Interaction of Lactoferrin with Ascorbate and the Relationship with Bleomycin-Dependent DNA Damage

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The interaction between bovine lactoferrin (bLF) and ascorbate (Asc) was investigated through malondialdehyde (MDA) formation in a solution containing DNA, bleomycin (BLM), and Fe$^{2+}$ or Asc. The inhibition by bLF on MDA formation in the presence of Asc was not changed even by adding carbonate or oxalate ions to the solution. The percentage inhibition by the hydrolysesates of bLF treated with pepsin, trypsin, and both enzymes on MDA formation was almost the same as that by the untreated bLF in the presence of Asc. The inhibition of MDA formation also occurred with the filtrate obtained from a solution containing bLF and Asc, but not with that from a solution of bovine serum albumin and Asc. The interaction of bLF and Asc was observed by gel filtration in a Sephadex G75 column. The binding amount of Asc was estimated to be 87 mol per molecule of bLF.

Lactoferrin (Lf) is a metal-binding single-chain glycoprotein. The molecule is folded into two globular lobes, representing the N-terminal and C-terminal halves (the N-lobe and C-lobe). Each lobe has a deep cleft between two dissimilar domains. In the internal cavity of the cleft, one specific site exists to strongly bind Fe$^{3+}$. The cavity allows sufficient flexibility for the accommodation of such anions as carbonate and oxalate.1)

We have reported that malondialdehyde (MDA) formation occurred in the solution containing DNA, bleomycin (BLM), and ascorbate (Asc), but was inhibited by adding 10 mM EDTA to the solution.2) It has been suggested that MDA formation is caused by the attack of hydroxyl radicals generated by the complex of BLM and Fe$^{2+}$ on DNA bases in the presence of oxygen.3-5) Therefore, we have considered that Asc actually behaves as a reductant of iron ion in the solution containing 11 μM iron as a contaminant from the reagents. Lf strongly inhibited MDA formation on BLM-dependent DNA damage, probably because of the presence of specific sites to bind Fe$^{3+}$. However, serum albumin (SA), α-lactalbumin, and β-lactoglobulin did not inhibit MDA formation. These proteins consisting of a single polypeptide chain have been also reported to bind metal ions,6-8) but such a cleft to incorporate metal ions and anions is absent in their molecules. Therefore, the internal cavity of the cleft in Lf molecule may play a role in inhibiting MDA formation.

This paper reports the inhibition of MDA formation by bovine Lf (bLF) in the presence of carbonate or oxalate ions together with Asc, and that by their enzymatic hydrolysesates in the presence of Fe$^{2+}$ or Asc. The interaction of bLF and Asc was further investigated by ultrafiltration and gel filtration of the solution containing these components. Bovine serum albumin (bSA), which does not inhibit MDA formation, was also used to compare with bLF.

Materials and Methods

Materials and reagents. Bovine apolactoferrin (bLF), presented by Nutritional Science Lab. of Morinaga Milk Industries Co.) was used after being chromatographed in an SP-Toyopearl 650C column (2.0 x 40.0 cm). Bovine serum albumin (bSA) and pepsin (10,000 units/g) were purchased from Wako Pure Chemical Industries, and trypsin (6,630 units/g) was from Sigma Chemical Industries. C-lobe prepared from bLF was presented by Professor K. Shimazaki, Hokkaido University. Each reagent of L-type amino acids and ascorbate (Asc) was obtained from Wako Pure Chemical Industries, and sodium carbonate (CO$_3$)$^2-$ and oxalate (CO$_2$)$^2-$ were from Tokyo Kasei Industries. Chelex 100 chelating resin was obtained from Bio-Rad Laboratories. Acrylamide, N,N'-methylene-bis(acrylamide), Coomassie Brilliant Blue R-250, dodecyl sodium sulfate (SDS) and MW-marker (Oriental Yeast Co.) were purchased from Wako Pure Chemical Industries, and N,N',N'-tetramethylnediamine was from Tokyo Kasei Industries.

BLM assay. The assay was done by the method of Matsue et al.2) Briefly, 0.1 ml of DNA (1 mg/ml), 0.02 ml of BLM (0.67 mM), and 0.1 ml of 2.5 mM Asc or 10.0 mM Fe$^{2+}$ were mixed in a plastic tube in that order, before adding a certain volume of a 24 mM phosphate buffer (pH 7.4, containing 0.15 M NaCl) to give a final volume of 1.0 ml. The other reagent solutions of bLF, CO$_3$)$^2-$ and (CO$_2$)$^2-$ and various amino acids were put into the tube before adding an Asc solution. The mixture of bLF and CO$_3$)$^2-$ or (CO$_2$)$^2-$ was left for 30 min at room temperature before being added to the reaction mixture. After the mixture of reagents was incubated at 37°C for 20 min in a shaking water bath (Advantec TC-1 incubator), 1.0 ml of TBA (1%, w/v; in a 50 mM NaOH) and 1.0 ml of HCl (25%, v/v) were added to the solution. The contents of the tube were transferred to a glass tube with a screw cap, and heated at 100°C for 15 min before being cooled. The resulting chromogen was extracted into 3.0 ml of 1-butanol, and the absorbance at 532 nm was measured with a spectrophotometer (Shimadzu Seisakusho, UV-190). The assay was done at 1.0 mM of Fe$^{2+}$ and 0.25 mM of Asc.

All water used in the experiments was treated in a Chelex column. DNA, BLM, and the reducing agents were dissolved in the phosphate buffer immediately before being used.

The amount of TBA-reactive substances was expressed as MDA, a calibration curve for converting the absorbance at 532 nm into MDA equivalents prepared by using 1,1,3,3-tetraethoxypropane by the method of Gajewski et al.5)

Influence of CO$_3$)$^2-$ or (CO$_2$)$^2-$ on bLF inhibition of BLM-dependent DNA damage. A solution of 1% bLF, 2.5 mM sodium carbonate, and 2.5 mM oxalate in the phosphate buffer was used for the assay. A mixture of 0.3 ml of 1% bLF and 0.1 ml of 2.5 mM sodium carbonate or oxalate was also used after it was left for 30 min at room temperature.
Enzymatic hydrolysis of bLf and bSA. The 1% solution of bLf or bSA adjusted to pH 3.0 with 0.1 N HCl was added to pepsin (the enzyme/protein ratio was 1:100), and was incubated at 37°C for 3h. Ten milliliters of the solution were then adjusted to pH 7.8 with 0.1 N NaOH and then trypsin was added at the same ratio, before adding the buffer to a final volume of 20 mL. Each solution of bLf and bSA treated with pepsin or trypsin was also prepared as already described. After the treatment, the same volume of the solution and 20% TCA was mixed and then left for 30 min at room temperature. The mixture was centrifuged for 20 min at 3000 rpm, and the absorbance of the supernatant (B) and the untreated bLf solution (A) at 280 nm were measured with the spectrophotometer. The percentage digestion was calculated as B/A x 100. The enzyme-treated solution was used for the BLM assay after being freeze-dried.

Binding of Asc to bLf and bSA molecules. Binding of Asc to bLf or bSA molecules was observed by ultrafiltration with a centrifuge tube (Ultrafree C3, Millipore Co.) and gel filtration in a Sephadex G75 column (2.5 x 40 cm). To examine whether Asc is absorbed to the filter membrane of a tube and oxidized during ultrafiltration, the BLM assay was first done by using the Asc solution and its filtrate. Asc was dissolved with the phosphate buffer to make the concentration of 0.1 to 0.5 mm. Each solution in a tube was centrifuged at 4000 rpm for 1 h (Himac Centrifuge Hitachi CT4D), the filtrate being used for the BLM assay.

Next, the effects of the filtrate obtained from the Asc solution containing bLf or bSA on MDA formation were observed. A certain volume of 1.0% bLf or bSA was mixed with 0.1 mL of 25 mm Asc in a tube, and the phosphate buffer was added to give a total volume of 1.0 mL. After this was left at room temperature for 30 min, 0.4 mL of the mixture was centrifuged as already described, and the filtrate was also used for the BLM assay.

Binding of Asc to the bLf molecule was examined by using the solution containing 0.1% bLf and 0.04 to 0.5 mm Asc. A linear relationship was found between the content of MDA formed and the Asc concentration in the reaction mixture with the Asc solution and its filtrate (Fig. 2). Therefore, the amount of free Asc in the solution containing Asc and bLf was obtained by measuring the content of MDA formed for the BLM assay with the filtrate. The amount of Asc bound to bLf was obtained by subtracting from the total amount of Asc in the solution for the amount of free Asc. The concentration of bLf and bSA in the solution was adjusted by using the extinction coefficient (1%, 1 cm) of 12.7 and 6.0 at 280 nm, respectively. A molecular weight of 83,100 was used for bLf.

Gel filtration was done by using a solution containing 1, 3, or 5 mg of bLf in 4.0 mL of the phosphate buffer with 0.5 mm Asc. The solution was put on a Sephadex G75 column, and was eluted with the phosphate buffer, monitoring at 280 nm (Atto Bio Mini UV monitor AC-5200S).

SDS-polyacrylamide gel electrophoresis. The electrophoreses of bLf and the enzymatic hydrolysates were done at a gel concentration of 12.5%, the proteins then being stained with Coomassie brilliant blue R-250.

Results and Discussion
Effects of CO$_3^{2-}$ and (COO$^{-}$)$_2$ on bLf inhibition of DNA damage

The formation of MDA caused by BLM-dependent DNA damage in the presence of such anions as CO$_3^{2-}$ and (COO$^{-}$)$_2$ together with Asc was observed as shown in Table I. The formation of MDA markedly increased in the presence of Asc, but did not occur in the presence of CO$_3^{2-}$ or (COO$^{-}$)$_2$ (No. 1, 2, and 3 in the table). Addition of the anions to the reaction mixture containing Asc did not influence MDA formation (No. 4 and 5). However, addition of bLf to the reaction mixture containing Asc strongly inhibited MDA formation (No. 6). In the reaction mixture containing bLf and Asc with CO$_3^{2-}$ or (COO$^{-}$)$_2$, the percentage inhibition of MDA formation was almost the same as that in the reaction mixture containing bLf and Asc (No. 7 and 9). Further, the mixture of both bLf and CO$_3^{2-}$ or (COO$^{-}$)$_2$ was added together with Asc to the reaction mixture. The percentage inhibition of MDA formation was almost the same as that in the reaction mixture containing bLf and Asc (No. 8 and 10). From these results, the inhibition of MDA formation might be not caused by incorporating Asc into a deep cleft of bLf.

Table I. Effects of Ascorbate (Asc), Carbonate (CO$_3^{2-}$) and Oxalate (COO$^{-}$)$_2$ Ions on Malondialdehyde (MDA) Formation in the Reaction Mixture Consisting of DNA and Bleomycin in the Absence or Presence of bLf

<table>
<thead>
<tr>
<th>No.</th>
<th>Solution</th>
<th>MDA (nmol/mL)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Asc</td>
<td>1.047</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>CO$_3^{2-}$</td>
<td>0.000</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>(COO$^{-}$)$_2$</td>
<td>0.008</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>Asc + CO$_3^{2-}$</td>
<td>1.057</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>Asc + (COO$^{-}$)$_2$</td>
<td>1.200</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>Asc + bLf</td>
<td>0.020</td>
<td>98.1</td>
</tr>
<tr>
<td>7</td>
<td>Asc + bLf + CO$_3^{2-}$</td>
<td>0.016</td>
<td>98.5</td>
</tr>
<tr>
<td>8</td>
<td>Asc + [bLf + CO$_3^{2-}$]</td>
<td>0.020</td>
<td>98.1</td>
</tr>
<tr>
<td>9</td>
<td>Asc + bLf + (COO$^{-}$)$_2$</td>
<td>0.082</td>
<td>92.2</td>
</tr>
<tr>
<td>10</td>
<td>Asc + [bLf + (COO$^{-}$)$_2$]</td>
<td>0.020</td>
<td>98.1</td>
</tr>
</tbody>
</table>

The concentrations of Asc, CO$_3^{2-}$, and (COO$^{-}$)$_2$ were 0.25 mm, and that of bLf was 3 mg/mL. The reagents in the parentheses were mixed and then kept for 1 h at room temperature.

Table II. Inhibitory Effects of Bovine Lactoferrin (bLf) and Serum Albumin (bSA) Treated with Pepsin, Trypsin, and Both Enzymes on MDA Formation for the Bleomycin Assay in the Presence of Fe$^{2+}$ or Ascorbate (Asc)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Enzyme used</th>
<th>Percentage digestion (%)</th>
<th>Inhibition (%) Fe$^{2+}$</th>
<th>Asc</th>
</tr>
</thead>
<tbody>
<tr>
<td>bLf</td>
<td>None</td>
<td>0</td>
<td>81.6 (94.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pepsin</td>
<td>54.5</td>
<td>68.3 (93.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>27.7</td>
<td>68.2 (97.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pepsin + trypsin</td>
<td>76.8</td>
<td>59.3 (93.7)</td>
<td></td>
</tr>
<tr>
<td>bSA</td>
<td>None</td>
<td>0</td>
<td>50.0 (182.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pepsin</td>
<td>47.2</td>
<td>19.0 (191.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>17.1</td>
<td>12.5 (209.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pepsin + trypsin</td>
<td>86.3</td>
<td>25.5 (198.8)</td>
<td></td>
</tr>
</tbody>
</table>

The concentrations of Fe$^{2+}$, Asc and the hydrolysates for the assay were 1.0 mm, 0.25 mm, and 3.0 mg/mL, respectively.

mixture containing bLf and Asc (No. 8 and 10). From these results, the inhibition of MDA formation might be not caused by incorporating Asc into a deep cleft of bLf.

Effects of enzymatic hydrolysis of bLf or bSA on BLM-dependent DNA damage

The enzymatic hydrolysis of bLf was further done to observe whether the inhibition of MDA formation by bLf is retained or lost by destroying the cleft structure of the protein.

The percentage digestion of bLf treated with pepsin, trypsin, and both enzymes was 54.5%, 27.7%, and 76.8%, respectively (Table II). Figure 1 shows the SDS–polyacrylamide gel electrophoretic patterns of the hydrolysates and the C lobe of bLf. The band corresponding to the untreated bLf and C-lobe still appeared in the trypsin-treated bLf (No. 3 in the figure), but not in the hydrolysates treated with pepsin and both pepsin and trypsin (No. 2 and 4). The percentage inhibition of MDA formation by the hydrolysates in the presence of Fe$^{2+}$ decreased with the increased digestion of bLf. However, despite the complete destruction of the N- and C-lobe of bLf, the hydrolysate treated with both enzymes retained 59.3% of the inhibition.
Although one molecule of bLF is found to bind two ferric ions at each one specific metal binding site in the cleft of the two lobes, the results imply that bLF may have another moiety to bind ferrous or ferric ions. Nagasaki et al. have suggested that LF binds iron at other site in addition to the metal binding sites. In our earlier experiments, the inhibition of MDA formation by bLF also happened in the reaction mixture containing excess Fe$^{2+}$ to bind at the specific sites.

In the presence of Asc, the percentage inhibition of MDA formation by the hydrolysates was almost the same as that by the untreated bLF. It appears that the inhibition of MDA formation by bLF is not associated with the cleft structure of the protein, and bLF may have another function to interact with Asc.

On the other hand, the percentage digestion of bSA by pepsin, trypsin, and both enzymes was 47.2%, 17.1%, and 86.3%, respectively. The inhibition of MDA formation in the presence of Fe$^{2+}$ was about 2 to 5 times more effective by the hydrolysates than by the untreated bSA. In contrast, in the presence of Asc, MDA formation was slightly enhanced by the hydrolysates. These phenomena may be caused by the occurrence of peptides and free amino acids in the hydrolysates.

Effects of various amino acids on MDA formation by BLM-dependent DNA damage

The effects of various amino acids on MDA formation were observed in the reaction mixture containing Fe$^{2+}$ or Asc. In Table III, all the amino acids did not inhibit MDA formation in the presence of Fe$^{2+}$. It is not clear why the inhibition of MDA formation by bSA is nevertheless enhanced by the enzymatic hydrolysis of this protein.

In the presence of Asc, the percentage inhibition of MDA formation was higher by basic and aromatic amino acids than by aliphatic and acidic amino acids. As the inhibition of MDA formation was not appreciably changed by the hydrolysates of bLF, the basic and aromatic amino acid residues constituting the peptide occurred or the molecule of this protein may participate in inhibition of MDA formation. However, it is still unknown whether the molecule of bSA and its hydrolysate containing these amino acids accelerate MDA formation.

Yamaguchi et al. have reported that the antioxidative activity of such dipeptides with an alanine residue at the N-terminus like Ala-His, Ala-Met, Ala-Tyr, and Ala-Trp on linoleic acid was stronger than the mixtures of these amino acids. Tsuge et al. have also purified such peptides as Ala-His-Lys, Val-His-His, and His-His-Ala-Asn-Glu-Asn with strong antioxidative activity from enzymatic hydrolysates of egg-white albumin. It is of interest that all the peptides other than Ala-Met contain basic and aromatic amino acids having higher inhibition of MDA formation in the presence of Asc.

Interaction between bLF or bSA and Asc

To distinguish between the different inhibitions of bLF and bSA on MDA formation, the interaction between bLF or bSA and Asc was investigated by ultrafiltration. The relationship between the content of MDA formed and the concentration of Asc was first observed by using the solution containing 0.1 to 0.5 mM Asc and its filtrate. The content
Interaction of Lactoferrin with Ascorbate

Fig. 3. Inhibitive Effects of the Ultrafiltrate from the Solution Containing Bovine Lactoferrin (bLF) or Bovine Serum Albumin (bSA) with Ascorbate (Asc) on Malondialdehyde (MDA) Formation of Bleomycin-Dependent DNA Damage.

The concentration of Asc was 2.5 mM. ○ bLF + Asc; ● bSA + Asc.

Fig. 4. Elution Pattern of Bovine Lactoferrin (bLF) or Bovine Serum Albumin (bSA) with Ascorbate (Asc) in a Sephadex G75 Column.

The concentration of Asc was 0.5 mM. Asc; Asc + bLF or bSA (1 mg/ml); Asc + bSA or bSA (3 mg/ml); Asc + bLF or bSA (5 mg/ml).

of MDA formed in the reaction mixture increased linearly with the increased concentration of Asc (Fig. 2). The content of MDA formed by using the filtrate was almost the same as that by the corresponding Asc solution. The results indicate that Asc is not adsorbed or bound to the filter membrane, and is not oxidized during ultrafiltration for 1 h.

The filtration from a 2.5 mM Asc solution containing different concentrations of bLF or bSA was also used for the BLM assay. In Fig. 3, the filtrate from the solution containing 0.1 to 0.5 mg of bSA per ml did not inhibit MDA formation. However, in the solution containing bLF, the inhibition of MDA formation by the filtrate was enhanced by the increased concentration of bLF. It is evident that Asc can be adsorbed or bound to the bLF molecule, but not to the bSA molecule.

The interaction between bLF or bSA and Asc was further observed by gel filtration of a 0.5 mM Asc solution containing different concentrations of each protein in a Sephadex G75 column (Figure 4). The height of the eluted peak of Asc decreased with the increased concentration of bLF, but did not change with that of bSA. It is also clear that Asc interacted with the bLF molecule, but not with the bSA molecule.

The molecule of bSA is a single polypeptide chain of 582 amino acid residues with a molecular weight of 66,267. The polypeptide chain is crosslinked by 17 disulfide bonds to form a series of nine loops and is grouped as three homologous domains. A rigid structure of bSA molecule existing in the form of a prolate ellipsoid may avoid binding of Asc. On the other hand, the flexible structure of bLF molecule including the deep cleft between two domains of each lobe may facilitate the incorporation of such anions as Asc.

Figure 5 shows the relationship between the ratio of bound Asc/free-Asc and bound Asc per 1 mol of bLF in the phosphate buffer. The amount of Asc binding to one mole of bLF molecules as a molecular weight of 83,100 is estimated by extrapolation to be 87 mol of Asc.

It is known that the prooxidant activity of Asc is induced by its ability to reduce transition metal ions by an one-electron transfer mechanism, which modifies proteins including enzymes. The level of Asc and bLF vary enormously in tissues and fluids. Human neutrophils contain Asc with Lf. However, most of the intracellular Asc has been reported not to bind, at least to protein with a molecular weight of more than 10,000. It is of interest if the binding of Asc together with metal ion to bLF molecule plays a role in antioxidative mechanisms in body fluids.
References


