Type and Location of SS Linkages in Human Hair and Their Relation to Fiber Properties in Water

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SYNOPSIS

The changes in the mechanical properties accompanying the reduction of disulfide (SS) linkages in hair were studied. A variety of extents of thiol groups were introduced into hair by treatments with thioglycolic acid and tri-*n*-butyl phosphine. The reduced fibers swollen with an aqueous 11*M* LiBr solution containing *N*-ethylmaleimide showed typical rubberlike elasticity in a solution composed of equal volumes of 8*M* LiBr and diethylene glycol mono-*n*-butyl ether. The crosslink density was determined from the shear modulus of the swollen fiber. It was found that the SS linkages can be divided into two groups: the intermolecular linkages group (SS_1 and SS_2) and the intramolecular linkages group (SS_3). The effect of the SS bond scission on the shear modulus of the reduced fibers in water was also studied. It was further found that intermolecular SS_1 linkages do not respond to the shear modulus of fiber in water, and the amounts of SS_1, SS_2, and SS_3 are about 35, 18, and 47% of the total cystine content in hair (623 μmol/g), respectively. With respect to the location of SS linkages in hair, important suggestions were obtained: the intermolecular SS_1 and SS_2 linkages are located in the microfibril and the matrix, and the former are more accessible to water than the latter; and SS_3 linkages are localized within the hydrophobic region of the matrix. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

Native disulfide (SS) bonds are principally covalent crosslinks in hair and form a 3-dimensional network structure with high crosslink density. The reduction of the crosslinks changes the mechanical properties and even the shape of the fiber. A crosslinked structure model of keratin fibers was proposed by Fraser et al.[1] They reported about the disulfide bonding within and between the subunits in microfibrillar proteins and discussed it on the basis of sequential and conformational structure of the proteins. Recently, there has been a great advance in the field of basic study on keratin structure. Microfibrils consist of low sulfur proteins similar to the structure of the intermediate filaments (IF) in epithelial cells. The matrix proteins consist of high-sulfur proteins, so-called intermediate filament associated proteins (IFAP).[2-5] They aggregate with each other to form macrofibrils. At present, the study on the actual aggregation structure through SS bonding remains as a future problem in keratin structural research.

A variety of physical measurements were employed to assess crosslinking in keratin.[6-10] However, there has been no reliable quantitative method for determination of the number of crosslinkages in keratin. Concerning the evaluation of the crosslinkages in wool, it was found that the fiber pretreated with LiBr solution containing *N*-ethylmaleimide (NEMI) shows a typical rubber elasticity in a diluent system composed of concentrated LiBr aqueous solution and diethylene glycol mono-*n*-butyl ether[11] and a determining method for the crosslink density of hair and the other keratin fibers was found.[12] On the basis of a rubber elasticity theory, the type of SS crosslinkages in microfibril and matrix components in keratin fibers has been discussed.

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Recently, the present authors[13] studied the location of SS bonds in the keratin structure by applying a two-phase model. The swollen keratin fiber consists of a mechanically stable phase of higher crosslinked domains originating from the high-sulfur matrix and a rubber phase with lower crosslink density originating from the low-sulfur microfibrils. On the supramolecular level, the study on the location and distribution of the SS linkages in keratin fiber has progressed considerably. It is of interest to study the effects of the scission of the SS linkages, located in different structural components, on mechanical properties of keratin fibers.

The aim of this study was to estimate the number and the type of the SS crosslinkages in microfibril and matrix components of hair, and to clarify the effect of the respective SS bond scission on the stress–strain property of the fibers in water.

EXPERIMENTAL

Materials

Hair from Japanese women was purified by washing with an aqueous solution of 1% sodium dodecyl sulfate for 10 min at 40°C, washing with distilled water, and then air drying.

Thioglycolic acid (TGA) and tri-*n*-butyl-phosphine (TBP) used as reducing agents were special reagent grade. NEMI used as a blocking agent of thiol groups was special reagent grade. Diethylene glycol mono-*n*-butyl ether (BC) was obtained by distillation as described in a previous article.[11]

Preparation of Reduced Hair

Purified hair fibers were reduced with a 0.50*M* TGA aqueous solution adjusted to pH 7 with aqueous ammonia for 2–300 min at room temperature, and then thoroughly washed with cold distilled water in a nitrogen atmosphere. The fibers were also reduced with a solution mixture composed of equal volumes of 0.1*M* borate-phosphate buffer solution (pH 8) and *n*-propanol containing a concentration range from 0.04*M* to 0.5*M* TBP for 1–24 h at room temperature, and then washed with cold distilled water in a nitrogen atmosphere. A portion of each sample of the reduced fibers was immersed in a $10^{-2}M$ NEMI solution at pH 8.0 to block the free SH groups to inhibit the thiol/disulfide interchange reaction.

Amino Acid Analysis

The reduced and unreduced fibers were immersed in a 0.5*N* monoiodo acetic acid solution (pH 8.0) for 22 h in a dark place to block the thiol groups, and then subjected to amino acid analysis. Lanthionine (Lan) and cystine (Cys) contents in hair samples were determined by a standard method with an amino acid analyzer (model 835, Hitachi Co.) according to the general procedure of Spackman et al.[14] Determination of sulfhydryl residues was carried out according to the procedure of Hirs.[15]

Preparation of Swollen Fibers

The reduced and unreduced fibers were treated with an excess of 11*M* LiBr solution containing $10^{-2}M$ NEMI at 90°C for 90 min and was subsequently immersed in a mixed solution composed of equal volumes of 8*M* LiBr and BC at room temperature. The swollen hair fibers thus prepared were subjected to mechanical tests.

Mechanical Tests for Swollen Fibers

The mechanical tests for swollen fibers were carried out by the method reported previously[12] using a model TCM 20S Tensilon (NMB Co.). The hair samples of about 20-mm length were extended up to about 20% at 2 mm/min in the mixed solution of equal volumes of 8*M* LiBr and BC at 50°C. Equilibrium stresses were obtained at any strains tested under this constant extension speed. After this extension test, the hair sample was washed thoroughly with distilled water in an unstrained state until the diameter of the hair sample no longer changed and was again extended at 2 mm/min in distilled water.

Determination of Crosslink Density and Shear Modulus in Water

According to the method reported in a previously,[13] the shear modulus G for TGA-reduced fibers and TBP-reduced fibers was measured under the equilibrium condition at 50°C.

From Gaussian chain statistics of the network system, the relation between equilibrium forces, τ, and extension ratios, α, is represented by eq. (1).[16]

$$\tau = G[\alpha - (1/\alpha^2)], \quad (1)$$

where τ is the stress referred to the swollen unstretched cross sectional area of the sample. Here, G can be written by eq. (2).

$$G = [\rho RT/M_c)v_2^{1/3}[1 - (2M_c/M)], \qquad (2)$$

where ρ is the density of the dry sample, R is the gas constant, T is the absolute temperature, v_2 is the volume ratio of the dry sample to the swollen sample, M_c is the number average molecular weight between crosslinks, and M is the average molecular weight of the polymer chain before crosslinking. Here, the M value was assumed to be 50,000 for the keratin system.[13] The crosslink density, ρ/M_c represents the number of chains in a unit volume of dry sample. The ρ/M_c values were estimated from the slope of the plot according to eqs. (1) and (2).

The shear modulus in water, G_w was determined from the slope of the plot according to eq. (1) within the range of 2% extension in water after the swollen samples were rinsed sufficiently with water.

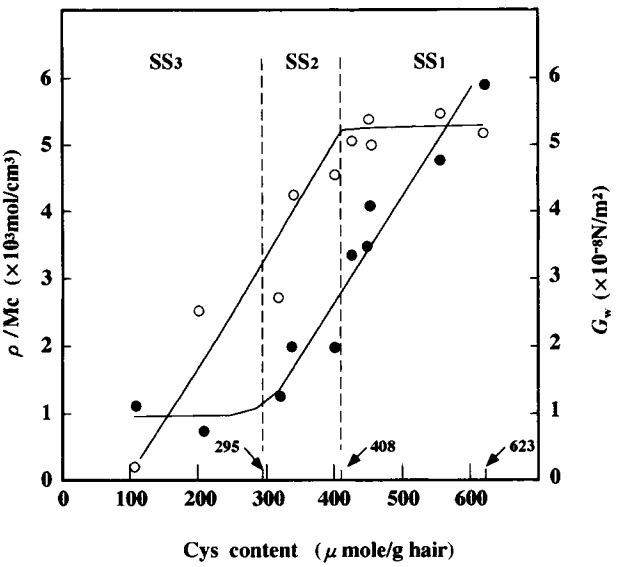

Figure 1 Relationships of (●) ρ/M_c and (○) G_w versus cystine content in TGA-reduced hairs.

RESULTS

Crosslink Densities and Shear Modulus in Water of TGA-Reduced Hair Fibers

Table I shows the results of the Cys content, v_2, G, and ρ/M_c values for TGA-reduced and unreduced fibers. The Cys content greatly decreased initially within 30 min and then decreased slowly. With the decreases of Cys content, the values of both v_2 and G decreased until ca. 180 min, and their decreasing tendencies are likely to be stopped with a further increase of the degree of reduction.

Figure 1 shows the relationships of ρ/M_c and G_w versus Cys content. From the relation between the profiles of the two curves, it is clearly suggested that there exist three different types of SS linkages controlling the mechanical properties of the fibers. The first type of SS linkage, corresponding to the SS groups, amounting to 215 μmol/g, is the range of Cys content from 623 to 408 μmol/g, which were reduced at a faster rate with TGA. The reduction of this type of SS groups results in a significant decrease of ρ/M_c and little or no changes of G_w. The second type of SS linkage is the SS groups in the range of Cys content from 408 to 295 μmol/g, namely, 113 μmol/g. During the reduction of these SS linkages, the values of both ρ/M_c and G_w decreased approximately parallel, with the SS groups being reduced at a slower rate with TGA. In contrast to the first type of SS linkages, the G_w values decreased, but ρ/M_c remained approximately constant with the decrease of the Cys content.

Consequently, in a broad sense, the mechanically effective intermolecular SS linkages, that is,

Table I Results Obtained for Unreduced and TGA-Reduced Hairs

Time of Reduction (min)	Cys Contents (μmol/g)	v_2	10^{-6} G (N/m²)	10^4 ρ/M_c (mol/mL)
Unreduced	623	0.863	15.0	59.3
2	556	0.649	11.0	47.6
5	453	0.608	9.15	40.7
10	450	0.512	8.27	34.7
30	427	0.333	7.34	33.5
90	402	0.306	4.30	19.8
120	338	0.289	4.25	19.8
180	320	0.262	2.61	12.6
240	207	0.237	1.47	7.43
300	110	0.249	2.28	11.1

328 μmol/g (=215 + 113), corresponding to 53% of Cys residues in hair, can be divided into two further types: one is mechanically effective only when the reduced fibers were swollen (SS_1), and another is effective either in the swollen state or in water (SS_2). The mechanically ineffective SS linkages in the swollen state can be estimated to be 295 μmol/g, which corresponds to 47% of total Cys content and acts as mechanically effective crosslinks in water (SS_3).

Crosslink Density and Shear Modulus in Water of TBP-Reduced Hair Fibers

Table II shows the results of the Cys content, v_2, G, and ρ/M_c values for TBP-reduced and unreduced fibers. The values of v_2 decreased significantly with the decrease of the Cys content. However, the decreasing tendency of G seemed to be less compared to that of v_2.

It was confirmed histochemically that TBP reduces the SS groups more rapidly in relatively hydrophobic regions of hair fiber.[17] Figure 2 shows the relationships of the ρ/M_c and G_w versus Cys content. It should be noted that in the initial reduction region, no change of the ρ/M_c values was observed until the Cys content reached about 510 μmol/g. However, the G_w decreased considerably. This behavior was very similar to the mechanical changes observed in the reduction of SS_3 by TGA. It was suggested, therefore, that the intramolecular SS linkages of hair fibers were preferentially reduced by TBP, and further that the reduction of the SS groups concerning the mechanical properties in water were different but specific between the reducing agents with different chemical properties, such as a hydrophilic or hydrophobic nature.

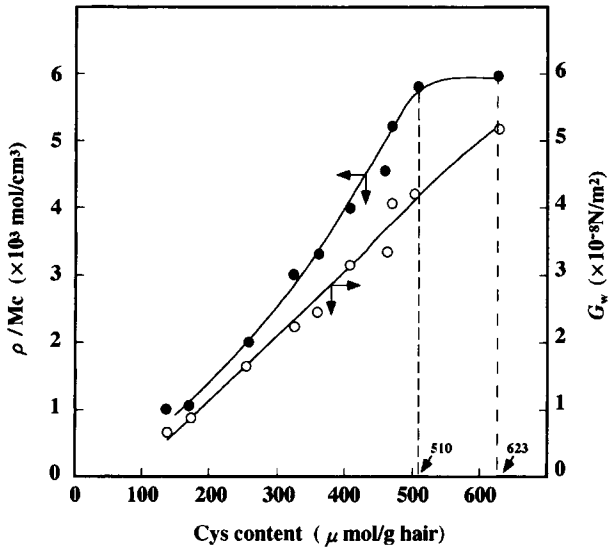

Figure 2 Relationships of (●) ρ/M_c and (○) G_w versus cystine content in TBP-reduced hairs.

DISCUSSION

Distribution of SS Crosslinkages in Hair

It was shown that TGA preferentially reduces the intermolecular linkages in the hydrophilic regions of keratin, while TBP reduces the intramolecular and mechanically ineffective SS linkages in the hydrophobic regions.[11] From the results obtained from the relationship between ρ/M_c and Cys content for the fiber reduced by TGA (Fig. 1), the ratio of intermolecular crosslinkages (SS_1 + SS_2) to intramolecular was estimated to be 53 : 47. The ratio for Lincoln wool was found to be 64 : 34.[18]

The matrix proteins contained a large amount of intramolecular crosslinkages and the volume frac-

Table II Results Obtained for Unreduced and TBP-Reduced Hairs

Concentration of TBP (M)	Time of Reduction (h)	Cys (μmol/g)	v_2	$10^{-6}\,G$ (N/m²)	$10^4\,\rho/M_c$ (mol/mL)
—	—	623	0.863	15.0	59.3
0.5	1	509	0.515	12.4	58.2
0.5	2	468	0.491	10.9	52.1
0.5	3	459	0.447	9.21	45.4
0.04	24	406	0.420	7.88	39.7
0.05	24	360	0.415	7.44	33.2
0.06	24	323	0.352	5.59	30.0
0.12	24	258	0.352	3.68	19.9
0.14	24	168	0.314	1.84	10.6
0.20	24	138	0.251	1.62	10.1

tion of the matrix components was proportionally increased with the increase of the Cys content in keratin.[13,19] It is well known that human hair is one of the most highly crosslinked keratin fibers with SS linkages. As compared with wool, the higher ratio of the intramolecular crosslinkages of hair can be considered to be reasonable.

Type and Location of SS Crosslinkages and Their Relation to Fiber Modulus in Water

It should be emphasized that the intermolecular crosslinks can be classified into two groups (Fig. 1): one is closely related to the modulus of the fiber in water, and the other is not. It is of great interest that the two groups of the intermolecular crosslinks play a different role in the fiber under stress in water. The shear modulus in water, G_w, could be taken as an index to estimate the hydration of denatured proteins by the reduction of SS linkages. The information about the hydrophobicity of the protein structures associated with the SS bonding was important to establish the relation between the crosslinking structure and functions of keratin fibers. The crosslinks other than SS crosslinkages, such as ionic and hydrogen bonds, are essentially important for mechanical properties of keratin fibers in water. Recent advances in the knowledge of the sequence and the structure of IF proteins shows that IF subunits are composed of a central α-helical rod domain and two nonhelical, hydrophilic terminal domains, so-called variable regions.[5,20]

The SS_1 groups may exist in the hydrophilic variable region of the IF proteins because they act as intermolecular bonds only when the fiber is swollen; therefore, the crosslinks other than SS bonds are broken. The content of the SS_1 was 215 μmol/g, corresponding to about 35% of the total Cys content of the hair. The percentage of the IF proteins in the macrofibril in human hair was reported to be about 50%,[13] and the Cys content in the variable region is $\sim$ 60% of the total Cys in the IF proteins, 200 μmol/g.[19,21] The Cys content in the variable region was calculated to be $\sim$ 60 μmol/g on the basis of whole hair protein. This implies that there must be a large amount of the SS_1 crosslinkages in IFAPs. The SS_1 linkages are closely related to the network modulus, G, but insensitively respond to the fiber modulus in water, G_w. From these characteristic properties of SS_1 linkages, it seems to be appropriate to consider that the SS_1 crosslinkages in IFAPs occur in the most hydrophilic region of the macrofibril as the type of crosslinkages between IF and IFAP or between IFAPs. With respect to this, it was reported that the nonhelical tails of IF proteins spill over into the aggregates of IFAPs, and their proteins may interact with each other through the SS linkages,[22] and intermolecular SS crosslinkages exist between the globular proteins.[13]

The SS_2 group was also attributed to the intermolecular crosslinkages in hair, because the reduction of SS_2 linkages results in a simultaneous decrease in G and G_w. It must be noted that the hydrophilicity of the environment of SS linkages was markedly different between the regions of SS_1 and SS_2 groups because they were sequentially reduced with TGA. Therefore, the hydrophilicity of the environment for the SS_2 group was expected to be less than that of the SS_1 group. It was supposed that the SS_2 crosslinkages were localized on a relatively hydrophobically ordered α-helical rod domain in the IF proteins. However, the amount of SS_2 linkages (113 μmol/g) was too large value to explain the crosslinking sites only limited in the α-helical rod domain, because the Cys residues of the α-helical section involved about 40% of the total Cys in IF proteins,[1] which corresponds to $\sim$ 40 μmol/g on the basis of whole hair proteins. This clearly shows that a part of the crosslinkages attributable to the SS_2 group exist in IFAPs such as the intermolecular crosslinks between the individual globular proteins. Thus, multiplicate crosslinking structures can be proposed in the macrofibril of hair. It is especially important that two types of intermolecular SS linkages are predicted to exist in IFAPs. However, further study is needed to make clear the number, type, and location of the crosslinkages of SS_1 and SS_2 groups in IFAPs.

The SS_3 linkages are clearly localized within the most hydrophobic environment in keratin, that is, the hydrophobic region of the globular matrix proteins. The SS_3 linkages are intramolecular, but effective to the extension modulus of fiber in water. These coincide with the result that the globular proteins behave like filler particles in hair.[23,24]

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