NOTE

Proton Nuclear Magnetic Resonance Spectroscopic Analysis of Polypeptide Models of the Elastic Network Phase of Wool Fibers: Defining Cys-Residues Capable of Serving as a Crosslinking Agent

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Received 23 October 2000; revised 12 March 2001; accepted 12 March 2001

ABSTRACT: A polymer network structure of the matrix phase of wool fibers was modeled by polypeptide systems containing fragments of high sulfur (HS) proteins with Cys-residues oxidized in pairs to form a network. Our previous studies have shown that Cys-residues belonging to the two characteristic repeats (dipeptide and pentapeptide) from HS proteins of the matrix have a low propensity to serve as crosslinks of the matrix. This study reveals that Cys-residues located outside the two repeats have a higher propensity to serve as crosslinks of the network than Cys-residues belonging to the repeats. Effective crosslinking by the nonrepeat Cys-residues, on the one hand, and formation of intrarepeat loops by the residues of the repeats, on the other hand, can explain well-known macroscopic properties of the matrix phase, such as its elasticity and ability to swell in solvents. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 82: 1824–1828, 2001

Key words: biopolymer networks; high sulfur proteins of wool; NMR

INTRODUCTION

To understand the roles that different Cys-residues of high sulfur (HS) proteins of wool play in the matrix network formation,[1–5] a structure of a number of Cys-containing peptides from the HS proteins had been studied earlier[6–8] in reduced and oxidized states. It had been reported[6–8] that Cys-residues belonging to the two characteristic repeats of the HS proteins, the dipeptide repeat and pentapeptide repeat,[3] exhibit quite unusual behavior that makes the participation of Cys-residues of the repeats in the formation of a network as a crosslinking agent rather unlikely. In this work, the studies discussed previously[6–8] are continued for peptides from the HS sequences that do not contain the repeats, and the behavior of the nonrepeat Cys-residues is compared with that of the Cys-residues of the repeats.

There is a consensus that the cortex of wool fibers includes semicrystalline microfibrils imbedded into the amorphous elastic interfilament matrix.[1–5] Cys-residues of HS proteins of the matrix are oxidized in pairs to give a network with disulfide bridges as crosslinks. Cys-residues are present at ~20 mol % in their sequences which are dominated by two repeats — a dipeptide Cys-Cys (two Cys are in the adjacent positions on a chain) and a pentapeptide Cys-Xxx-Pro-Yyy-Cys (where Xxx and Yyy frequently are Gln and Thr, respectively). It has been shown[6–8] that Cys-residues of the repeats prefer to form intrarepeat loops to inter-

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Journal of Applied Polymer Science, Vol. 82, 1824–1828 (2001)

chain crosslinks and that this preference is exceptionally high for the dipeptide repeat. It has been also discussed[6–8] that the formation of an intrarepeat loop should effectively eliminate its two Cys-residues as potential half-crosslinks of a network. The latter may help to reconcile a long-standing controversy between the high number of oxidized Cys-residues (~20%) in the matrix and its high elasticity that is typical of a slightly crosslinked network of an order of 1% of a crosslinking agent or less. However, a question arises what specific Cys-residues perform the function of crosslinks in a weakly crosslinked network of the matrix. To answer this question we studied fragments of HS proteins containing only those Cys-residues of the sequences that do not belong to the repeats. Two peptides, TCLQTSGCETGCG (peptide 1, res. 67–79 in the B2 family of proteins) and ICSSVGTCGSSCGQPTCS (peptide 2, res. 12–29 from the same sequence) were investigated. Following the approaches developed earlier,[6–9] Cys-residues of these peptides were oxidized under two sets of conditions, in dilute solution (to avoid interchain contacts) and in bulk (to introduce the interchain contacts). Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) was used to monitor changes on oxidation. The most important result of this work is that Cys-residues outside the repeats, unlike Cys-residues of the repeats, [6] behave in a quite common way and are capable of forming interchain disulfide bonds in a concentrated phase. This result suggests Cys-residues outside the repeats can serve as crosslinks of the matrix network.

EXPERIMENTAL

Peptides

A 13-residue peptide **1** and an 18-residue peptide **2** from the sequences of the HS proteins of the matrix were synthesized and purified [>90% pure as determined by high-performance liquid chromatography (HPLC)] and characterized by liquid chromatography–mass spectrometry (LC-MS) and HPLC by Multiple Peptide Systems, Inc. (San Diego, CA).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Ac-T C L Q T S G C E T G C G-NH_2 (peptide 1, res. 67–79, B2 proteins)

Ac-I C S S V G T C G S S C G Q P T C S-NH_2 (peptide 2, res. 12–29, the same proteins)

The primary structure of peptides **1** and **2** was subsequently confirmed by our NMR experiments.

Oxidation

The experimental procedures in this paper are similar to those we applied for the repeats containing peptides in our earlier work.[6–8] Peptides **1** and **2** were oxidized in two different ways — in solution and in bulk. In the first case, oxygen was slowly bubbled into a 5 mM solution of a peptide in dimethylsulfoxide (DMSO; the peptides are not soluble in water) for 24 h at 25 °C. The completion of the oxidation was verified by the absence of the NMR signals for the reduced form. For the oxidation in bulk, the polypeptide system was slightly swollen in DMSO (or water) at 3.5–4.5 mg of peptide in 10–12 μL of the solvent and kept in the oxygen atmosphere for 48 h. The oxidation was performed directly in the NMR tube, and then NMR spectra of the unpurified sample were taken. The samples for liquid-state two-dimensional (2D) NMR studies were ~5 mM of a peptide in DMSO-d_6. To maintain the similar molar concentration for different samples needed for comparison, the prepared solution was divided into two or three equal parts that were placed into separate NMR tubes. One sample was kept unchanged and used as a control, and the other ones were oxidized under different conditions (or first dried, then oxidized).

NMR Spectroscopy

For 2D experiments, 350–512 incremental free induction decays (FIDs) of 1000 complex points were collected in a phase-sensitive mode. For TOCSY or clean-TOCSY,[10] the length of the soft 90° pulse was ~20 μs. The MLEV mixing time was ~50 ms. A nuclear Overhauser enhancement spectroscopy (NOESY) sequence at 200 ms mixing time was employed for the DMSO-d_6 solutions; all nuclear Overhauser effects (NOEs) were negative. Total correlation spectroscopy (TOCSY), double-quantum-filtered correlation spectroscopy (DQF-COSY), and NOESY pulse sequences were used for assignment.[11] Shifted cosine bell and skewed cosine bell functions were used for apodization. The signal of the residual water was presaturated in all spectra. For 2D spectra, the data were zero-filled to 1024 before the second Fourier transform.

RESULTS AND DISCUSSION

The assignment of ^{1}H NMR spectra of peptides **1** and **2** by conventional 2D NMR techniques (i.e., TOCSY, 2QF-COSY, and NOESY) was straightforward and for the amide region is given in Figures 1 and 2. Sharp differences between the initial linear peptides **1** and **2** and the systems obtained as the result of their oxida-

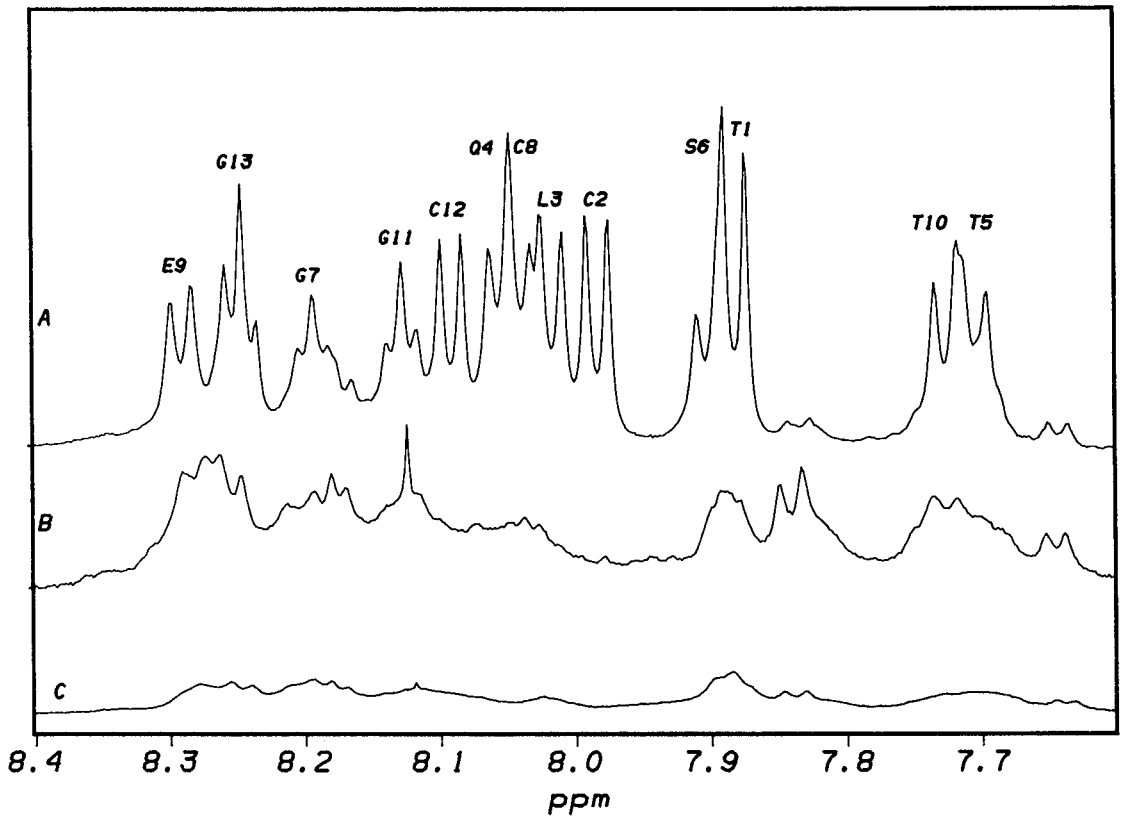

Figure 1 ^{1}H NMR spectra of (A) the initial linear peptide **1**, (B) peptide **1** oxidized in solution, and (C) the system obtained by the oxidation of peptide **1** in bulk. Each of the three samples have a similar amount of a peptide (i.e., ~5 mM in DMSO). The spectra were obtained under similar spectroscopic conditions.

tion can be seen in their 1D NMR spectra (Figures 1 and 2). The initial linear peptide **1** (spectrum 1A) is compared with the same peptide oxidized in solution (spectrum 1B) and with the peptide system obtained by oxidation of peptide **1** in bulk (spectrum 1C). The three samples have similar initial amounts of peptide **1** — the oxidation in solution and the oxidation in bulk were performed directly in the NMR tubes, and no peptide was added (or removed) in the process to any of the three tubes. The spectra were obtained under the identical spectroscopic conditions.

The samples were not similar in terms of their homogeneity. The solutions of initial peptide **1** was transparent and did not contain a visible precipitate, whereas the attempts to dissolve the peptides oxidized in bulk in different solvents (DMSO, DMFA, methanol, ethanol, chloroform, water at different pH) were not successful. The peptides oxidized in solution did not have visible precipitates.

The difference in the phase composition of the samples is reflected in the appearance of their NMR spectra. Whereas spectrum A is well resolved and the intensity of the lines is normal for linear peptides at similar concentrations, the intensity of spectra C are low and the lines are broad; that is, the signals practically vanish from spectrum C. The behavior of peptide **2** is similar to that of peptide **1** (Figure 2). Spectra of the samples oxidized in solution also have low intensity and broad lines prohibiting assignment (spectra 1B and 2B), though these lines are not as broad as in spectra 1C and 2C. These features are different from those we observed earlier under the identical conditions in the peptides containing the repeats.[6–8] Spectra for the peptides with repeats oxidized in solution had narrow lines and intensity normal for small peptides. For the dipeptide repeat, the latter was true even for oxidation in bulk.[6]

Line broadening on oxidation of peptides **1** and **2** can be caused, in general, by a number of factors, which include network formation, linear polymerization, or precipitation followed by possible conformational change induced by formation of an intramolecular cyclic structure. The latter two factors, however, seem unlikely to be dominant for the following reasons. Previous studies performed on similar peptides[6–8] have shown that the formation of intramolecular loops leave them soluble in DMSO and even in water, and they have normal NMR spectra with narrow lines typical of

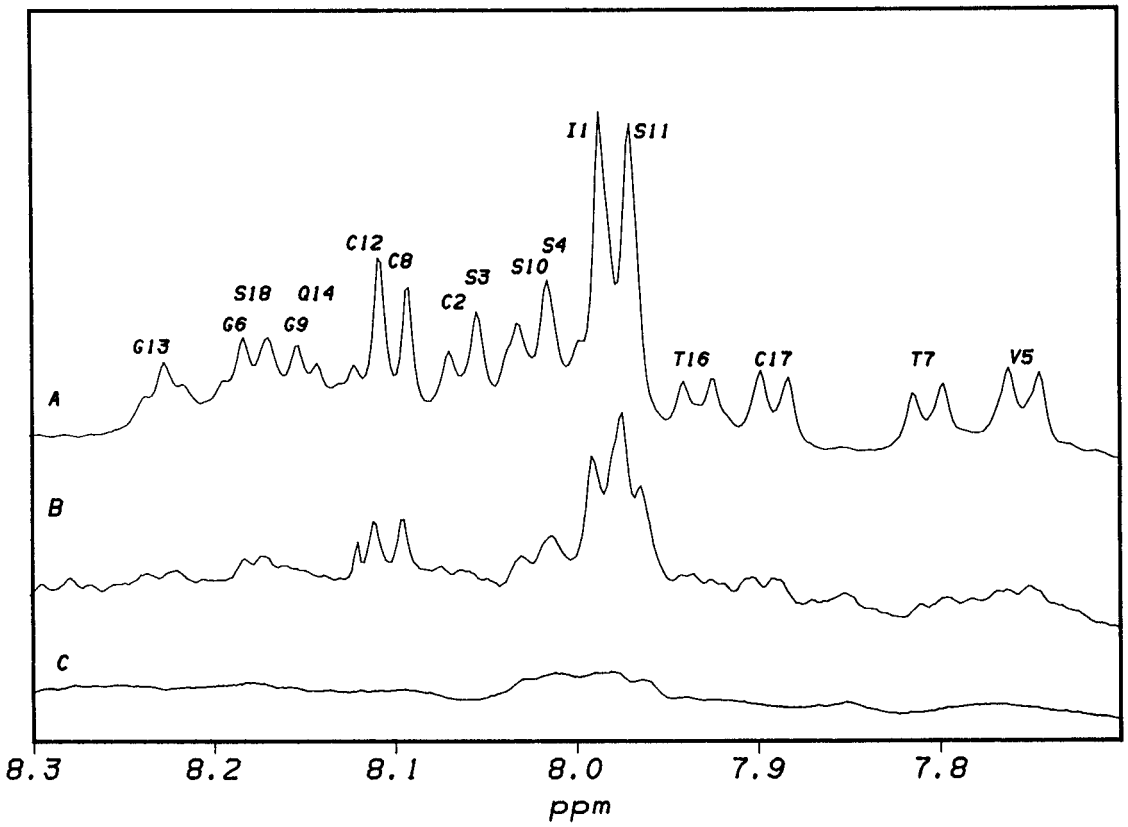

Figure 2 ^{1}H NMR spectra of (A) the initial linear peptide **2**, (B) peptide **2** oxidized in solution, and (C) the system obtained by the oxidation of peptide **2** in bulk. For experimental conditions see the Experimental section and the caption to Figure 1.

short peptides. The broad lines in spectra 1C and 2C, as well as in spectra 1B and 2B, suggest the presence of bonds different from only intrapeptide ones. Additionally, large linear sequences of the B2 family are also known to be soluble after the network structure of intermolecular disulfide bonds is destroyed.[1–5] However, as already mentioned, in the current work and in our previous reports,[6–8] our attempts to dissolve the precipitates in a range of organic solvents and in water at different pH were not successful. This result suggests that the structures formed here by oxidation are, most likely, not linear polymers, but rather networks. The presence of interchain bonding due to oxidation is also supported by the fact that the visible precipitates were formed only during the oxidation in bulk, where interchain contacts are plentiful, but not in solution, where interchain contacts are substantially reduced. More support for the notion that the precipitates are networks comes from swelling and solid-state NMR studies[6]of a precipitate of a similar nature. A ^{15}N NMR line for a similar peptide labeled with ^{15}N was found to have a shape typical of a slightly-to-moderately crosslinked polymer network (ref. 6, Figure 6). Though the details of the structure of the products of oxidation of peptides **1** and **2** remain to be studied by different methods, the presented data combined with the results of the previous studies of similar peptides[6–8] suggest that interchain crosslinking can be expected to be a major factor causing line-broadening here. Intrapeptide bonding and linear polymerization, most likely, occur at a certain level as well.

Peptides **1** and **2** behave in a quite mundane fashion by forming interchain bonds in bulk and some interchain bonds in solution. This behavior should be expected for short random-coiled peptides carrying more than two Cys-residues. Though there is nothing unusual about it, this result is important because an ordinary behavior of Cys-residues in peptides **1** and **2** is different from the special behavior of Cys-residues belonging to the repeats studied earlier.[2, 3, 6–8]

As shown earlier.[6] Cys-residues of the repeats prefer an intrarepeat disulfide bonding to the intermolecular one. Therefore, Cys-residues of the dipeptide repeat and pentapeptide repeat can be effectively eliminated as potential crosslinks because their participation in the formation of intrarepeat miniloops. Because only ~15% of all Cys-residues in the B2 proteins are located outside of the repeats, the remaining majority of 85% of the potential crosslinks of the network can be eliminated by oxidation. Therefore, relatively few Cys-resi-

dues can serve as crosslinks of the network. This inability to serve as crosslinks can explain the high elasticity of the matrix and its ability to swell in solvents despite a high content (20%) of the potential crosslinking agent (Cys-residues). Because two Cys-residues are needed to form a link, the effective content of the crosslinking agent can be estimated at ~1.5%. This estimate is in the range of a slightly-to-moderately crosslinked system, which is in agreement with the experimentally observed properties of the matrix.

It should be mentioned that for oxidation of some hypothetical enzymatic nature, the results could be quite different from those obtained here for oxidation by air.

CONCLUSION

The presented data suggest that Cys-residues of the HS proteins of wool outside the repeats differ from Cys-residues inside the repeats in terms of their ability to form interchain disulfide bonds. The nonrepeat Cys-residues are better candidates for serving as a crosslinking agent of the network of the matrix. The existence of several types of Cys-residues in terms of their propensity to serve as crosslinks of the matrix network explains the elasticity of the matrix and its ability to swell in solvents. The detailed structure of oxidation products of peptides from the HS proteins of wool needs further investigation.

ACKNOWLEDGMENT

This work was supported by grant 99-27-07400-M98P1 from the United States Department of Commerce/National Textile Center. I thank Michael Zimmerman and Bryan Frieman for their help with preparation of the samples and processing the data.

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