Radiolytic Degradation of Cystathionine Irradiated with Monochromatic Soft X-rays at the K-shell Resonance Absorption of Sulfur

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The degradation of cystathionine was investigated using irradiation with monochromatized synchrotron X-rays at 2472 eV and 2466 eV. 2472 eV corresponds to the energy of the K-shell resonance absorption peak of the sulfur atom. The energy at 2466 eV was adopted as a reference. Cystathionine has a sulfur atom which joins two amino acid residues. This form is useful for analyzing the effect of degradation that originates from photoexcitation in the sulfur atom. The degradation products of cystathionine were detected by high performance liquid chromatography (HPLC). Of the products present, α-aminobutyric acid was produced threefold on irradiation with 2472 eV X-rays, as compared with the value for irradiation at 2466 eV and the same exposure. Almost the same amount of glycine was produced at the two irradiation energies. The yields of these two products were analyzed on the basis of the amount of cystathionine degraded and the number of photons absorbed by sulfur and the other elements in cystathionine. Cleavage of a bond was shown to depend on whether the bond includes an atom that absorbs the X-ray photon.

INTRODUCTION

The relation between energy deposition on a specific molecule and its biological consequence was studied in order to understand the biological effects of electromagnetic radiation and their underlying molecular processes. The process of radiolysis by ionizing radiation cannot be analyzed completely because of difficulty in identifying the atom in the molecule that absorbs the radiation energy. An exceptional case is the absorption of X-ray energy in a defined atom by inner-shell photoelectric absorption. After inner-shell
photoabsorption occurs in elements of low atomic number, the atom emits Auger electrons with ranges of less than a few micrometers, the atom eventually undergoing multiple ionization. Studies on the effects of inner-shell photoabsorption have been very few, however, because of the lack of an intense soft X-ray source with a continuous spectrum. Synchrotron radiation, however, has become available as a source of intense soft X-rays that permit use of the desired photon energy with an appropriate monochromator.

Halpern and Stöcklin\(^1\) reported that the radical yield in solid 5-bromodeoxyuridine irradiated with monochromatic X-rays increased near the absorption edge of bromine. They suggested the importance of multiple-charged atoms in this increase. Deiodination in irradiated iodoamino acids in a solid also has been reported to increase discontinuously at the K-absorption edge of iodine\(^2\). In neither case was change in the reaction pathways associated with inner-shell absorption of a specific atom suggested. In the gas phase, however, change in the fragmentation pattern of the molecule has been reported to depend on the photo-absorbing atom in the molecule\(^3\). The study presented here is focused on whether the fragmentation pattern of a molecule in a solid depends on the photo-absorbing atom.

As the target molecule, we used cystathionine, an amino acid with a sulfur atom. The cystathionine molecule is composed of two amino acid moieties joined by a sulfur atom (Fig. 1). Cystathionine gives various amino acid products depending on the site of breakage. These products are readily analyzed by the usual HPLC. We observed changes in the spectrum of products associated with the photoabsorption by sulfur when irradiated with monochromatic synchrotron soft X-rays. The relation between the cleaved bond and the atom which absorbs the X-ray photon is discussed.

**MATERIALS AND METHODS**

*Chemicals and preparation*

The irradiated material was cystathionine (Fig. 1) which is comprised of two amino acid products:

- Glycine
- Ethyl cysteine
- Alanine
- Methyl cysteine
- Alamine
- Homocysteine
- α-aminobutyric acid
- Cysteine

![Molecular formula of cystathionine](https://example.com/cystathionine.png)

**Fig. 1.** Molecular formula of cystathionine. The relation of each cleavage site to the expected products is shown by arrows.
acids, alanine and α-aminobutyric acid, joined together by a sulfur atom. The first group of radiolytic products is made up of fragments that have a hydrogen atom attached to the cleaved site (Fig. 1). The second group is composed of fragments having the hydroxyl group, instead of hydrogen; e.g., serine and homoserine.

The cystathionine and other amino acids used as the expected degradation products were obtained from Sigma Co. (St. Louis, U.S.) and used without further purification. Special grade chemicals from Wako Chemical Industries, Ltd. (Osaka, Japan) were purchased for the high performance liquid chromatography (HPLC) buffer preparations. All the solutions used were prepared with double-deionized, distilled, ultrapure water (resistance, 18MΩ).

**Monochromatic soft X-rays**

Synchrotron radiation at the Photon Factory (Tsukuba, Japan) was used as the light source to irradiate the sample with monochromatic soft X-rays. This radiation was monochromatized in an InSb double crystal monochromator at the beamline 11B. The beam size at the sample position was adjusted to 6(h)×4.5(v) mm² by a slit system. The soft X-ray intensity was measured in an ionization chamber after passing the beam through thin films into air. The intensity at the sample position was estimated taking into account the absorptions of the air and the films in the beam path. The fluence rate at the sample position was $2.1 \times 10^{18}$ photons per minute per m² (about $2.6 \times 10^2$ C/kg/min or 1MR/min) for a ring current of 200 mA. The energy resolution was about 2 eV at 2.5 keV. Contamination of the higher harmonics was less than 0.1%. The energy of the monochromatized soft X-rays was calibrated using the photoelectron emission spectrum of molybdenum disulfide.

**Selection of irradiation energies**

To determine the irradiation energies, the absorption spectrum of cystathionine was measured at the K-shell absorption edge of sulfur. A thin layer of cystathionine (surface density $5 \times 10^{-2}$ kg/m²) was formed on a thin film (5 μm) of mylar. This sample was placed just in front of the ionization chamber in the beam path. The values obtained are given in Fig. 2. A sharp absorption peak was observed at 2472 eV (as reported by Dehmer³) together with the K-shell absorption edge structure of sulfur. We selected the soft X-ray energy of this peak as one irradiation energy because of its large absorption cross section; the other energy selected was 2466 eV, which was not absorbed by the K-shell of sulfur. The two energies are so close that the absorption cross sections of all the atoms constituting cystathionine (except sulfur) are considered to be the same.

**Photoabsorption cross section of cystathionine**

This cross section at 2466 eV was calculated by summing up the cross sections of individual elements⁶ that had been multiplied by the number of atoms in the molecule. We estimated the cross section of cystathionine at the observed resonance absorption peak, 2472 eV, as in an earlier report⁷. First, the difference in the absorption cross section was calculated for the energies above and below the K-edge of sulfur using the values of Scofield⁹. The calculated difference then was multiplied by the experimentally obtained ratio (2.6)
of the peak height of the resonance absorption to the absorption difference between 2460 and 2500 eV (Fig. 2). The cross section obtained for the sulfur resonance peak was added to the calculated cross sections of cystathionine at 2460 eV, giving absorption cross sections for the molecule of $3.3 \times 10^{-23}$ m$^2$ at 2472 eV and $8.7 \times 10^{-24}$ m$^2$ at 2466 eV.

Irradiation of the sample

A 5 µl sample of the cystathionine solution (10 mg/ml in water) was dried in a 4(h)×2(v) mm$^2$ area on an aluminum sample plate. Each plate was set on a sample holder, then placed in a sample irradiation chamber and irradiated in vacuo. The surface density of the sample was $6.25 \times 10^{-3}$ kg/m$^2$. Secondary electron equilibrium was considered to hold when the sample was irradiated with 2.5 keV X-rays because the maximum range of the secondary electrons is less than $3 \times 10^{-4}$ kg/m$^2$.

On absorption, the intensity of the monochromatic soft X-rays decreased. The respective transmittances of the sample at 2472 and 2466 eV were calculated to be 0.767 and 0.864, based on the values for the mass energy coefficient ($\mu en/\rho$) of cystathionine. The ($\mu en/\rho$) at 2466 and 2472 eV was calculated by the method used to obtain the absorption
cross section, but using the μen/ρ value for each element\(^9\). The exposure values described hereafter are adjusted for attenuation in the sample.

**Product analysis with HPLC**

Irradiated samples were analyzed in an HPLC system (Type LC-6A, Shimadzu) equipped with an ion-exchange column (Li type, Shimpack ISC-07/S1504Li, Shimadzu) in a temperature-controlled box to detect the cystathionine degradation products. Two lithium citrate buffers with different pHs were used for the mobile phase in order to obtain a gradual change in pH from 2.6 to 10.0 within 140 minutes. The solution eluted was mixed with a fluorescent o-phthal aldehyde (OPA) solution after which the fluorescent products detected in a fluorescent spectrophotometer were used as a measure of the quantity of amino acid present (OPA method\(^10\)). The detection limit under our conditions was about 10 pmol.

**Retention times of fragments of cystathionine**

The authentic amino acids expected as fragmentation products were injected into the ion-exchange column to determine their retention times. The names of the amino acids are given on the abscissa (retention time) of the chromatograms obtained (Fig. 3). Of these amino acids, the retention times of cysteine and methylcysteine were almost the same, as were those of alanine and ethylcysteine. When a mixed solution of cysteine and methylcysteine (or alanine and ethylcysteine) was injected, the peaks could not be separated. No homocysteine was found under the analysis conditions used. The conversion factors used to obtain the amount of amino acid in the peak area were obtained by measuring the peak areas of the quantities of the various authentic amino acids.

**RESULTS**

On irradiation of the sulfur K-shell resonance absorption peak (2472 eV), the color of the samples changed from white to yellow, the yellow becoming deeper as exposure increased. This color change was not observed on irradiation at an energy just below the peak (2466 eV) for the maximum exposures used. We next analyzed the degradation products of cystathionine using HPLC.

The chromatograms of a sample irradiated at the resonance absorption peak (exposure, \(3.77\times10^4\) C/kg) and one irradiated below the peak energy (exposure, \(4.59\times10^4\) C/kg) are shown in Fig. 3. Various products were presented as peaks on these chromatograms as well as the remaining cystathionine. Using the peak area of the remaining cystathionine we determined the degradation rate of the sample. At the resonance absorption peak, this rate was 1.9 times greater than the value for energy below the peak energy. The amount of degraded cystathionine at an exposure of \(5\times10^4\) C/kg was calculated to be 33 nmol at the resonance absorption peak, and 17 nmol at energy below the peak energy.

All the expected products shown in Fig. 1 were presented on the chromatograms. The
integrated peak areas increased with exposure, except for methionine and serine which were separate even on chromatograms of unirradiated samples. The spectrum of the peak height at 2472 eV (Fig. 3) clearly differs from that at 2466 eV.

In the case of glycine and aminobutyric acid, the peaks of which are separate from the others on the chart, the production efficiencies calculated are shown in column one of Table 1. The production efficiency of α-aminobutyric acid per exposure at 2472 eV was 3.0-fold the value at 2466 eV, whereas the efficiency of glycine at 2472 eV was almost the
same as at 2466 eV. The efficiencies obtained were used to calculate the amounts of α-aminobutyric acid and glycine produced at an exposure of $5 \times 10^4$ C/kg in order to get the fraction of the products per degraded cystathionine molecule. The amount of aminobutyric acid was 7.4 nmol $(1.5 \times 10^{-13} \text{ mol-kg/C}) \times 5 \times 10^4 \text{ (C/kg)}$ at 2472 eV and 2.5 nmol at 2466 eV. The ratio (%) of the aminobutyric acid produced to the amount of degraded cystathionine was 22% at 2472 eV; 1.5-fold the value (15%) at 2466 eV. For glycine, however, the ratio (0.7%) at 2472 eV was smaller than the value (1.2%) at 2466 eV. These ratios are given in Table 2. The analyses clearly show that the product yields depend on the irradiation energy even though the difference may be as small as 6 eV.

Many unidentified peaks were presented on the chromatograms, most of which increased with exposure. As indicated by the arrows in Fig. 3, two clear peaks were present on the chromatogram at the resonance absorption peak that were not distinct below the peak energy.

DISCUSSION

The chromatograms of the solid cystathionine irradiated with monochromatic soft X-rays showed that the degradation pattern at the K-shell resonance absorption peak of sulfur (2472 eV) differs from that at the energy (2466 eV) just below the resonance peak (Fig. 3). This was confirmed by calculating the production efficiencies per unit exposure of aminobutyric acid and glycine (column 1, Table 1) and the production efficiencies based on the amount of cystathionine degraded (Table 2). The coloration of the irradiated sample at the resonance absorption peak also supports this difference.

We estimated the quantum yield of the products in order to determine the change in the fragmentation mechanism. The production cross sections shown in column 2, Table 1 were obtained by dividing the production efficiencies by the photon flux per exposure. The values were converted using the absorption cross sections to the product yield per photon absorbed in cystathionine at both energies (column 3, Table 1). The yield for aminobutyric acid at 2472 eV is a little less than that at 2466 eV although the production efficiency per unit exposure at 2472 eV was threefold that at 2466 eV because of the larger cross section at 2472 eV. The yield for glycine at 2472 eV is one-third that at 2466 eV. We also estimated the yield per photon absorbed only by the sulfur K-shell, taking advantage of the fact that the absorption cross sections of atoms are almost constant at the two irradiation energies, except that of the sulfur K-shell. The yield was obtained from the difference in the production cross sections (column 2, Table 1) at 2472 and at 2466 eV divided by the photoabsorption cross section of the sulfur K-shell, as shown in the equation:

$$(\text{Yield by sulfur K-shell photoabsorption}) = \frac{(\text{Production cross section at 2472 eV}) - (\text{Production cross section at 2466 eV})}{(\text{K-shell resonance absorption cross section of sulfur})}$$
Table 1. The production efficiency per unit exposure, production cross section, quantum yield, and yield for the resonance absorption of the sulfur of $\alpha$-aminobutyric acid and glycine.

<table>
<thead>
<tr>
<th>Product</th>
<th>Efficiency per unit exposure ($\times 10^{-15}$ mol-kg/C)</th>
<th>Production cross section ($\times 10^{-24}$ m²)</th>
<th>Quantum yield</th>
<th>Yield for sulfur resonance absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-aminobutyric acid</td>
<td>2472eV 2466eV</td>
<td>2472eV 2466eV</td>
<td>6.9 8.8</td>
<td>5.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.8 4.0</td>
<td>7.4 6.1</td>
<td>0.22 0.69</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 2. Ratios of the amount of product to the amount of the decrease in cystathionine on monochromatic soft X-ray irradiation.

<table>
<thead>
<tr>
<th>Product</th>
<th>Ratio of the amount of product to the amount of cystathionine decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-aminobutyric acid</td>
<td>2472eV 2466eV</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.7 1.2</td>
</tr>
</tbody>
</table>

The yields obtained are listed in the last column of Table 1. The yield at 2466 eV irradiation can be regarded as the yield per photon absorbed by atoms other than sulfur because the contribution of the sulfur L-shell is small in the total photoabsorption cross section.

The yield of aminobutyric acid produced by sulfur K-shell absorption (5.9) was somewhat less than the yields produced by other atoms (8.8). For glycine, the yield produced by sulfur K-shell absorption (0.05) was much smaller than that produced by the absorption by other atoms (0.69). These values indicate that (1) the C-C bond cleavage that produces glycine as a product almost never occurs in sulfur K-shell photoabsorption and (2) the cleavage efficiency of the S-C bond is not sensitive to the atomic site of photoabsorption in the molecule. The distinct decrease in the yield of glycine is evidence of the change in the degradation pathway associated with the inner shell photoabsorption of sulfur.

That the yield of $\alpha$-aminobutyric acid in both cases of absorption exceeds unity appears to indicate the participation of photoelectrons, Auger electrons from neighboring molecules, or radical reactions in the production of $\alpha$-aminobutyric acid. Such processes
would mask the specific effect of sulfur inner-shell photoabsorption. For a detailed analysis of the degradation process, the complete identification of the peak present in the chromatogram would be necessary.

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REFERENCES