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Molecular Mechanisms of the Cytotoxicity of Human α-Lactalbumin Made Lethal to Tumor Cells (HAMLET) and Other Protein-Oleic Acid Complexes*

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Background: The α-lactalbumin-oleic acid complex has a unique apoptotic activity for selectively killing tumor cells.
Results: We identified the oleic acid-binding site in the human- and goat-α-lactalbumin complexes by two-dimensional NMR.
Conclusion: Oleic acid is bound to a loosely organized hydrophobic core of the proteins in the molten globule state.
Significance: The results are crucial for understanding the molecular mechanisms of the cytotoxicity of the protein-oleic acid complexes.

Although HAMLET (human α-lactalbumin made lethal to tumor cells), a complex formed by human α-lactalbumin and oleic acid, has a unique apoptotic activity for the selective killing of tumor cells, the molecular mechanisms of expression of the HAMLET activity are not well understood. Therefore, we studied the molecular properties of HAMLET and its goat counterpart, GAMLET (goat α-lactalbumin made lethal to tumor cells), by pulse field gradient NMR and 920-MHz two-dimensional NMR techniques. We also examined the expression of HAMLET-like activities of complexes between oleic acid and other proteins that form a stable molten globule state. We observed that both HAMLET and GAMLET at pH 7.5 were heterogeneous, composed of the native protein, the monomeric molten globule-like state, and the oligomeric species. At pH 2.0 and 50 °C, HAMLET and GAMLET appeared in the monomeric state, and we identified the oleic acid-binding site in the complexes by two-dimensional NMR. Rather surprisingly, the binding site thus identified was markedly different between HAMLET and GAMLET. Furthermore, canine milk lysozyme, apo-myoglobin, and β_2-microglobulin all formed the HAMLET-like complex with the anti-tumor activity, when the protein was treated with oleic acid under conditions in which their molten globule states were stable. From these results, we conclude that the protein portion of HAMLET, GAMLET, and the other HAMLET-like protein-oleic acid complexes is not the origin of their cytotoxicity to tumor cells and that the protein portion of these complexes plays a role in the delivery of cytotoxic oleic acid molecules into tumor cells across the cell membrane.

HAMLET (human α-lactalbumin made lethal to tumor cells), first discovered by the Lund group in Sweden (1, 2), is a complex formed by α-lactalbumin (α-LA)[5] and oleic acid (OA), and has a unique apoptotic activity by which it selectively kills tumor cells. The α-LA portion of HAMLET is in a molten globule (MG)-like conformation under physiological conditions (2), and hence this is an intriguing example in which the MG-like state of the protein exerts a biological activity, relating to the tumor cell killing activity, which is different from the original biological activity in the native state, *i.e.*, the specificity-modifier activity of the lactose synthase system for α-LA (3). In human milk, α-LA is the most abundant whey protein (3), and OA is the most abundant fatty acid and occurs as a triacylglyceride (4). The acidic environment of the stomach of a breast-fed infant is thus very favorable for production of HAMLET, because α-LA forms the MG state at acidic pH (5), and a significant amount of milk triacylglycerides are digested by lingual and gastric lipases in the stomach, releasing OA (4, 6, 7). The generally held idea that gastric lipase preferentially hydrolyzes short and medium chain fatty acids was recently questioned, and the main fatty acids released by gastric lipase in the stomach were OA and palmitic acid (6, 7). It is thus quite reasonable to postulate that HAMLET is produced in the breast-fed infant

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[5] The abbreviations used are: α-LA, α-lactalbumin; OA, oleic acid; MG, molten globule; HLA, human α-lactalbumin; GLA, goat α-lactalbumin; PFG, pulse field gradient; PBMC, peripheral blood mononuclear cell; HSQC, heteronuclear single quantum coherence; CML, canine milk lysozyme; AMG, apomyoglobin; B2M, β_2-microglobulin.

stomach, protecting infants from tumor development (2, 8). HAMLET has also been used successfully for cancer therapy in treating glioblastomas (9), skin papillomas (10), and bladder cancer (11).

Despite these beneficial features of HAMLET, the molecular mechanisms of the formation and stabilization of HAMLET, as well as the structure, including the OA-binding site, of HAMLET are not well understood. Several issues, including (i) whether the OA binding to the MG-like α-LA is specific or nonspecific, (ii) where the OA-binding site is located in α-LA in the MG-like state, (iii) how many OA molecules are bound to α-LA in HAMLET, and (iv) whether HAMLET is monomeric or oligomeric, remain obscure. To address these issues, here we studied the molecular properties of HAMLET and its goat counterpart, GAMLET (goat α-lactalbumin made lethal to tumor cells), by reversed phase HPLC, pulse field gradient (PFG) NMR, and 920-MHz ultrahigh magnetic field NMR techniques. We also examined the expression of HAMLET-like anti-tumor cell activities of complexes between OA and other proteins (canine milk lysozyme, apo-myoglobin, and β_2-microglobulin) that form a stable MG state under acidic conditions.

We observed that seven or eight OA molecules were bound to an α-LA molecule in HAMLET and GAMLET and that both HAMLET and GAMLET at pH 7.5 were heterogeneous, being composed of the native protein, the monomeric MG-like state, and the oligomeric species. At pH 2.0 and 50 °C, both HAMLET and GAMLET appeared in the monomeric MG-like state, and we identified the OA-binding site in both complexes in this state by 920-MHz two-dimensional NMR spectra. Rather surprisingly, the OA-binding site thus identified was markedly different between HAMLET and GAMLET despite their essentially identical anti-tumor activities. The results thus indicate that no unique binding site of OA in the protein is required for expression of the anti-tumor cell activities. Furthermore, canine milk lysozyme, apo-myoglobin, and β_2-microglobulin all formed the HAMLET-like complex when the protein was treated with OA under conditions in which their MG states were stable, and all these complexes exhibited the HAMLET-like anti-tumor activities, although the strength of the activity was dependent on the protein species. From these results, we conclude that the protein portion of HAMLET, GAMLET, and the other HAMLET-like protein-OA complexes is not the origin of their cytotoxicity to tumor cells and that the protein portion of these complexes plays a role in delivering cytotoxic OA molecules into tumor cells across the cell membrane.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—^{15}N-Labeled and [^{13}C,^{15}N]-double-labeled human α-LA (HLA) and goat α-LA (GLA) were expressed in *Escherichia coli* BL21(DE3)/pLysS cells in M9 minimal medium as inclusion bodies using expression plasmids pHLAC and pSCREEN-LA, respectively, and purified as described previously (12, 13); pHLAC was originally a gift from Peter S. Kim. Authentic HLA and GLA were purified from fresh human and goat milk as described previously (13). Human milk was obtained from individual donors, after signed informed consent. The procedure was approved by the human ethics committee of the National Institute for Physiological Sciences, National Institutes of Natural Sciences (Okazaki, Aichi, Japan). Goat milk was obtained from the National Livestock Breeding Center (Nagano, Japan).

Also used were the N49D variant of canine milk lysozyme expressed using a *Pichia pastoris* expression system and purified in a previous study (14) and the β_2-microglobulin expressed in *E. coli* and purified in a previous study (15). Apo-myoglobin, which was prepared from horse heart myoglobin (Sigma) by the method of Teale (16) was a gift from K. Kamagata.

Preparation of HAMLET, GAMLET, and the Other Protein-OA Complexes—The following buffer systems were used for preparation of the protein-OA complexes: 20 mM glycine-HCl buffer (pH 2.0) for HLA, GLA, and canine milk lysozyme; 20 mM acetate buffer (pH 4.0) for apo-myoglobin; and 20 mM citrate buffer (pH 3.6) for β_2-microglobulin. The concentrations of the proteins were all 3 mg/ml, and the 120 molar excess of OA compared with the molar amount of each protein was directly suspended into the protein solution with sonication in a TOMY model UD-211 sonicator, and each protein-OA mixture was kept at 50–60 °C for 10 min. After cooling the mixture to room temperature, the excess OA molecules were carefully removed by centrifugation. We then exchanged the buffers of the resultant protein-OA complexes with 50 mM NH_4HCO_3 by ultrafiltration and lyophilized the complexes. HAMLET and GAMLET were also prepared by the original method of Svensson *et al.* (2) using an OA-conditioned DEAE-Triacryl M (Bio-Sepra, France) column.

Antitumor Activities of the Protein-OA Complexes—L1210 cells and peripheral blood mononuclear cells (PBMCs) were used as tumor and normal cells for the cell killing activity assays of the protein-OA complexes. The cells were first washed with PBS and resuspended at a density of 2×10^6 cells/ml in RPMI 1640 medium without FBS. The cell culture was seeded in a 96-well plate. The protein-OA complex to be tested was added to the cell culture at a final protein concentration ranging from 1 to 10 μM for L1210 cells and 10 or 20 μM for PBMCs. After incubation for 30 min at 37 °C in 5% CO_2, 10 μl of FBS was added to the culture. After an additional 4-h incubation at 37 °C in 5% CO_2, the cell viabilities were evaluated by the trypan blue assay.

HPLC Analysis—The molar ratio (stoichiometry) of OA to α-LA in HAMLET and GAMLET was determined in an LC-8020 HPLC system (TOSOH) using an octadecyl-4PW column (TOSOH) at 30 °C. The mobile phase consisted of two solutions (A and B): A, 0.1% TFA in H_2O, and B, 0.1% TFA in acetonitrile. A linear gradient with a flow rate of 1.0 ml/min was used as follows: first held at 75% A/25% B from 0 to 5 min and then ramped to 0% A/100% B from 5 to 75 min. The column was then re-equilibrated back to 75% A/25% B for 5 min. OA and the protein in the HAMLET and GAMLET complexes were first dissociated by guanidine hydrochloride at 6 M. 200 μl of the protein and OA mixture thus obtained (α-LA concentration, 75–110 μM) was injected into the column, and α-LA and OA were detected by absorbance at 280 and 215 nm, respectively.

PFG-NMR Spectra—In PFG-NMR experiments, HAMLET (or GAMLET) was dissolved at a final α-LA concentration of 0.3 mM in PBS (pH 7.5) that contained 10% D_2O and 0.05%

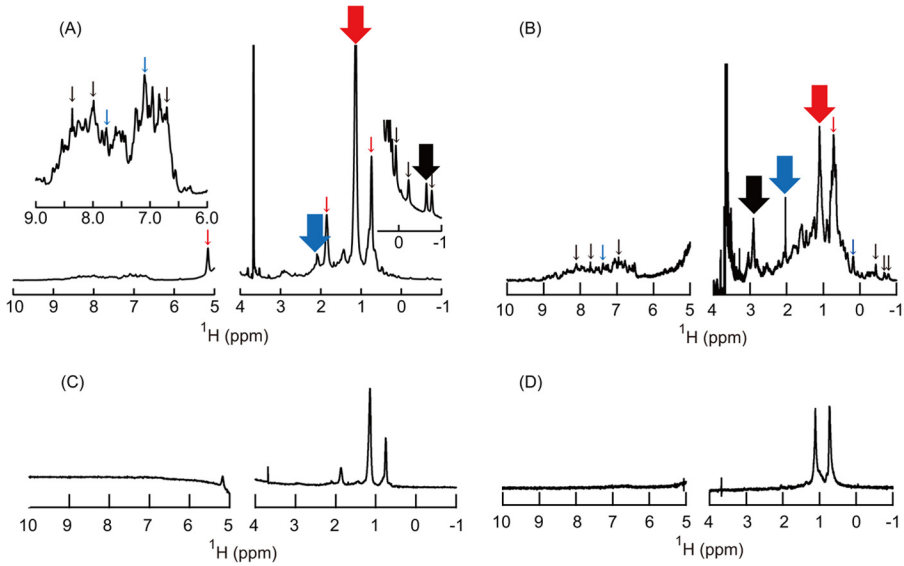

FIGURE 1. **The PFG-NMR spectra of HAMLET (*A* and *C*) and GAMLET (*B* and *D*) at pH 7.5 and 25 °C.** The spectra in *A* and *B* are those at a field gradient strength of 0.69 G/cm, and the spectra in *C* and *D* are those at 69 G/cm. *Arrows* indicate the NMR signals used for the analysis. *Arrows* of the same color (*black*, *blue*, or *red*) indicate NMR signals that gave the same R_h values (see text). The *large arrows* indicate the NMR signals used to draw the decay curves in Fig. 2.

dioxane. PFG-NMR measurements were performed on a Bruker Avance 500 spectrometer with a bipolar longitudinal eddy-current-decay pulse sequence as described previously (15, 17). Sixteen spectra were acquired while changing the strength of the diffusion gradient (g) from 5 to 100% of the maximum power level. The NMR signal intensity (S), normalized by the intensity at zero field gradient (S_0), is related to g as follows,

$$S/S_0 = \exp(-d \cdot g^2) \qquad \text{(Eq. 1)}$$

where d is the observed decay rate, which is proportional to the diffusion coefficient of the molecule. We fitted the signal decays of dioxane, HAMLET, and GAMLET to Equation 1 and estimated the hydrodynamic radii (R_h(Complex)) of the complexes (HAMLET and GAMLET) by Equation 2 (18),

$$R_h(\text{Complex}) = \frac{R_h(\text{dioxane}) \times d(\text{dioxane})}{d(\text{Complex})} \qquad \text{(Eq. 2)}$$

where d(dioxane) and d(Complex) are the decay rates of the NMR signal obtained from dioxane and the complex (HAMLET or GAMLET), respectively, and R_h(dioxane) is the hydrodynamic radius of dioxane, 2.12 Å.

Two-dimensional ^{1}H-^{15}N Heteronuclear Single Quantum Coherence (HSQC) Spectra—Two-dimensional ^{1}H-^{15}N HSQC spectra of HLA and GLA in the MG state (pH 2.0) and the spectra of HAMLET and GAMLET at pH 2.0 were collected at 50 °C on a JEOL ECA-920-MHz NMR spectrometer. We acquired 32 transients for each of 256 t_1 points, and the sweep widths in t_1 and t_2 were 2476 and 14988 Hz, respectively. To achieve the backbone resonance assignment of HLA and GLA in the MG state (pH 2.0) and GAMLET at pH 2.0, HNCACB and CBCACONH experiments were performed at 50 °C. All of the NMR spectra were processed and analyzed by NMRPipe and NMRView.

RESULTS

Preparation of HAMLET and GAMLET—We prepared HAMLET and GAMLET by two different methods: (i) a heat treatment method (19), in which the mixture of α-LA and OA was heated at pH 2.0 and 50–60 °C for 10 min, followed by removal of excess OA and lyophilization, and (ii) the original column method, in which HAMLET and GAMLET were prepared by column chromatography using an OA-preconditioned ion exchange column (2). The anti-tumor activities of the HAMLET and GAMLET complexes prepared by the two methods were identical to each other (data not shown). The complexes prepared by the two methods were also essentially identical to each other with respect to their NMR spectra and the hydrodynamic properties measured by the PFG-NMR technique (see below). In the present study, we used the complexes prepared by the heat treatment method unless otherwise stated. The stoichiometries of protein and OA in HAMLET and GAMLET were determined by reversed phase HPLC analysis, and 8.5 and 6.9 OA molecules were bound to an α-LA molecule in HAMLET and GAMLET, respectively.

Hydrodynamic Radii of HAMLET and GAMLET—To investigate the oligomeric state of HAMLET and GAMLET, we measured the effective hydrodynamic radii (R_h) of these protein complexes by the PFG-NMR technique. Fig. 1 (*A* and *B*) shows the ^{1}H NMR spectra of HAMLET and GAMLET, respectively, at a field gradient strength of 0.69 G/cm, and these spectra are compared with those at the largest field gradient strength (69 G/cm) (Fig. 1, *C* and *D*) (pH 7.5 and 25 °C). The spectrum of HAMLET (Fig. 1*A*) is very similar to the spectrum previously reported (20). Both the spectra of HAMLET and GAMLET exhibit broad peaks at 0.745 and 1.375 ppm, which may arise from the bound OA molecules (2, 20), as well as the native protein peaks in the aromatic and up-field regions, although the peak intensities of the bound OA are smaller in GAMLET than in HAMLET. In the spectra at the largest field gradient strength, essentially all proton signals from the native proteins had vanished, whereas the broad signals from the bound OA were still observed, indicating the presence of at least two components that had significantly different hydrodynamic radii.

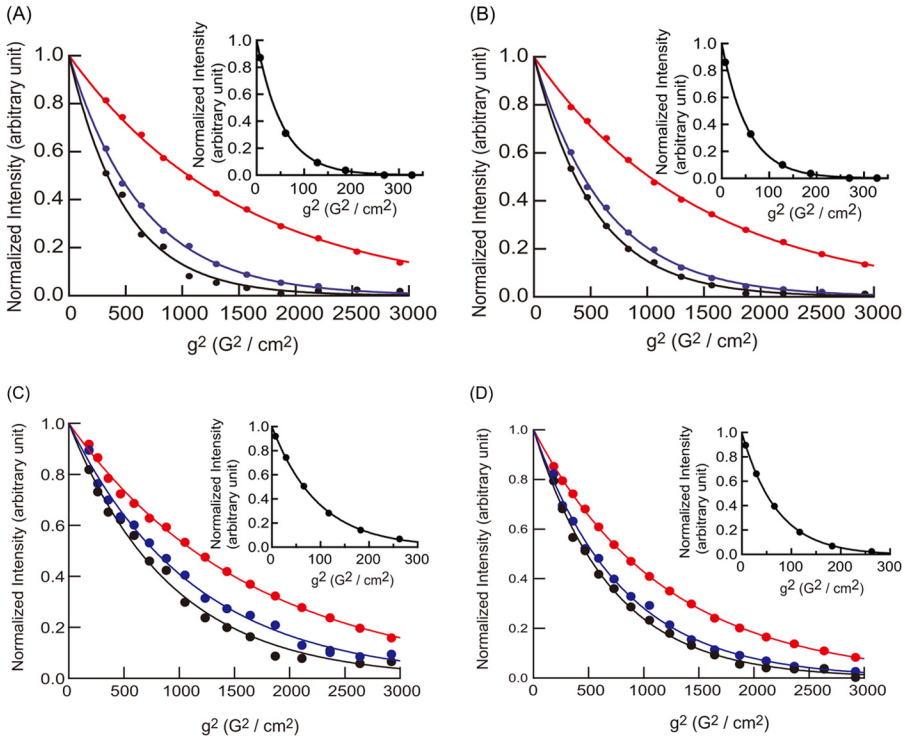

FIGURE 2. *A* and *B*, the PFG-NMR analysis of HAMLET (*A*) and GAMLET (*B*) (pH 7.5 and 25 °C). The intensities of the NMR signals indicated by the *large black*, *blue*, and *red arrows* in Fig. 1 are shown as a function of the square of the field gradient strength. *C* and *D*, same analysis results for HAMLET (*C*) and GAMLET (*D*) in the presence of 2 M urea (pH 7.5 and 25 °C). The *solid lines* represent the theoretical curves obtained by fitting the experimental data to a single exponential function. The *inset* in each panel shows the decay curve for dioxane.

The peak intensities of the individual NMR signals were investigated as a function of the square of the field gradient strength. Fig. 2 (*A* and *B*) shows the typical decay curves thus obtained, and we calculated the R_h values from the decay curves using the R_h value of the internal reference compound, 1,4-dioxane, of 2.12 Å (18). For both HAMLET and GAMLET, the NMR signals are classified into three groups as indicated by *arrows* of three different colors (Fig. 1, *A* and *B*). For HAMLET, the *black*, *blue*, and *red arrow* signals gave R_h values of 19.8 ± 1.1, 24.5 ± 0.4, and 61.0 ± 1.3 Å, respectively. Similarly for GAMLET, the signals indicated by *black*, *blue*, and *red arrows* gave R_h values of 20.0 ± 0.9, 23.6 ± 0.6, and 57.1 ± 0.4 Å, respectively. Therefore, for both HAMLET and GAMLET, the *black arrow* signals were those from the native proteins. The R_h value of native α-LA, previously measured by sedimentation (21), dynamic light scattering (22), and PFG-NMR techniques (23), ranges from 18.8 to 19.7 Å, and hence it is in excellent agreement with the values from the *black arrow* signals. The theoretical R_h values calculated from the x-ray crystallographic structures (Protein Data Bank codes 1B9O and 1HFY) of native HLA and GLA using the program HYDROPRO (version 7c) (24), are 20.6 and 19.9 Å, respectively, again in excellent agreement with the values shown above. The R_h values (23–25 Å) obtained from the *blue arrow* signals are 10–20% larger than the value (20.8–20.9 Å) for the monomeric MG state of α-LA (21–23). The *blue arrow* signals may thus represent a monomeric MG-like state with the R_h value increased by 10–20% by bound OA molecules. The R_h values (61.0 and 57.1 Å for HAMLET and GAMLET) obtained from the *red arrow* signals indicate that these signals represent an oligomeric protein-OA complex that may consist of 13–17 protein molecules; this estimate was based on the volume ratios calculated from the radii of the monomeric and oligomeric protein-OA complexes. As a result, both HAMLET and GAMLET are composed of three components, the native protein, the monomeric MG-like state, and the oligomeric protein-OA complex. Because the NMR signals of OA were observed mostly as *red arrow* signals, most OA molecules were bound to the oligomeric complexes at pH 7.5 and 25 °C. At least one of the *red arrow* signals appeared to be OA, and thus, if OA was in rapid exchange, it might adversely affect the determination of R_h. However, this is very unlikely, because the exchange between free OA and bound OA was slow even under a harsher condition (pH 2.0 and 50 °C) (see below). We also carried out the PFG-NMR experiment for the HAMLET and GAMLET prepared by the column method and obtained essentially the same results (data not shown).

We also performed the PFG-NMR experiments in the presence of 2 M urea, and the results again indicated the presence of three components, the native species, the monomeric complex, and the oligomeric complex (Fig. 2, *C* and *D*). The R_h values of native α-LA and the monomeric complex were essentially identical to those shown above in the absence of urea. However, the R_h values of the oligomeric species became significantly smaller, 36.5 ± 1.4 and 35.0 ± 1.6 Å for HAMLET and GAMLET, respectively, at 2 M urea, indicating significant dissociation occurring in the presence of urea.

We further carried out the PFG-NMR experiments at pH 2.0 and 50 °C, the conditions used for preparation of HAMLET and GAMLET by the heat treatment method. Under these conditions, all the NMR signals, including those of the protein and

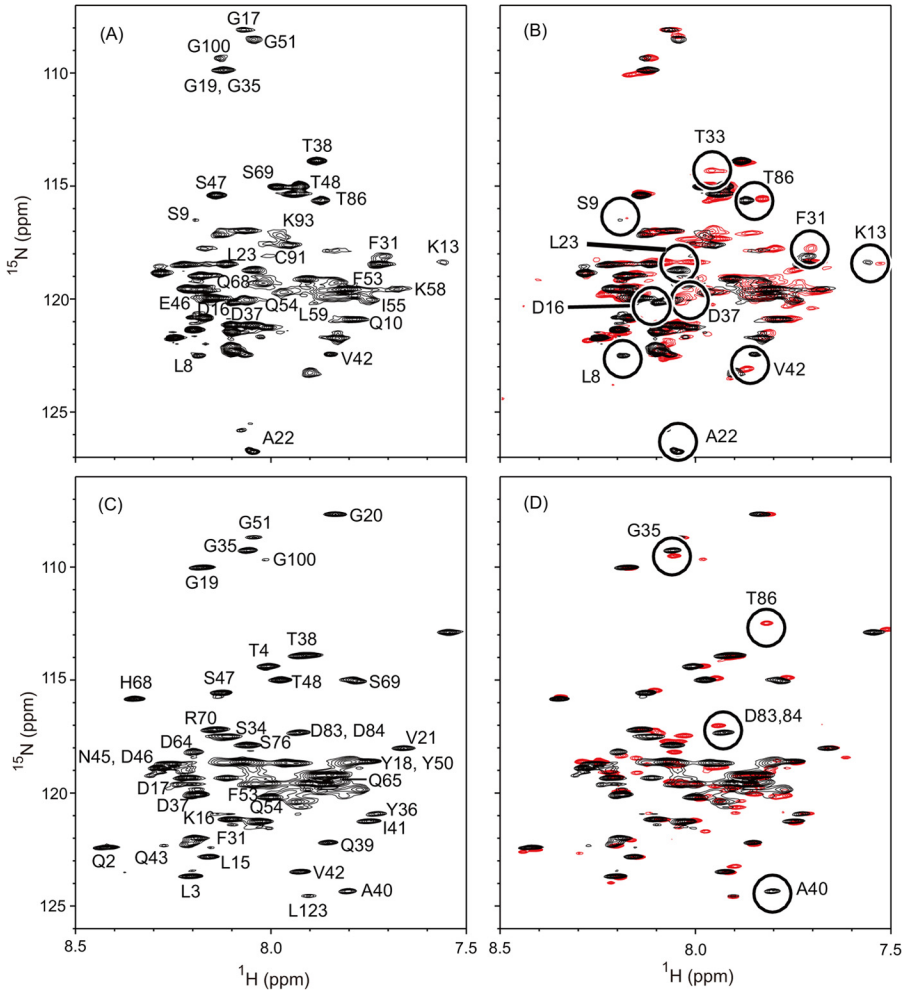

FIGURE 3. **The ^{1}H-^{15}N HSQC spectra of HLA (*A*) and GLA (*C*) in the free MG state, and the spectra of HAMLET (*B*) and GAMLET (*D*) (pH 2.0 and 50 °C).** In *B* and *D*, the spectra of HAMLET and GAMLET, shown in *red*, are superimposed on the spectra in the free MG state, shown in *black*. The NMR signal assignments are indicated with a one-letter amino acid code and residue number. The ^{1}H, ^{13}C, and ^{15}N chemical shifts at 323 K have been deposited in the BioMagResBank under accession numbers BMRB-11515, BMRM-11517, and BMRM-11523. The cross-peaks that show a difference between the free MG state and the α-LA-OA complex (HAMLET or GAMLET) are *circled* in *B* and *D*.

bound OA, exhibited decay curves essentially identical to those of the monomeric MG-like state at neutral pH shown above, and the R_h values of HAMLET and GAMLET thus obtained were 25.0 ± 0.3 and 24.8 ± 0.8 Å, respectively. Observation of the coincident decay curves for the protein and bound OA signals indicated that OA was not in rapid exchange.

Identification of the OA-binding Site in HAMLET and GAMLET—To identify the OA-binding site in HAMLET and GAMLET, we measured ^{1}H-^{15}N HSQC spectra of the monomeric MG-like state of HAMLET and GAMLET and compared these spectra with those of the free MG state of HLA and GLA without the bound OA. The HAMLET and GAMLET complexes were prepared from ^{15}N-labeled HLA, ^{15}N-labeled GLA, and [^{13}C,^{15}N]-double-labeled GLA, and their anti-tumor activities were identical to those of the complexes prepared from the nonlabeled proteins (data not shown). The HSQC spectra were measured at pH 2.0 and 50 °C, conditions under which both HLA and GLA are fully in the MG state, and the HAMLET and GAMLET complexes are in the monomeric MG-like state. The backbone assignment of [^{13}C,^{15}N]-double-labeled HLA and GLA in the MG state and [^{13}C,^{15}N]-double-labeled GAMLET was obtained by three-dimensional CBCACONH and HNCACB experiments recorded on a 920-MHz NMR instrument. We assigned 53 of the 121 nonprolyl residues of HLA in the MG state and 40 of the 121 nonprolyl residues of GLA (Fig. 3, *A* and *C*). For HLA, an additional assignment of 33 residues was provided by C. Redfield (25). For GAMLET, we assigned cross-peaks that were significantly affected by OA binding.

Fig. 3 (*B* and *D*) shows ^{1}H-^{15}N HSQC spectra of HAMLET and GAMLET, respectively, overlaid on the spectra of HLA and GLA in the MG state. Many cross-peaks appear at the same positions between HAMLET and HLA and between GAMLET and GLA, indicating that HAMLET and GAMLET were in the monomeric MG-like state under these conditions. However, several cross-peaks show clear differences. The cross-peaks of Leu-8, Ser-9, Lys-13, Asp-16, Ala-22, Leu-23, Phe-31, Thr-33, Asp-37, Val-42, and Thr-86 were different between HAMLET and HLA in the MG state (Fig. 3*B*), and the chemical shift values were significantly different for these cross-peaks. Similarly, the cross-peaks of Gly-35, Ala-40, Asp-83, Asp-84, and Thr-86 were different between GAMLET and GLA in the MG state (Fig. 3*D*). The chemical shift values were significantly different

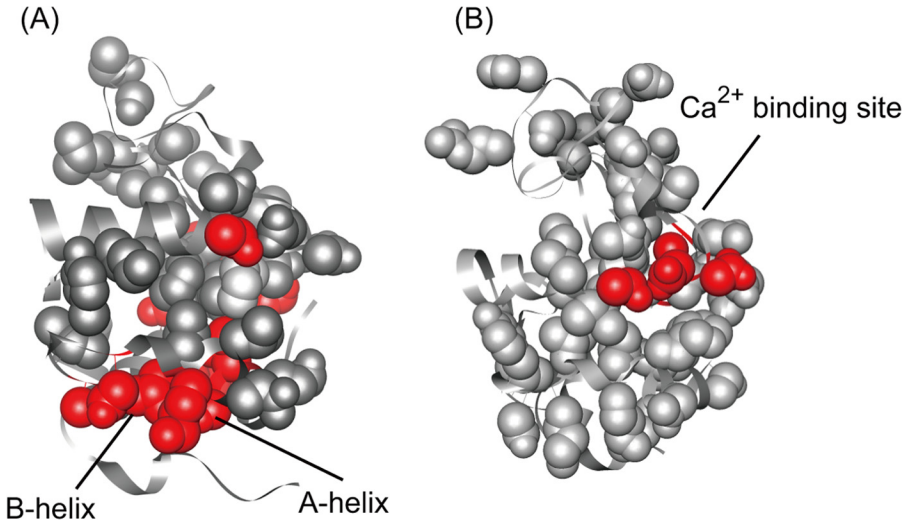

FIGURE 4. **The OA-binding sites of HLA in HAMLET (*A*) and of GLA in GAMLET (*B*) as determined by differences in cross-peaks between the free MG state and the α-LA-OA complex (HAMLET or GAMLET; Fig. 3, *B* and *D*).** The amino acid residues represented by the space-filling model are those whose cross-peaks in the ^{1}H-^{15}N HSQC spectra are assigned (Fig. 3, *A* and *C*). The *red* residues indicate the OA-binding site of each protein.

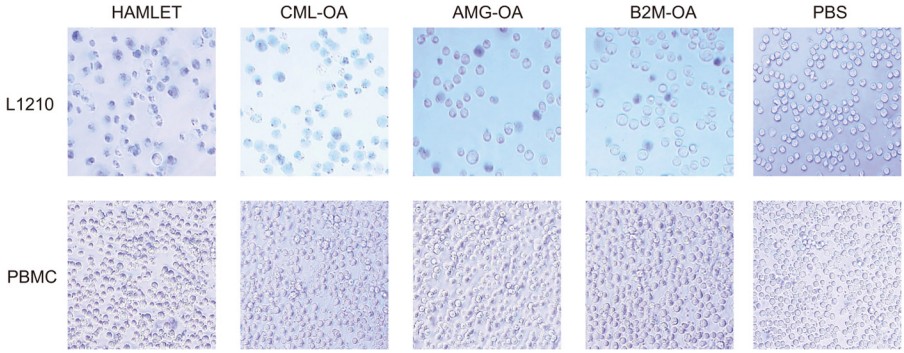

FIGURE 5. **Anti-tumor activities of HAMLET, CML-OA, AMG-OA, and B2M-OA as measured by the trypan blue assay.** The pictures show images of L1210 cells and PBMCs obtained with an optical microscope after 4 h of incubation with the protein-OA complexes. Each complex at the final protein concentration of 10 μM was applied to the culture of L1210 cells. 20 μM of HAMLET, CML-OA, and B2M-OA and 10 μM of AMG-OA were applied to the culture of PBMCs. *PBS* indicates the control in which the PBS buffer solution was added to the culture.

for Gly-35, Asp-83, and Asp-84, the cross-peak of Ala-40 disappeared in GAMLET, and the cross-peak of Thr-86, absent in the acidic MG state, appeared in GAMLET.

From the above results, we identified the OA-binding site in HAMLET and GAMLET. Fig. 4 shows the three-dimensional structures of native HLA and GLA, and the amino acid residues whose cross-peaks are significantly affected by OA binding are shown in *red*. Rather surprisingly, the OA-binding site was quite different between HAMLET and GAMLET. In HAMLET, most of the residues affected by OA binding are within the A- and B-helices (Fig. 4*A*). On the other hand, the residues affected by OA binding in GAMLET are located near the Ca^{2+}-binding site of the protein and hence at the interface between the α- and β-subdomains (Fig. 4*B*). It is thus concluded that the OA-binding site is located at quite different places, between the A- and B-helices in HAMLET and at the interface between the α- and β-subdomains in GAMLET, although both the protein-OA complexes show essentially the same anti-tumor activities.

Anti-tumor Activities of the Protein-OA Complexes Other than HAMLET and GAMLET—The difference in the OA-binding site between HAMLET and GAMLET indicates that a unique common OA-binding site is not required for expression of anti-tumor cell activity. This fact led us to investigate possible expression of anti-tumor activities in the complexes between OA and the other proteins that form the stable MG state. We chose canine milk lysozyme, apo-myoglobin, and β_2-microglobulin. Canine milk lysozyme, a Ca^{2+}-binding lysozyme, is homologous to α-LA and forms the MG state at pH 2 (26, 27). Apo-myoglobin and β_2-microglobulin form the MG state at pH 4 (15, 28, 29). We prepared the OA-protein complexes (denoted by CML-OA, AMG-OA, and B2M-OA for the canine milk lysozyme-, apo-myoglobin-, and β_2-microglobulin-OA complexes) by the same method as used for HAMLET and GAMLET, *i.e.*, by heat treatment of the mixture of each protein and OA at 50–60 °C for 10 min under the conditions in which the MG state was stable, followed by removal of excess OA and lyophilization. We applied these complexes to L1210 leukemia cells to examine the anti-tumor cell activities of the complexes, and the viability of tumor cells was determined by trypan blue staining. In the presence of 10 μM of each complex, CML-OA, AMG-OA, and B2M-OA exhibited 100, 45, and 15% of the anti-tumor cell activities observed in the presence of 10 μM HAMLET, respectively (Fig. 5). Therefore, all the complexes between OA and the proteins examined exhibited similar anti-tumor activities, although the degree of activity was dependent on the protein species. To test the specificity of cell

killing activities of these complexes, we also applied the complexes to normal cells, PBMCs. The results are also included in Fig. 5, and all the protein-OA complexes tested did not kill PBMCs at a high concentration (20 μM for HAMLET, CML-OA, and B2M-OA and 10 μM for AMG-OA), suggesting that the cell killing activities of these complexes were tumor cell-specific.

DISCUSSION

The PFG-NMR data in the present study clearly indicate that both the HAMLET and GAMLET complexes are composed of three components, *i.e.*, the native protein (R_h = 20 Å), the monomeric MG-like state (R_h = 24 Å), and the oligomeric species (R_h = 57–61 Å) under the native conditions (pH 7.5 and 25 °C). Under the acidic condition (pH 2.0) at 50 °C, both HAMLET and GAMLET appeared in the monomeric MG-like state, and to identify the OA-binding site, we examined the ^{1}H-^{15}N HSQC spectra of the HAMLET and GAMLET complexes with those of the proteins in the free MG state without bound OA. Rather surprisingly, the OA-binding site thus identified was markedly different between the two protein complexes, indicating that no unique binding site of OA in the protein is required for expression of the anti-tumor cell activities. That the expressions of the anti-tumor activities by CML-OA, AMG-OA, and B2M-OA were similar further indicates that the formation of the anti-tumor complex with OA is not peculiar to α-LA.

Whether HAMLET or the other α-LA-OA complexes that have similar anti-tumor activities are monomeric or oligomeric has been a subject of controversy. Spolaore *et al.* (30) recently reported that the bovine α-LA-OA complex (BAMLET) is oligomeric with an R_h value of 37 Å as measured by dynamic light scattering and gel filtration chromatography, and the oligomeric species displayed cytotoxicity toward tumor cells. Their results are thus fully consistent with the present results, although the molecular size of the complex is smaller in their complex. On the other hand, Svanborg and co-workers (20, 31) have reported on the basis of their PFG-NMR experiments that HAMLET exists in the monomeric form with an R_h value of 26.9 Å. The discrepancy between their results and ours is rather surprising, because both investigations used the same PFG-NMR techniques. The only significant difference in the experimental condition was the presence of 2 M urea in their experiments. We thus performed the PFG-NMR experiments in the presence of 2 M urea (Fig. 2, *C* and *D*) and observed significant dissociation occurring in the presence of urea. However, HAMLET and GAMLET still consisted of three components, the native species, the monomeric complex, and the oligomeric species, at 2 M urea. It is thus concluded that 2 M urea has a significant impact on the oligomeric state of HAMLET and that under the physiological condition in the absence of urea, HAMLET is mostly oligomeric. It is also suggested that the oligomeric state of HAMLET is fragile and easily dissociated by 2 M urea. The fragile nature of the oligomeric α-LA-OA complex was also reported in BAMLET: (i) The oligomeric BAMLET complex was dissociated by dilution and the subsequent gel filtration (30), and (ii) the major signals observed in the mass spectrometric analysis of originally aggregated BAMLET were reported to be monomeric under physiological conditions (32). Furthermore, the anti-tumor complex originally prepared from human milk was reported to be oligomeric (33), supporting our conclusion that HAMLET is oligomeric under physiological conditions.

Although the OA-binding site is markedly different between HAMLET and GAMLET, the binding site in each complex reasonably corresponds to the most structured region in the MG state of each protein. From previous hydrogen/deuterium exchange studies, the peptide NH protons most highly protected in the MG state of HLA were located in the A- and B-helices (34), whereas they were located in the C-helix in the MG state of GLA (13). These regions thus coincide with the OA-binding sites in HAMLET and GAMLET (Fig. 4). Because the structured region in the MG state may correspond to a loosely organized hydrophobic core accessible to solvent water, the above results strongly suggest that OA is not recognized by a specific structure but rather is recognized by nonspecific hydrophobic interactions in the loosely organized hydrophobic core in the MG state. The location of the OA-binding site in the A- and B-helices in HAMLET may not be inconsistent with the hydrogen/deuterium exchange and limited proteolysis studies of HAMLET reported by Casbarra *et al.* (35), in which they observed that the α-subdomain was resistant to proteolysis whereas the β-subdomain was more flexible and susceptible to proteolysis. The A- and B-helices of HLA are already significantly protected in the MG state (34), and the OA binding to these helices further stabilizes the helical structures and makes the α-subdomain more resistant to proteolysis.

Our conclusion that OA molecules in HAMLET and GAMLET are recognized by nonspecific hydrophobic interactions led us to examine the possible expression of similar anti-tumor activities in the complexes between OA and the other proteins that form the stable MG state. As expected, all of the complexes examined, CML-OA, AMG-OA, and B2M-OA, expressed similar anti-tumor activities (Fig. 5). The anti-tumor activity of CML-OA may not be surprising, because a similar Ca^{2+}-binding lysozyme, equine milk lysozyme, exerts cytotoxicity to tumor cells when complexed with OA (36, 37). As to AMG-OA, Tolin *et al.* (38) mentioned the formation of a cytotoxic complex of apo-myoglobin and OA in their recent paper on BLA fragment-OA complexes. The anti-tumor activity of B2M-OA is, however, a new finding of the present study. β_2-Microglobulin is a β protein with a typical immunoglobulin fold (39), and hence it is structurally quite different from α-LA and lysozyme, which are α-helix-rich $\alpha+\beta$ proteins (3), and also from apo-myoglobin, which is a typical all-α protein (40). Recently, it was also reported that the OA complexes with bovine β-lactoglobulin and pike pervalbumin induced the tumor cell death and that the mechanisms of the cell death by these protein-OA complexes were analogous to those by HAMLET (41, 42).

The nonspecific nature of the OA binding to a protein in the MG state may produce variations in the binding stoichiometry between OA and the protein, depending on solution conditions, the preparation methods of the complexes, and the protein species. For HAMLET, the stoichiometries so far reported thus ranged from less than 1 to 9 (the number of the OA mol-

ecules per an HLA molecule) (20, 43–45), and the stoichiometries in HAMLET and GAMLET prepared by the present method were 8 and 7, respectively.

The expression of similar anti-tumor cell activities by the complexes formed between OA and a variety of proteins in the MG state strongly suggests that the protein portion of these complexes may not be the origin of their cytotoxicity to tumor cells. Furthermore, there is now ample evidence that OA itself is cytotoxic and promotes apoptosis in various tumor cells (46–51), and the mitochondrion-related apoptotic behaviors, such as reduction in mitochondrial membrane potential and cytochrome *c* release, observed after treatment with OA were also observed in the HAMLET-induced apoptosis in tumor cells (52–54). Recently, Brinkmann *et al.* (55) also reported that the cell killing mechanisms of the BAMLET complex and of OA alone examined by flow cytometry were very similar to each other. It is thus strongly suggested that the protein portion of HAMLET, GAMLET, and the other HAMLET-like protein-OA complexes probably works as a delivery carrier of the cytotoxic OA to tumor cells (30, 38). It is becoming increasingly evident that not only the rigid native structures but also the flexible MG structures are biologically functional, and many of the functional MG states are related to translocations of proteins or hydrophobic ligands across membrane bilayers (56–61), and the interaction of HAMLET with artificial and natural membranes has been reported (62–64). We thus conclude that the MG-like state in HAMLET, GAMLET, and the other related complexes plays a role in transferring the hydrophobic OA molecules into tumor cells across the cell membranes.

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