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ESR Spectrum Attributed to Trisulfide Neutral Radical [RSS(R)S•R] of Protein Observed for α-Keratin Present in White Human Hair

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ESR spectrum attributed to the trisulfide neutral radical of protein (RSS(R)S•R, $g_1 = 2.055$, $g_2 = 2.035$, and $g_3 = 2.000$) is observed for the first time for α-keratin present in completely dried white human hair exposed to near-UV radiation under a helium atmosphere.

The photoexcited state of tryptophan is considered to be an important electron source for the reduction of a disulfide bond in proteins.[1] The one-electron reduction of the disulfide bond results in the formation of sulfur-centered radicals. ESR spectra attributed to the thiyl[2] (sulfanyl) and disulfide radicals[3] have already been observed for proteins exposed to UV radiation. The trisulfide radical is expected to be an intermediate species formed during the exchange reaction of disulfide bonds, which have been regarded as a regulatory switch of protein functions. However, the ESR characteristics of the radical are still unknown.[4] Recently, we have reported that the disulfide radicals of α-keratin were stable in the matrix of completely dried white human hair (eq 1).[5] The disulfide radical is expected to be a source of the thiyl radical of α-keratin. In the present study, ESR measurements are performed on human hair exposed to near-UV radiation in order to detect the trisulfide radicals of α-keratin.

$$\mathrm{RSSR} + \mathrm{e}^- \leftrightarrow [\mathrm{RSSR}]^{-\bullet} \overset{\mathrm{H}^+}{\leftrightarrow} \mathrm{R^{\bullet}SSHR} \leftrightarrow \mathrm{RS^{\bullet}} + \mathrm{HSR} \quad (1)$$

Untreated human white hair (Beaulax Co., Ltd.) was washed with 5.0% sodium dodecyl sulfate and completely dried in a desiccator over P_2O_5.[6] The water content in the dried hair was minimized (4.5% by weight).[6] A lock of 100 hairs (33 mm, 19.0–21.0 mg) was bundled up with a copper film ($3.0 \times 8.0 \times 0.1\ \mathrm{mm^3}$) and was degassed in vacuo. The sample was then sealed in an ESR quartz cell under dry helium atmosphere and was exposed to near-UV radiation at 77 K.[5] A Xe-lamp (UVF-203S, Sanei Electric, wavelength; 255 to 390 nm, 0.78 $\mathrm{W\,cm^{-2}}$), equipped with a band-pass filter (RU-340, Sanei Electric), was used as the source of near-UV radiation. The sample was annealed at 313, 353, and 373 K by using a temperature control system (DVT2, JEOL). This system was used in combination with the Xe lamp to expose the hair to near-UV radiation at 373 K. ESR spectra were recorded at 77 K by using an X-band ESR spectrometer (TE-300, JEOL). 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl (Sigma Aldrich) was used as the primary standard of the total spin concentration.[7] ESR parameters were estimated as the mean value of three independent ESR measurements.

Before UV-radiation, the dried hair exhibited a weak isotropic ESR signal attributed to the organic radical at $g = 2.005$ (data not shown). Next, the ESR spectrum of the hair that was exposed to near-UV radiation at 77 K for 30 min after annealing at 313 K for 15 min was recorded. As shown in Figure 1a, the observed ESR spectrum revealed the presence of an anisotropic species with rhombic symmetry (defined as species I, $g_1 = 2.060$, $g_2 = 2.026$, $g_3 = 2.000$, and $A_2 = 0.8$ mT) and an almost isotropic species in the free spin region (defined as species II, $g = 2.005$).[5] In addition, a couple of weak shoulders were also recognized around 2.05 and 2.03, which could arise from a new paramagnetic species (defined as species III). After the duplicate integration of the ESR spectrum was carried out, the total spin concentration was estimated to be ca. 5.6×10^{17} (spin $\mathrm{g^{-1}}$).[8] The ESR parameters of species I was analogous to the disulfide radical of α-keratin present in human hair[5] and in nail[3] ($g_1 = 2.061$, $g_2 = 2.025$, $g_3 = 2.000$, $A_2 = 0.8$ mT). The possible structure of the radical was formulated to be either that of a disulfide neutral radical (RS•SHR) or that of a perthiyl radical (RSS•). On the basis of the proton doublet splitting, the structure is now confirmed to be that of the disulfide neutral radical. According to the *g*-value and ESR line-shape, species II

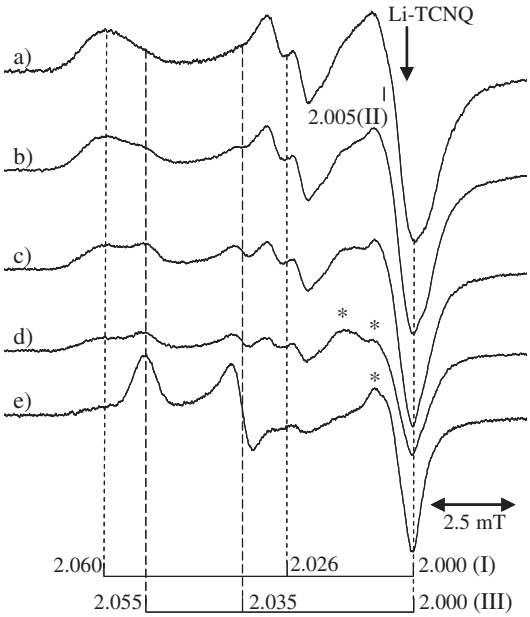

Figure 1. ESR spectra observed at 77 K for white human hair exposed to near-UV radiation at 77 K. Spectrum recorded after annealing the sample at (a) 313 K for 15 min, (b) 333 K for 15 min, (c) 353 K for 15 min, and (d) 373 K for 15 min. (e) ESR spectrum recorded at 77 K for the hair exposed to near-UV radiation at 373 K. The asterisks represent the free radicals derived from the protein present in human hair.

is safely classified as the anion radical of the disulfide group of α-keratin ($[RSSR]^{-\bullet}$).[3]

Next, the sample was annealed at 333 K for 15 min and ESR measurements were performed at 77 K. The observed ESR signal intensity of the both species I and II was slightly reduced, but the weak ESR signals of species III were still detected, as depicted in Figure 1b. The total spin concentration decreased to 5.0×10^{17} (spin g^{-1}) during annealing. The sample was then annealed again at 353 and 373 K for 15 min each. As shown in Figures 1c and 1d, the ESR signal intensity of species I and II further decreased, and the total spin concentration also decreased to 3.8×10^{17} and 2.7×10^{17} (spin g^{-1}), respectively. In contrast, ESR signal of species III exhibited a line shape typical of the rhombic symmetry. These results indicated that species III is a particular paramagnetic species formed during thermal decomposition of the disulfide radicals (species I and II).

In order to obtain a well-resolved ESR spectrum of species III, further ESR measurements were preformed by changing the conditions of near-UV radiation to the hair. When the hair was exposed to near-UV radiation at 373 K for 30 min, the ESR spectrum of species III could be recorded with weak signals attributed to species I, as depicted in Figure 1e. Thus, the g-values of species III were evaluated to be $g_1 = 2.055$, $g_2 = 2.036$, and $g_3 = 2.00$. In this case, the proton doublet splitting in the g_2 component completely disappeared, suggesting that species III had no protons near the radical center unlike species I.

The g-separation of species III was slightly smaller than that of species I, but the averaged g-value of species III ($g_{ave} = 2.029$) was in excellent agreement with that of species I ($g_{ave} = 2.030$). This provided experimental evidence that species III had an electronic structure that resembled that of species I. In addition, the g-values of species III agreed well with those of trisulfide anion radical ($S_3^{-\bullet}$) that was trapped in the cavity of zeolite.[9] Recently, the g-values of a radical trapped in a cavity of sodalite was accurately evaluated to be $g_1 = 2.051$, $g_2 = 2.038$, and $g_3 = 2.002$, by performing W-band ESR (94.5 GHz) measurements.[10] In terms of the g-parameters, species III can be classified as a trisulfide radical composed of two disulfide bonds, where the unpaired electron is expected to be located in the σ^* orbital of the terminal sulfur atom.[11]

As described above, species III was considered to be a paramagnetic species formed by the thermal decomposition of species I and II. The S–S bond of the disulfide radicals essentially tend to cleave to form thiyl radical.[12] Because the bond order of the S–S bond of species I was weakened by at least half compared to that of a normal disulfide bond, the S–S bond can be thermally cleaved to the thiyl radical. However, under aerobic conditions, the thiyl radical readily reacts with oxygen to form cysteic acids.[13] Under the anaerobic condition used in the present study, the extended life-time of the thiyl radical might enable the addition of the neighboring disulfide bond to form the trisulfide neutral radical. In addition, the glass transition of the dried hair ($T_g \approx 360$ K)[6] also affects the formation of the S–S bond linkage between the thiyl radical and disulfide moiety. Therefore, species III is formulated to be RSS(R)S$^{\bullet}$R, where R is the amino acid residue of cystein present in α-keratin (eq 2). Taking into account the resonance structures of species III, the bond-order of the S–S bond can be reduced by at least a quarter compared to that of a normal disulfide bond. The S–S bond order of species III can be larger than that of species I. This trend is consistent with that obtained from the observation results; that is, above 353 K, species III is more stable than species I.

$$\begin{aligned} R^1S^{\bullet} + R^3SSR^4 &\leftrightarrow R^1SS(R^3)S^{\bullet}R^4 \\ &\uparrow\downarrow \\ R^1S^{\bullet}S(R^3)SR^4 &\leftrightarrow R^1SSR^3 + {}^{\bullet}SR^4 \end{aligned} \qquad (2)$$

The trisulfide neutral radical is often thought to be an intermediate formed during the exchange reaction of disulfide bonds; this reaction proceeds via an addition and elimination sequence, as expressed in eq 2.[4] The results of fast pulse radiolysis revealed that the trisulfide neutral radical derived from cystein exhibited an absorption maximum around 380 nm.[14] However, so far, ESR spectrum ascribed to the trisulfide neutral radical has never been detected for proteins and small molecules. Consequently, ESR spectrum of species III observed for α-keratin present in white human hair is the first example for the detection of ESR spectrum attributed to the trisulfide neutral radical in protein. The present findings have confirmed the formation of the trisulfide neutral radical by the reaction of a thiyl radical and disulfide group in protein. It is, therefore, concluded that the trisulfide neutral radical is a probable intermediate species in the exchange reaction of disulfide linkage in protein. Further investigations on the chemical reactivity of the radical are now in progress.

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