Reduced[f]	3	1.8	3	—	0.101	—	—	—	—	—	—

[a] The densities for hair, wool, and reduced hair, ρ were assumed to be 1.30 g/mL.
[b] The number of intermolecular crosslinks calculated from the M_c value by assuming tetrafunctional crosslinker.
[c] The value of ϕ'_d for the untreated hair sample was designated as ϕ'_{d0} (=0.565) in the text.
[d] In micromoles per gram of LS protein.
[e] In micromoles per gram of whole hair protein (see the text).
[f] The swollen samples of the reduced wool fibers were too weak in strength to measure the stress–strain property and to determine the shear modulus, G.

reduced hairs is dropped to about 1/22 as compared to that value of the untreated hair. The ϕ_d values are virtually zero. This indicates that domain structures almost disappear in the reduced fibers. The crosslink density is decreased from 3.42×10^{-4} mol/mL of the unreduced hair to 0.88×10^{-4} mol/mL. It is noteworthy that for SS content below 123 μmol/g, the number of mechanically effective crosslinks is substantially the same. This means that a considerable amount of SS bonds, at least 123 μmol/g, does not contribute to the stresses originating from the strained network, and they act as intramolecular linkages.

With respect to the rate of reduction for SS groups, an important suggestion is also obtained that the intermolecular bonds react much faster than the intramolecular bonds; and finally the intramolecular SS bonds are almost completely reduced with TBP. A large amount of the intermolecular crosslinks other than SS links exists in the hair; and the amount of unknown crosslinks reaches 33.8 μmol/g ($=10^6/2M_c$) on the basis of whole hair protein, because no domains are substantially included in the reduced hair. This value corresponds to about 5.8% of the total SS content in the untreated hair (581 μmol/g). This calculation shows that relatively higher amounts of lysinoalanine and isopeptide linkages may exist in the hair proteins, because the Lan content in the untreated hair is only 7.9 μmol/g (Fig. 1). No such crosslinks are included, however, in the wool keratin (Table II).

Structural Parameters for RKCNH Fibers

Table III shows the results of the characterization of the swollen network for the RKCNH. The observed values for G and v_2 gradually increase with an increase of the time of KCN treatment. It is also shown that these increasing tendencies are caused by the increases of the amount of S crosslinks (ρ/M_c), the volume of domains (ϕ_d), and the value of the shape factor (κ).

Figure 9 shows the relationships of ϕ_d and κ versus time of treatment. As compared with the values of either ϕ_d or κ for KCNH fibers, as shown by curves (a or b), significant decreases are observed for RKCNH fibers. These are clearly due to the reduction of the SS bonds in the domains. It is suggested that the formation of S crosslinks in the matrix domains does not occur before 1 h treatment with aqueous KCN, but commences with a steep increase after 1 h, and then gradually increases thereafter. This suggests further that the conversion reactions of SS to S bonds occur much faster in the regions of the LS microfibrils than those in the HS matrix. As shown in Figure 1(a), the Lan content in the KCNH fiber prepared by 1 h treatment reaches 65.4 μmol/g, which corresponds to about 20% of the reactive Cys content (326 μmol/g) in the hair at 50°C (Table I). The major amount of this Lan is considered to be involved in the LS proteins of hair. The κ versus time relationship shows that the shape of domains in the network crosslinked with only S links

tends to become more elliptical with an increase of the time of KCN treatment. This gives additional evidence that the SS bonds located in the matrix proteins are converted to S bonds in the latter stage of the reaction.

The volume fraction of domains in unswollen fibers, ϕ'_d, can be calculated by

$$\phi'_d = \phi_d/v_2 \tag{9}$$

where v_2 is the volume fraction of polymer in swollen fiber. The values of ϕ'_d are shown in Table II. The domain volume in the human hair is 56.5% of the total volume of the fiber. This figure is larger than that of the Lincoln wool that contains 36.0% in volume. These values are comparable to the volume of the HS matrix in the respective keratin fiber.[10,11,27,28] The value of ϕ'_d for each RKCNH fiber in Table III shows the apparent contribution of S bonds to the formation of domain structure. The values of ϕ'_d increase with an increase of the LAN content in the latter stage of reaction (Fig. 1). It should be noted, however, that a perfect reformation of domain structure through S bonds cannot be attained, even though the conversion reaction is substantially completed by the treatment with aqueous KCN for 10 h. The decrease in the volume of domains due to the reduction of the SS bonds remaining in KCNH fibers may result in the production of noncrosslinked or nonrigid globular matrix materials. It seems to be appropriate to consider that the RKCNH fibers contain a considerable amount of noncrosslinked proteins originating from a portion of the globular matrix. We must, therefore, evaluate the true crosslink density in the LS proteins from the present results obtained for the system including the noncrosslinked materials.

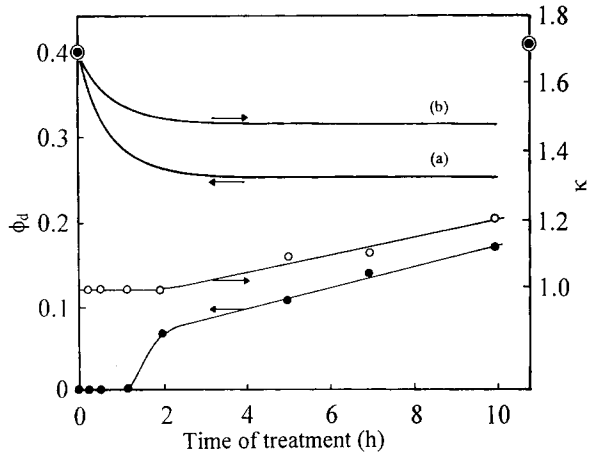


Figure 9 Relationships of ϕ_d and κ for completely reduced KCN-treated hair (RKCNH) fibers versus time of treatment with aqueous KCN at 50°C: (●) ϕ_d, (○) κ. Curves a and b transformed from Figure 8 represent the respective lines of ϕ_d and κ for KCNH fibers: (◉) untreated hair.

Crosslink Density in LS Proteins

Considering a system composed of crosslinked polymer and its homopolymer, the crosslink density of the system including homopolymer, ρ/M_c, can be shown by

$$\rho/M_c = \rho_1 V_1/M_{cl} \tag{10}$$

where V_1 is the volume fraction of the network polymer, ρ is the density of the materials composed of network polymer and homopolymer, and ρ_1 is the density of the network polymer with the number average molecular weight of the chain, M_{cl}, respectively.

We can apply eq. (10) for the calculation of the molecular weight of the network chain in the present

Table III Results Obtained for Completely Reduced KCN-Treated Hair (RKCNH) Fibers

Length of Treatment (h)	10^{-5} G (N/m^2)	v_2	10^4 ρ/M_c[a] (mol/mL)	M_c (g/mol)	κ	ϕ_d	ϕ'_d[b]
0	5.61	0.193	0.88	14800	1.00	0.00	0.00
0.25	6.56	0.217	0.92	14100	1.00	0.00	0.00
0.5	13.0	0.250	1.25	10400	1.00	0.00	0.00
1	19.3	0.283	1.51	8600	1.00	0.00	0.00
2	32.9	0.345	1.78	7300	1.00	0.063	0.183
5	47.8	0.360	2.20	6600	1.10	0.101	0.280
7	74.8	0.374	2.28	5700	1.10	0.135	0.361
10	94.2	0.457	2.20	5900	1.20	0.164	0.359

RKCNH samples were prepared from hair treated with KCN for different amounts of time.
[a] The densities for hair and RKCNH fibers, ρ, were assumed to be 1.30 g/mL.
[b] The volume fraction of domains in the dry sample.

Table IV Results Obtained from Eq. (11) for RKCNH Fibers

Length of Treatment (h)	$10^4\ \rho/M_{cl}$[a] (mol/mL)	M_{cl}[b] (g/mol)	$10^6/2M_{cl}$[c] (μmol/g)	$[S]_{inter}^{LS}$[d] (μmol/g)	$[S]_{inter}$[e] (μmol/g)
0	2.03	6400	78.1[f]	0	0
0.25	2.13	6100	82.0	3.9	1.7
0.52	2.89	4500	111	33.0	14.4
1	3.51	3700	135	56.9	24.8
2	3.33	3900	128	49.9	21.7
5	3.61	3600	139	60.9	26.5
7	3.33	3900	128	49.9	21.7
10	3.25	4000	125	46.9	20.4

RKCNH samples were prepared from hair treated with KCN for different amounts of time.
[a] Crosslink density in LS proteins calculated by assuming the densities of the dry samples, ρ = 1.30 g/mL.
[b] The number average molecular weight between crosslinks in LS proteins calculated by eq. (11).
[c] The total number of intermolecular (S + X) crosslinks in micromoles per gram of LS protein.
[d] The number of intermolecular S crosslinks calculated by eq. (12) in micromoles per gram of LS protein.
[e] The number of intermolecular S crosslinks calculated by eq. (13) in micromoles per gram of whole hair protein.
[f] The number of intermolecular X crosslinks in LS protein $[X]_{inter}^{LS}$.

keratin system, including a disrupted portion of the domains. Assuming the similarity of the densities for the component polymers, eq. (10) can be rewritten as

$$M_{cl} = \{(1 - \phi'_{d0})/(1 - \phi'_d)\}M_c \qquad (11)$$

where ϕ'_{d0} and ϕ'_d are the volume fractions of domains in the untreated and the RKCNH fibers, respectively. Here, M_{cl} represents the number avarage molecular weight of the chain in the LS proteins and the value of ϕ'_{d0} is 0.565 as shown in Table II. The results obtained from eq. (11) are shown in Table IV. The relationship of ρ/M_{cl} versus time of KCN treatment is also shown in Figure 10. It is of interest that the calculated values of ρ/M_{cl} increase sharply up to 1 h and then level off around the value obtained for the hair itself. This suggests that: the conversion reactions of SS to S bonds occur between LS proteins; the formation of intermolecular S crosslinks commences at the initial reaction step and is substantially completed within 1 h; and the intermolecular SS bonds change to S bonds at the same sites as those of the original SS bonds.

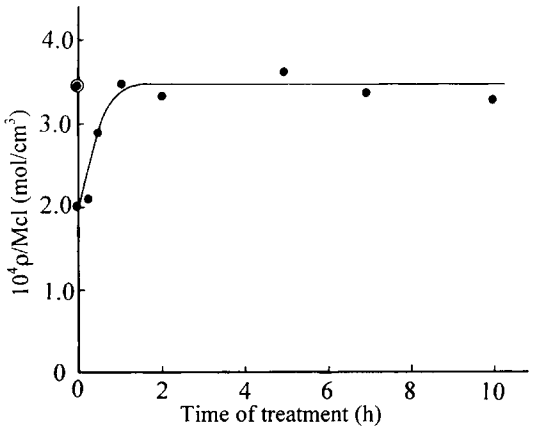


Figure 10 Relationship of the crosslink density, ρ/M_{cl}, in LS protein of RKCNH fibers versus time of treatment with aqueous KCN: (◉) untreated hair.

DISCUSSION

Crosslinking Structure of LS proteins

Concerning the location of the unknown crosslinks amounting to 33.8 μmol/g of hair protein (Table II), it is uncertain whether they occur in the microfibrillar region of the LS proteins or the globular matrix region of the HS proteins or both. Lincoln wool does not contain such stable crosslinks against the reducing agent. However, it should be noted that the total content of the intermolecular crosslinks in the wool is approximately similar to that of the hair keratin (see Table II). Recent advance in the knowledge of IF proteins shows that the amino acid sequence and compositions of LS proteins are very similar among keratins.[8,29] Considering the similarity in chemical and physical structure of LS protein in wool and hair, the assumption that the stable and unreducible crosslinks reside in the LS proteins seems to be appropriate for the present hair keratin. According to this assumption, the crosslink density of the unknown crosslinks, X, can be calculated by

using eq. (11) for the completely reduced hair. The calculated value of ρ/M_{cl} is 2.03×10^{-4} mol/mL corresponding to 78.1 μmol/g of LS protein as the number of intermolecular crosslinks (see Table IV). This figure also corresponds to about 60% of the total number of intermolecular crosslinks in the LS proteins of hair (132 μmol/g) (see Table II). It might be presumed, therefore, that the crosslinks in LS protein of the hair keratin have been occupied by the stable and unreducible crosslinks amounting to about 60% of total intermolecular crosslinks in place of the SS links. Thus, the total number of intermolecular crosslinks in the LS proteins of RKCNH fibers (fourth column of Table IV) can be divided into two parts as shown by

$$10^6/2M_{cl} = [S]^{LS}_{inter} + [X]^{LS}_{inter} \quad (12)$$

where $[S]^{LS}_{inter}$ and $[X]^{LS}_{inter}$ in micromoles per gram of LS protein are the numbers of the intermolecular S crosslinks formed by the KCN treatment and the intermolecular X crosslinks in the untreated hair, respectively. Here, the value of $[X]^{LS}_{inter}$ is 78.1 μmol/g of LS protein (Table IV). The values of $[S]^{LS}_{inter}$ obtained from eq. (12) are also shown in the fifth column of Table IV. This result suggests that the conversion reactions of intermolecular SS crosslinks into S links substantially complete in the LS proteins within 1 h.

It is of interest to compare the Lan content with the amount of intermolecular S crosslinks produced by the KCN treatment, $[S]_{inter}$, on the basis of whole hair protein. The values of $[S]_{inter}$ in micromoles per gram of whole hair protein can be calculated as above by

$$[S]_{inter} = (1 - \phi'_{d0})[S]^{LS}_{inter} \quad (13)$$

The results are shown in the last column of Table IV, and their plots against the time of KCN treatment are also shown in Figure 1. During the reaction before 1 h, where the formation of the intermolecular S bonds substantially completes within the LS proteins, the proportion of the formation of intermolecular crosslinks is only about 25% of the Lan content at a definite time. Above 1 h, the values of $[S]_{inter}$ are approximately constant, with 23.0 μmol/g as the average value. This calculation suggests that the intermolecular SS bonds in LS protein are converted to the corresponding S bonds in the earlier stage of the reaction, while a large amount of intramolecular S bonds are formed from the SS bonds in the matrix protein over the whole reaction range.

In fact, the amount of the intermolecular SS bonds in LS protein of the untreated hair, $[SS]^{LS}_{inter}$, can be evaluated as 53.9 μmol/g (=132 − 78.1), where the 132 is the total amount of intermolecular crosslinks in LS protein (Table II) and the 78.1 is the value of $[X]^{LS}_{inter}$. Therefore, on the basis of whole hair protein, the amount of intermolecular SS crosslinks, $[SS]_{inter}$ in micromoles per gram of whole hair protein can be calculated by

$$[SS]_{inter} = (1 - \phi'_{d0})[SS]^{LS}_{inter} \quad (14)$$

The value obtained for $[SS]_{inter}$ is 23.5 μmol/g of whole hair protein. This figure nearly equals the value of $[S]_{inter}$ in the latter stage of the reaction, that is, 23.0 μmol/g, which corresponds to only about 7% of the total amount of reactive Cys, 326 μmol/g (Table I).

Crosslinking Structure of HS Protein

Significant decreases in the size and the volume of domains also occur in the faster reaction rate region (Fig. 8) within ca. 1.5 h. This may be due to the deaggregation of globular matrix proteins resulting from SS bond scission between the HS proteins through the conversion reactions of intermolecular SS bonds to intramolecular S bonds. With respect to this, it was suggested that from the swellability and mechanical properties of keratins, some interchain SS links must be present between the matrix proteins.[30] The number of intermolecular crosslinks between globular matrix proteins might be evaluated by using the aforementioned data. The total number of intermolecular crosslinks in LS protein was 132 μmol/g, corresponding to 66% of the total SS content in the LS proteins (200 μmol/g) that was determined to be approximately constant among keratins.[31,32] For present hair keratin, the SS content in LS protein of the intact hair can be estimated to be ca. 35 μmol/g (=23.5/0.66) on the basis of whole hair protein, provided that the intermolecular SS links occur in the same ratio for the SS content as described above. Here, it could be presumed that all of the SS links in the LS proteins, amounting to 35 μmol/g, react much faster than those in the HS proteins. After about 1.5 h, leveling-off tendency of the ϕ_d values was observed. This fact indicates that the deaggregation of domain ceased. As shown in Figure 1, the SS crosslinks transform into S crosslinks amounting to ca. 100 μmol/g after 1.5 h. This suggests that the conversion reactions of SS bonds between the globular proteins occur predominantly within 1.5 h, and about 65 μmol/g (=100 − 35) of

SS crosslinks change into S crosslinks accompanying the change of the type of the crosslink, namely, the intermolecular SS links to intramolecular S links. Accordingly, the amount of the crosslinks between the intact globular HS proteins, $[SS]^{HS}_{inter}$, is calculated to be 115 μmol/g HS protein ($=65/\phi'_{d0}$, where ϕ'_{d0} is 0.565).

The number of moles of the SS crosslinks between the globular matrix proteins can be calculated as $10^{-6} \cdot [SS]^{HS}_{inter} \cdot M_{HS}$, where M_{HS} is the average molecular weight of HS proteins, that is, 20,000.[33] The calculated value is 2.3 mol per HS protein molecule. The SS content in the globular HS protein is also calculated to be 966 μmol/g [$=(581 - 35)/\phi'_{d0}$] (Table II), which corresponds to ca. 19 mol per HS protein molecule. Thus the amount of SS links between globular HS proteins is only about 12% of the SS content in the HS protein, and the crosslinking sites of 4.6 mol may present on the surface region of an individual globular protein. These minor parts of the crosslinks play an important role in the mechanical properties of the swollen keratin, because the crosslinks between the HS proteins result in an increase in the volume of the domains acting as reinforcing filler particles.[10] However, the marked decrease observed for the values of G with the time of treatment seems to be unexplained only by the filler effect. A part of the HS matrix proteins may be associated with the Cys rich, terminal nonhelical regions of the LS protein via SS bonds, resulting in the formation of a continuous phase in the swollen network. When such intermolecular SS bonds change into intramolecular S bonds, a considerable decrease in G is also expected. Further work is needed for the evaluation of the amount of the SS links between HS and LS proteins, which may be included as a portion of the quantity accounted for by the SS links between HS proteins. It should be further emphasized that the major parts of SS bonds in the HS proteins are intramolecular linkages.

The reactions occurring above 1.5 h are related presumably with the SS bonds located within the matrix proteins. After the KCN treatment for 10 h, the Lan content in the hair reached ca. 305 μmol/g and unreactive Cys was ca. 270 μmol/g (see Fig. 1). Accordingly, the intramolecular S bonds amounting to ca. 205 μmol/g (=305 − 100) were formed from SS bonds within the globular HS proteins. Therefore, the amount of the intramolecular SS bonds within HS protein in the intact hair was ca. 475 μmol/g (=270 + 205), which corresponds to ca. 82% of the total Cys content in the hair keratin (581 μmol/g).

Number, Type, and Location of Crosslinks in Hair

The distribution of the crosslinks in the hair is shown in Figure 11. The figures designated in parentheses in the figure are the percentage values referred to the total number of crosslinks based on LS, HS, and whole keratin proteins, respectively. As shown in Figure 11(a), the percentage values of X and SS intermolecular crosslinks in the LS proteins are 39 and 27%, respectively. However, these figures are likely to be underestimated, because the distribution of SS crosslinks is not random and some Cys residues occur in sites too near to play a role as different intermolecular crosslinks in the rubbery network.[11] We can expect, therefore, that the number of intramolecular links is less than the value of 34%. The amount of the unknown X crosslinks present in the intact hair is likely to be varied by the history of the hair, because the Lan and lysinoalanine crosslinks are produced from hair washing under alkaline conditions[24,34] and heat drying with a hair dryer under wet conditions.[35]

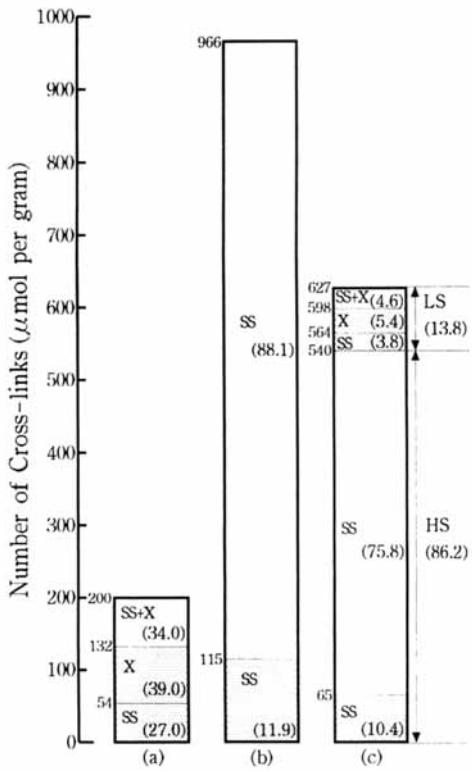


Figure 11 Changes in the distribution of SS and X crosslinks in LS, HS, and whole hair (LS + HS) proteins: (a) LS protein, (b) HS protein, (c) LS + HS protein. Shaded and unshaded regions denote intermolecular and intramolecular crosslinks, respectively. The figures designated in parentheses are the percentage values referred to the total number of crosslinks in the respective protein.

As shown in Figure 11(b), the numbers of intra- and intermolecular crosslinks in the HS protein are 88 and 12% of the total SS content in the corresponding protein (966 μmol/g), respectively. There still remains the possibility for the occurrence of the intermolecular links between LS and HS proteins, and the number of this type of crosslinks might be included in the number of intermolecular SS crosslinks between HS proteins estimated as above.

The number and type of crosslinks based on whole keratin protein are shown in Figure 11(c). The percentage of the number of crosslinks in LS protein is 13.8% and the value in HS protein is 86.2% of the total crosslinks in the hair (627 μmol/g). The percentages for inter- and intramolecular bonds are 19.6 and 80.4%, respectively.

The pattern of the crosslinks in the microstructures of human hair became considerably clearer from the chemical and physical analysis of the reaction of hair with aqueous KCN. However, the reactivity was too high to obtain further detailed information about the distribution of crosslinks within and between the microfibril and the matrix components, such as the SS crosslinks in the α-helical rod domain and the terminal regions of the microfibril protein. Further work is needed to explore these problems.

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