Molecular Simulation of Bovine β-Lactoglobulin Adsorbed onto a Positively Charged Solid Surface

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To obtain detailed insight into the mechanism of β-lactoglobulin (β-Lg) adsorption to a stainless steel surface at acidic pH, the adsorption of positively charged β-Lg to a positively charged surface (Au (100) surface with virtual positive charge) was simulated using classical molecular dynamics. The initial orientation and position of β-Lg on the surface were determined using Monte Carlo simulation using the implicit water system. Molecular dynamics simulation with the explicit water system was conducted for a 5 ns simulation time to monitor β-Lg adsorption. To investigate surface charge density effects on adsorption of β-Lg, the positive charge number per Au atom on the (100) surface, *C*, was varied from 0 to +0.0250|e|. Stable adsorption occurred in MD simulations when *C* was equal to or less than +0.0200|e|. Among these surface Au charge conditions, no large difference was observed in the vertical separation distance between the surface and the protein's center of mass, and the orientation angle. This fact indicates that the main interactions contributing to the adsorption were van der Waals interactions. The protein domain contacting the surface was near Thr125, agreeing with previous experimental studies. Considering simulation results and those previous experimental studies suggests a detailed adsorption mechanism of β-Lg at acidic pH: β-Lg molecule is adsorbed initially with the specific part of 125−135th residues close to the surface by van der Waals interactions. Simultaneously or subsequently, side carboxylic groups of acidic amino acid residues near the surface in 125−135th residues dissociate, leading to firmer adsorption by attractive electrostatic residue−surface interaction.

1. Introduction

Adsorption of proteins onto solid surfaces is an important issue in various fields. During food and drug manufacturing processes, proteinaceous deposits are known to be formed often on equipment surfaces. Deposits of this kind potentially act as a nutrient for product spoilage organisms and pathogens. Therefore, the equipment surface must be cleaned regularly to remove them. Furthermore, removal of deposits is necessary to prevent equipment damage and impaired performance. In general, equipment cleaning requires much water, chemicals, heat energy, and time. To raise cleaning efficiency, understanding the mode of deposit formation is important.

Bovine β-lactoglobulin (β-Lg) is a small protein of 162 amino acid residues. It is one of the whey proteins of cow's milk that is known as a major component of proteinaceous deposits formed on the surface of dairy manufacturing equipment such as heat exchangers of milk sterilizers. Adsorption of milk protein such as β-Lg onto a solid surface is the initial and controlling step in deposit formation.[1,2] Therefore, better knowledge of adsorption characteristics of β-Lg on solid surfaces is expected to be a key factor for improvement of dairy equipment cleaning processes.

Adsorption behavior of β-Lg from aqueous solutions onto solid surfaces has been investigated for solid surfaces of several kinds: chromium,[3,4] stainless steel,[1,5] silicon,[6−8] silica substances,[5,9,10] polysulfone,[9] and polystyrene.[11] Reportedly, adsorption isotherms of β-Lg molecules at room temperature on surfaces of stainless steel,[1] chromium,[3] and silicon[7,8] show Langmuir-like shape, which indicates that β-Lg molecules are adsorbed onto the surface until a monolayer is formed. Effects of temperature,[1,12,13] pH,[1,6,12] protein concentration,[1,12] and the flow rate of the sample solution[13] on the adsorption of β-Lg have also been investigated in many studies using various techniques such as ellipsometry, depletion experiments, and neutron reflection.

In spite of those many studies, the mode and mechanism of β-Lg adsorption on solid surfaces remains to be clarified. For understanding the adsorption mechanism of β-Lg in detail, an important clue is molecular information of adsorbed β-Lg, such as the domain of the protein molecule mainly contributing to its adsorption.

It has been reported that β-Lg adsorbs irreversibly on stainless steel surfaces at acidic pH,[14] although both β-Lg and the stainless steel surface have a positive charge around this pH regime.[15,16]

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Recent adsorption experiments using peptide fragments from β-Lg by tryptic digestion obtained information about the adsorption domain of β-Lg on stainless steel surfaces at acidic pH (3.3).[14] Several peptides show a strong affinity to the stainless surface. A typical peptide fragment is Thr-Pro-Glu-Val-Asp-Asp-Glu-Ala-Leu-Glu-Lys (residues 125−135). Furthermore, β-Lg adsorbed onto stainless steel at pH 3.0 was also subjected to lysyl endopeptidase treatment, and the course of fragmentation was compared with that observed for β-Lg in solution.[17] The results revealed a clear difference for a fragment composed of residues 102−135; it was released readily in solution but only slightly from the β-Lg adsorbed onto the stainless steel surface. From these results, the adsorption domain of β-Lg at acid pH was estimated to be the 125−135th amino acid residues.

Although the experimental studies described above have revealed important and relevant information, the studies' respective approaches limited their ability to probe adsorption phenomena at the molecular level. Particularly, it remains unclear why the adsorption domain of β-Lg at acid pH is located around the 125−135th amino acid residues.

Molecular simulation is a direct method to investigate complex systems at the molecular level. The limited applicability of many experimental methods to protein adsorption makes this problem suitable for investigation using a computer simulation such as molecular dynamics (MD) or Monte Carlo (MC) simulations. Recently, several adsorption simulations of proteins or protein fragments have been conducted as advances in computational power and simulation techniques.[18−22] In spite of their importance in dairy manufacturing processing, however, few reports describe computer simulation of β-Lg adsorption.

The objective of this study was to provide molecular information of adsorbed β-Lg on stainless steel surfaces in acidic aqueous solutions using a classical MD simulation. Combining simulation results with those of previous experimental studies, we expect to gain more detailed insights into the adsorption mechanism at acidic pH.

Both β-Lg and stainless steel surface are charged positively at acidic pH.[15,16] Therefore, the simulation conducted in this study was an adsorption of a positively charged protein to a positively charged solid surface. As the protein molecule, we used β-Lg, with an equivalent positive charge number to that in aqueous solution at pH 3. As the surface, Au atoms with virtual positive charges in the (100) surface were adopted. It is preferable to use stainless steel as the adsorption surface because it is often used as a material for dairy equipment. However, detailed chemical structure models of stainless steel that is used for molecular simulation were not available. Therefore, in place of stainless steel, we used surface Au atoms having virtual positive charge numbers on the the (100) surface. Consequently, our simulation results might not correspond directly to the experimental results obtained using stainless steel. However, considering that understanding of the adsorption phenomena of β-Lg at the molecular level is insufficient now, we believe that the approach used here will give hints related to the adsorption state of β-Lg.

The simulation method used here is based on that of Zhou et al.[19] First the orientation and position of β-Lg to the surface were determined in an implicit water system using the MC simulation at the condition where β-Lg structure was rigid. Then MD simulations were conducted to monitor the adsorption state of β-Lg on the surface.

2. Materials and Methods

2.1. Molecular Modeling of Materials. The crystal structure of β-Lg (PDB ID 3BLG) was used as a starting point of simulation. After removal of water molecules, hydrogen atoms were added using the GROMACS pdb2gmx tool.[23,24]

The deprotonation state of dissociable amino acid residues in β-Lg at pH 3.0 in the protein model was determined as follows. The pK_a value of each dissociable residue was first obtained using the empirical pK_a prediction program PROPKA.[25] Table 1 shows the predicted pK_a values and calculated apparent degrees of deprotonation of each dissociable residue at pH 3.0. The residue was judged to be deprotonated in the protein model if the apparent deprotonation ratio was 0.5 or more. In other cases, the residue was determined to be protonated. Table 1 also shows protonation states of each dissociable amino acid residue according to the criteria described above. The protein model has a net charge of +17|e|. It was within +2|e| difference from the PROPKA prediction.

Using pdb2gmx, protein model topology with protonation or deprotonation of dissociable residues was prepared. The potential parameters for β-Lg were from the OPLS-AA force field[26] implemented in GROMACS. The OPLS-AA includes the bonded interaction terms representing the energy of deformation of bond length, bond angles, and dihedral angles, and the nonbonded terms: the Lennard-Jones potential (van der Waals interaction) and electrostatic potential between atom-based charges.

Surfaces for β-Lg adsorption were modeled using the Au (100) surface with positive surface charge densities, as explained above. The Au (100) was constructed by stacking a single unit of face-centered cubic lattice of Au into a plane. The unit lattice length was 0.41 nm. The Au atom mass was set to 196.96655. The charge of each Au atom belonging to the (100) surface, C, was set to +0.0025|e|. According to the Gouy−Chapman model,[27,28] the charge of +0.0025|e| gives rise to an approximate surface potential of ca. 20 mV in 0.01 M NaCl solution, which is comparable to the experimental zeta potential values of stainless steel at 0.01 M NaCl, pH 3.[16] Furthermore, to investigate the effects of charge density of the surface on adsorption behavior of β-Lg, surfaces with $C = 0$, +0.0125|e|, +0.0200|e|, and +0.0250|e| per each Au atom belonging to the (100) surface were also prepared. The force field for Au atoms was a classical combination of Lennard-Jones potential[29] (van der Waals interaction) and electrostatic potential between atom-based charges, as described in earlier studies.[18,30]

Originally, the OPLS-AA force field was not intended to be used for adsorption simulation and combination to the Au parameter. It is noteworthy that much work remains to be done to validate a

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Table 1. Deprotonation State of Dissociable Amino Acid Residues in β-Lg Model

		prediction by PROPKA			protein molecular model	
residue number	residue type	pK_a	degree of deprotonation	apparent charge per each residue in |e|	deprotonation state	charge per each residue
1	N-ter	7.65	0.00	1.00	protonated	1
8	LYS	10.50	0.00	1.00	protonated	1
11	ASP	3.65	0.18	−0.18	protonated	0
14	LYS	10.43	0.00	1.00	protonated	1
20	TYR	13.27	0.00	0.00	protonated	0
28	ASP	2.43	0.79	−0.79	deprotonated	−1
33	ASP	4.22	0.06	−0.06	protonated	0
40	ARG	13.51	0.00	1.00	protonated	1
42	TYR	6.88	0.00	0.00	protonated	0
44	GLU	5.50	0.00	0.00	protonated	0
45	GLU	4.71	0.02	−0.02	protonated	0
47	LYS	10.22	0.00	1.00	protonated	1
51	GLU	3.97	0.10	−0.10	protonated	0
53	ASP	3.23	0.37	−0.37	protonated	0
55	GLU	4.01	0.09	−0.09	protonated	0
60	LYS	9.59	0.00	1.00	protonated	1
62	GLU	4.14	0.07	−0.07	protonated	0
64	ASP	4.01	0.09	−0.09	protonated	0
65	GLU	4.50	0.03	−0.03	protonated	0
66	CYS	99.99	0.00	0.00	protonated	0
69	LYS	10.36	0.00	1.00	protonated	1
70	LYS	10.08	0.00	1.00	protonated	1
74	GLU	4.34	0.04	−0.04	protonated	0
75	LYS	10.50	0.00	1.00	protonated	1
77	LYS	10.50	0.00	1.00	protonated	1
83	LYS	10.50	0.00	1.00	protonated	1
85	ASP	3.80	0.14	−0.14	protonated	0
89	GLU	6.26	0.00	0.00	protonated	0
91	LYS	10.50	0.00	1.00	protonated	1
96	ASP	2.69	0.67	−0.67	deprotonated	−1
98	ASP	1.56	0.96	−0.96	deprotonated	−1
99	TYR	10.07	0.00	0.00	protonated	0
100	LYS	10.01	0.00	1.00	protonated	1
101	LYS	10.29	0.00	1.00	protonated	1
102	TYR	14.99	0.00	0.00	protonated	0
106	CYS	99.99	0.00	0.00	protonated	0
108	GLU	3.05	0.47	−0.47	protonated	0
112	GLU	4.25	0.05	−0.05	protonated	0
114	GLU	4.85	0.01	−0.01	protonated	0
119	CYS	99.99	0.00	0.00	protonated	0
121	CYS	10.56	0.00	0.00	protonated	0
124	ARG	11.10	0.00	1.00	protonated	1
127	GLU	3.98	0.09	−0.09	protonated	0
129	ASP	2.91	0.55	−0.55	deprotonated	−1
130	ASP	3.75	0.15	−0.15	protonated	0
131	GLU	4.43	0.04	−0.04	protonated	0
134	GLU	4.5	0.03	−0.03	protonated	0
135	LYS	10.43	0.00	1.00	protonated	1
137	ASP	3.30	0.33	−0.33	protonated	0
138	LYS	10.43	0.00	1.00	protonated	1
141	LYS	10.5	0.00	1.00	protonated	1
146	HIS	6.51	0.00	1.00	protonated	1
148	ARG	11.90	0.00	1.00	protonated	1
157	GLU	3.98	0.09	−0.09	protonated	0
158	GLU	4.71	0.02	−0.02	protonated	0
160	CYS	99.99	0.00	0.00	protonated	0
161	HIS	3.77	0.15	0.85	protonated	1
162	C-ter	3.41	0.28	−0.28	protonated	0
				Total charge: 15.11		Total charge: 17

certain protein force field for simulation of protein adsorption, as pointed out by Agashe et al.[20]

2.2. MC Simulation. The initial orientation of β-Lg on the surface was determined using MC simulation with implicit water, as in the work of Zhou et al.[19]

First, one β-Lg molecule was placed with a random orientation so that its center of mass (COM) was 2.59 nm above the Au (100) surface. Then, the protein molecule was translated and rotated around its COM. During MC simulation, the protein structure was kept rigid. The MC system temperature was set to 300 K. Cut-off lengths of 1.0 and 1.7 nm were used, respectively, for the Lennard-Jones (van der Waals interaction) and electrostatic potential. As reported by Sun et al.,[31] a sigmoidal distance-dependence dielectric constant D was used for calculation of

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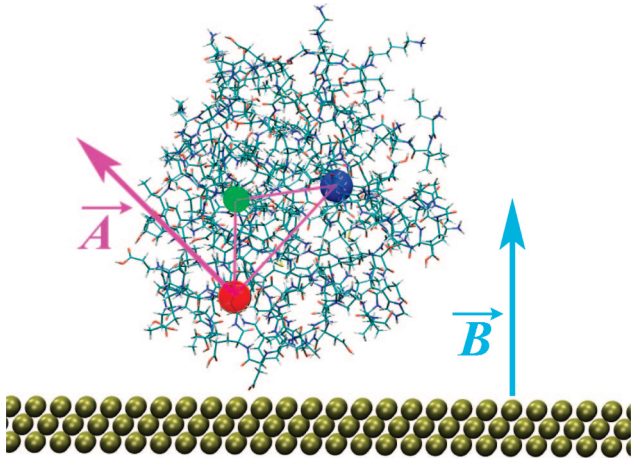

Figure 1. Description of the protein orientation angle θ. The tan space-filled representation is for gold. The space-filled ball representations are for S in Cys66 (red), S in Cys106 (blue), and α-C in Cys121 (green). Vector $\vec{\mathbf{A}}$ is normal to the plane containing three atoms of β-Lg, S in Cys66, S in Cys106, and α-C in Cys121. Vector $\vec{\mathbf{B}}$ is normal to the gold surface. The protein orientation angle θ is defined as the angle formed by these two vectors.

electrostatic interaction energy according to Mehler and Solmajor, as[32]

$$D = A + \frac{B}{1 + k\mathrm{e}^{-\lambda Br}}$$

where $B = \varepsilon_0 - A$, $\varepsilon_0 = 78.4$, $A = -8.5525$, $k = 7.7839$, $\lambda = 0.03627\mathrm{nm}^{-1}$, and r is the distance between two atoms. One million MC steps were carried out. Preliminary MC simulations revealed that β-Lg often translated far away from the Au surfaces with $C = +0.0125|e|$, $+0.0200|e|$, and $+0.0250|e|$ before it approached the surface. Therefore, to reduce the computation time, the translational motion of β-Lg was restrained for these Au surfaces so that the COM did not separate by more than 4.59 nm above the surface. The trajectories for MC simulation were analyzed using FORTRAN software produced by the authors. The change of vertical separation distance (VSD) between the surface and the COM of protein molecule was monitored to verify whether the protein molecule maintains its position close to the surface. To track the protein orientation with respect to the Au (100) surface, the protein orientation angle θ was also monitored: θ was defined as the angle at which the normal vector to the plane containing three atoms of β-Lg (S in Cys66, S in Cys106, and α-C in Cys121) forms with the normal vector to the gold surface, as presented in Figure 1.

The orientation and position of β-Lg with the least protein-surface interaction potential energy was selected as an initial orientation of subsequent MD simulation.

2.3. MD Simulation. All MD simulations were done using the MD program suite GROMACS on a quad-core processor Core 2 Quad cluster. The MD simulation systems were constructed with periodic boundary conditions with the following unit box size given as X × Y × Z, where the X and Y axes are parallel to the Au (100) surface plane and where Z is normal to the Au (100) surface plane, 10.25 nm × 9.84 nm × 10.25 nm. Also, β-Lg was placed on the Au (100) surface, then SPC water was added. The Cl^- and Na^+ ions were added to the simulation cell to produce a NaCl concentration of 0.01 M. Subsequently, to maintain system neutrality, chlorine ions or sodium ions were added to the simulation box again.

Using the GROMACS MD program suite, the system was energy-minimized using the steepest descent method, with the protein bond length and Au atoms fixed. Then the initial velocity of each atom was assigned from a Maxwell−Boltzmann distribution at 10 K. Subsequently, the temperature of the entire system was raised from 10 to 300 K at 2.90 K/ps. After the temperature reached 300 K, MD simulation with NVT ensembles was performed for 5 ns of simulated time. The temperature control was done using the Nose-Hoover method.[33,34] Positions of gold atoms were fixed during the simulation. As for the protein, only bond lengths were fixed according to results of precedent studies,[20,35] which enabled a time step of 2 fs to be used for the MD simulation. The cutoff distances used for the van der Waals and the electrostatic interactions were 1.0 and 1.7 nm, respectively, as in the MC simulation. The resultant trajectory data were saved at every 2000 steps (4 ps).

For data analyses of MD, FORTRAN software and the GROMACS suite analysis tools were used. The trajectories were analyzed to monitor the VSD and the orientation angle θ as well as the MC simulation using the "g_dist" and "g_sgangle" tools in GROMACS. To obtain information related to amino acid residues contributing to β-Lg adsorption, amino acid residue containing the closest protein atom to the Au (100) surface was evaluated as the function of simulation time using the combination of "g_dist" and the authors' FORTRAN software.

During MD simulations, the protein is not treated as a rigid body; it has internal motions. If large structural changes continue in the protein molecule during the MD simulation, the θ described above will be influenced considerably by structural changes of the protein, which is a source of inaccuracy for estimating the protein orientation to the surface. Consequently, the root-mean-square deviation (rmsd) of the protein structure compared to the energy-minimized structure was also calculated to check structural change in the β-Lg molecule during the MD run. This was done using the "g_rms" tools in GROMACS.

3. Results

3.1. MC Simulation. Figures 2 and 3 respectively show typical changes of VSD and θ during MC simulations. For the conditions of $C = 0|e|$, $+0.0025|e|$, and $+0.0125|e|$, the VSD and θ approach specific values (VSD of ca. 1.7 nm, θ of ca. 110°) at very early MC steps and stabilized at those values. The minimum distances between the protein atoms and the Au (100) surface were around 0.2 nm when the VSD took these values. We repeated the MC simulations five times using different initial protein orientation for each surface charge condition. All repetitions engendered similar results for $C = 0|e|$, $+0.0025|e|$, and $+0.0125|e|$; the initial orientation and position of β-Lg for MD were easily determined according to the criteria described above.

In the MCs at $C = +0.0200|e|$ and $+0.0250|e|$, the values of θ and the VSD fluctuated more intensively than $C = 0$, $+0.0025|e|$, and $+0.0125|e|$. As stated in the Materials and Methods section, the translational motion of β-Lg was restrained for $C = +0.0125|e|$, $+0.0200|e|$, and $+0.0250|e|$ so that the COM did not separate by more than 4.59 nm above the surface. This means that the value of VSD was limited within 4.59 nm. As shown in Figure 2d,e, the VSD often visited this restrained value. When the VSD was around 4.5 nm, the θ changed wildly and did not show any tendency of taking specific value. At $C = +0.0200|e|$, a tendency of the θ value being ca. 20° or ca. 110° was recognized for some repetitions, although these values were not as stabilized as those for $C = 0|e|$, $+0.0025|e|$, and $+0.0125|e|$. Therefore, for subsequent MD, we selected the two orientations with the least protein−surface interaction potential energy, respectively, from those around both θ of ca. 20° and θ of ca. 110°. Regarding the condition of $C = +0.0250|e|$, the θ value tended to be ca. 20° with large fluctuation of θ for some repetitions. The orientation and position of β-Lg with the least protein−surface interaction potential energy was also observed at ca. 20°. Then we selected

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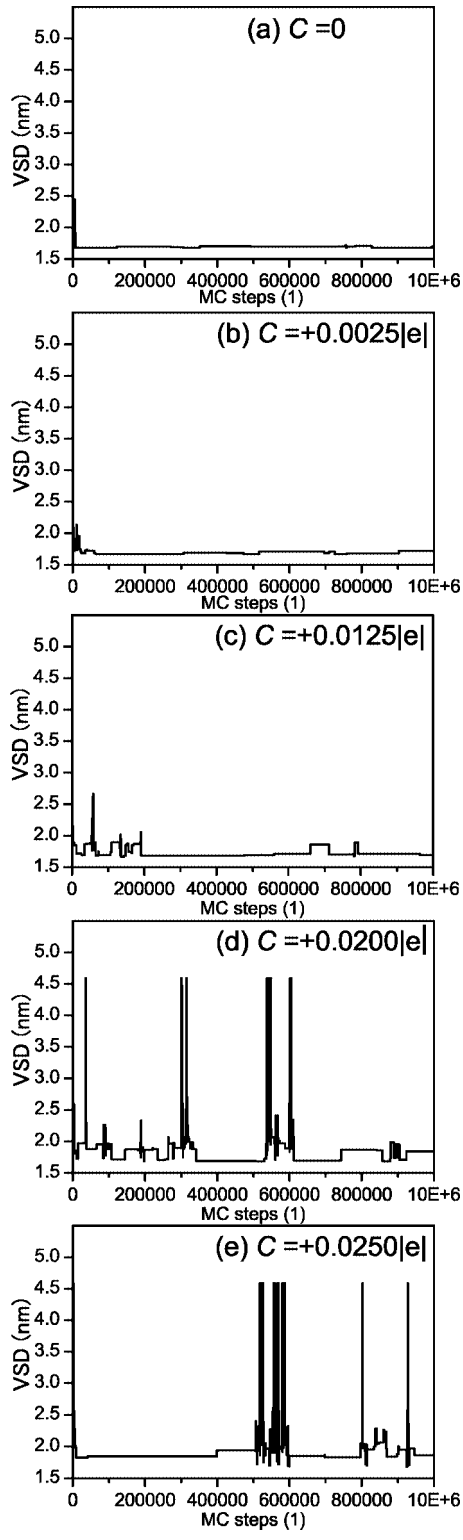

Figure 2. Change of the VSD during MC simulations. Charges assigned to individual Au atoms belonging to the (100) surface, *C*, were (a) zero, (b) +0.0025|e|, (c) +0.0125|e|, (d) +0.0200|e|, and (e) +0.0250|e|.

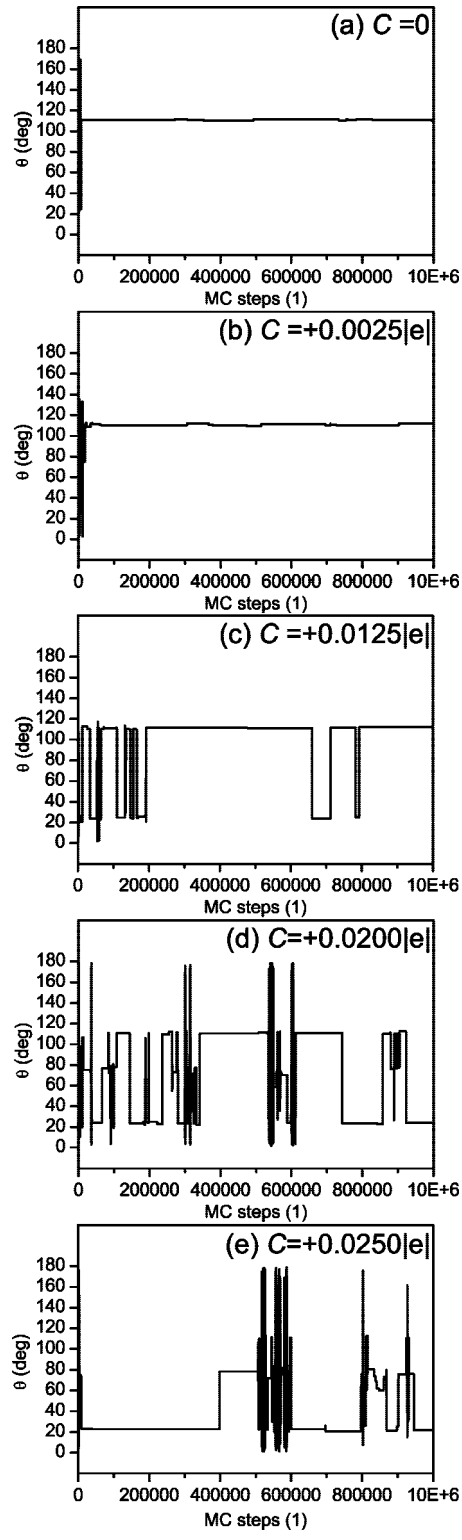

Figure 3. Change of the protein orientation angle θ during MC simulations. Charges assigned to individual Au atoms belonging to the (100) surface, *C*, were (a) zero, (b) +0.0025|e|, (c) +0.0125|e|, (d) +0.0200|e|, and (e) +0.0250|e|.

the orientation and position as initial ones for following MD simulations.

3.2. MD Simulation. Results of rmsd obtained during the MD simulations are shown in Figure 4 as a function of simulation time. An initial rapid increase in rmsd was observed in the first 500 ps. Subsequently, the rate of increase of rmsd decreased and tended to stabilize for most conditions. In some conditions, such as $C = +0.0250|e|$ and $+0.0125|e|$, the rmsd continued to increase gradually, even after 500 ps. However, the values of rmsd were ca. 0.23 nm or less during 5 ns of simulation time. The resolution of the original 3BLG file is 0.256 nm.[36] Therefore, the protein molecules retained their overall structure during the simulation, which reflects that the calculated value of the protein orientation

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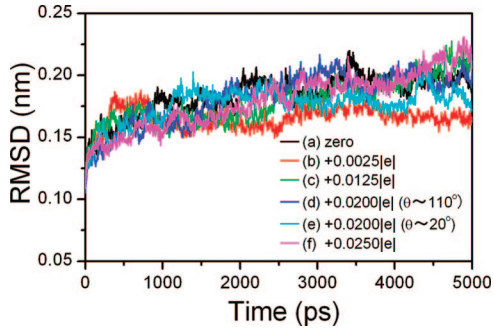

Figure 4. rmsd of the protein structure of β-Lg compared to the energy-minimized crystal structure during MD simulation. Charges assigned to individual Au atoms belonging to the (100) surface, *C*, were (a) 0, (b) +0.0025|e|, (c) +0.0125|e|, (d) +0.0200|e| with an initial θ of ca. 110°, (e) +0.0200|e| with an initial θ of ca. 20°, and (f) +0.0250|e|.

angle θ is useful as an index of protein orientation to a surface.

Figures 5a−f and 6a−f show VSD and θ as a function of the simulation time.

For $C = 0$, +0.0025|e|, +0.0125|e|, and +0.0200|e|, the initial θ of ca. 110° was stable throughout the simulation time as well as the initial VSD (VSD of ca. 1.7 nm, θ of ca. 110°; Figures 5a−d and 6a−d). During simulation, the minimum distances between the protein atoms and the Au (100) surface were also stable around 0.2 nm (data not shown). These show that the β-Lg molecule was adsorbed with orienting itself with a specific part of the protein surface close to the Au surface over the course of the 5 ns simulation. Furthermore, the values of θ were almost equal among the three conditions, indicating that the part of the protein surface close to the Au (100) surface was similar.

On the Au surface of $C = +0.0200|e|$ with an initial θ of ca. 20°, the value of VSD increased and θ changed significantly. Consequently, the protein molecule translated away from the surface with rotational motion during the simulation.

Similarly, both VSD and θ changed markedly; no stabilization tendency was observed on the Au surface with $C = +0.0250|e|$. The time courses of VSD and θ indicate that the protein translated away from the surface during the first 3 ns and then exhibited an approach to the Au surface for the remainder of the simulation, with continuous rotational motion. Stable adsorption of the protein was not observed.

The distributions of occupation time for the closest amino residues are shown in Figure 7a−f. On Au (100) surfaces with $C = 0$, +0.0025|e|, and +0.0125|e| (Figure 7a−c), the Thr125 residue was at the top position among all amino residues. In fact, Thr125 was also second after Pro50 on the Au surface of $C = +0.0200|e|$ with an initial θ of ca. 110° (Figure 7d). Figures 5 and 6 show that the protein molecule oriented itself with its specific protein surface area near the surface. Therefore, these results suggest that the domain of the protein molecule in contact with the surface was located around Thr125 for these surface and initial θ conditions. Through careful observation of the trajectory animations, we confirmed this. Figure 8 depicts a typical snapshot of β-Lg close to the Au surface with $C = +0.0025|e|$ as an example. As the figure shows, the predicted segment mainly contributing β-Lg adsorption to the stainless steel surface at acidic pH in experimental studies[14,17] is also shown. Interestingly, it was located near Thr125.

At conditions of $C = +0.0200|e|$ with an initial θ of ca. 20°, and for +0.0250|e|, both Ile29 and Asp64 were the closest to the surface for the longest time during the simulation (Figure 7e,f). However, the results in VSD (Figure 5e,f) and θ (Figure 6e,f) portray that the protein molecule was not located stably near the Au surface for these two conditions. For this reason, we inferred that the residues described above did not contribute protein adsorption to the surface.

4. Discussion

In MD simulations, the stable adsorption parts of protein on the Au (100) surfaces with $C = +0.0025|e|$, +0.0125|e|, and +0.0200|e| were almost identical to that with $C = 0$, which indicates that the main interactions contributing the adsorption were from the van der Waals interaction because the difference of Au surface charge density did not affect the protein adsorption parts. Increasing the Au surface charge to +0.0250|e| caused unstable protein positions and orientations, probably because repulsive electrostatic interactions between positive charges on

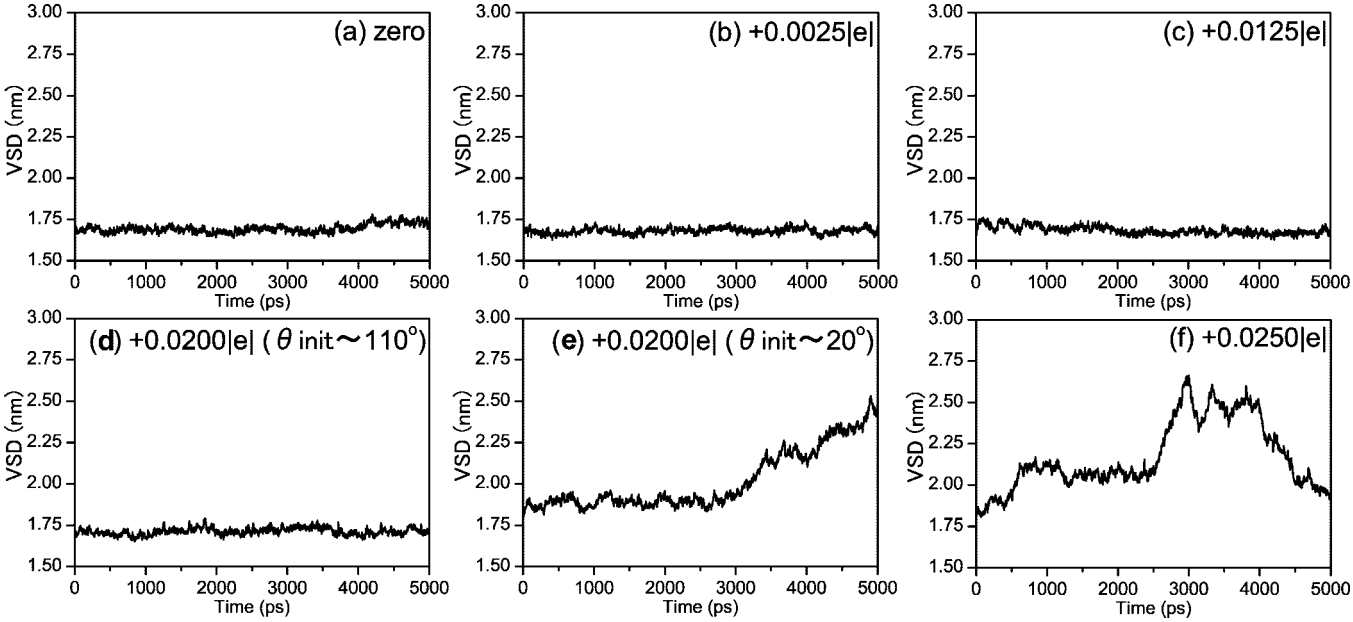

Figure 5. Change of the VSD during MD simulations. Charges assigned to individual Au atoms belonging to the (100) surface, *C*, were (a) 0, (b) +0.0025|e|, (c) +0.0125|e|, (d) +0.0200|e| with an initial θ of ca. 110°, (e) +0.0200|e| with an initial θ of ca. 20°, and (f) +0.0250|e|.

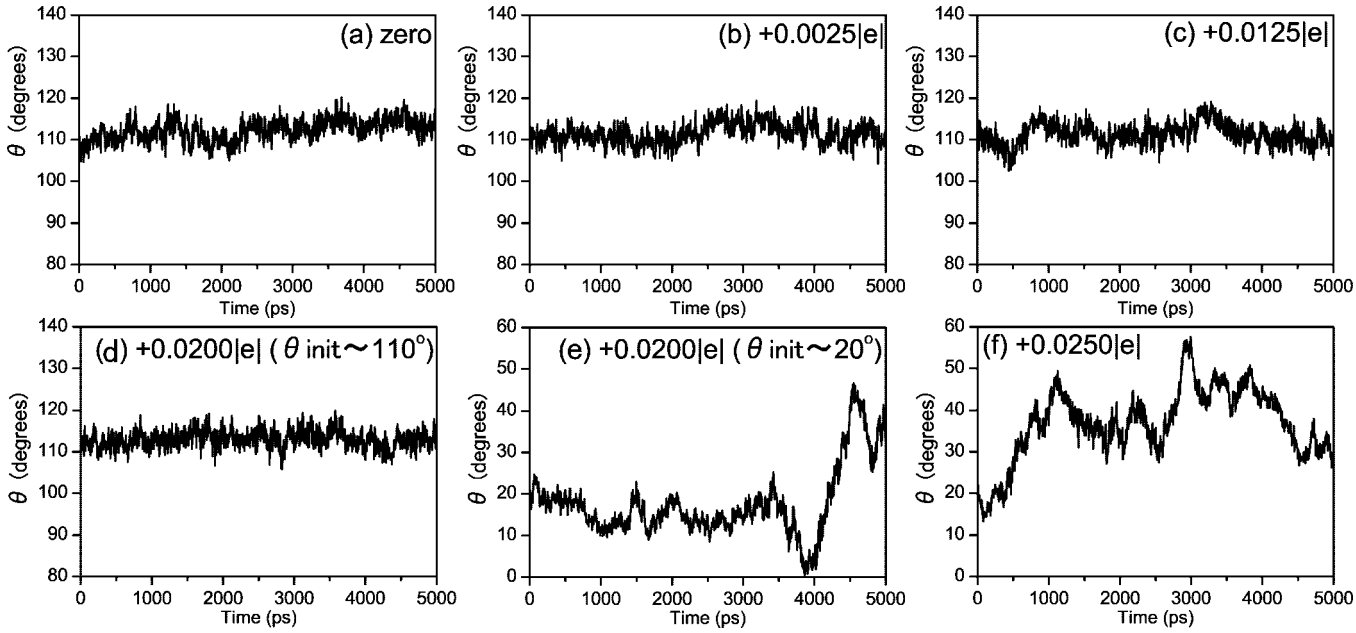

Figure 6. Change of the protein orientation angle θ during MD simulations. Charges assigned to individual Au atoms belonging to the (100) surface, *C*, were (a) 0, (b) +0.0025|e|, (c) +0.0125|e|, (d) +0.0200|e| with an initial θ of ca. 110°, (e) +0.0200|e| with an initial θ of ca. 20°, and (f) +0.0250|e|.

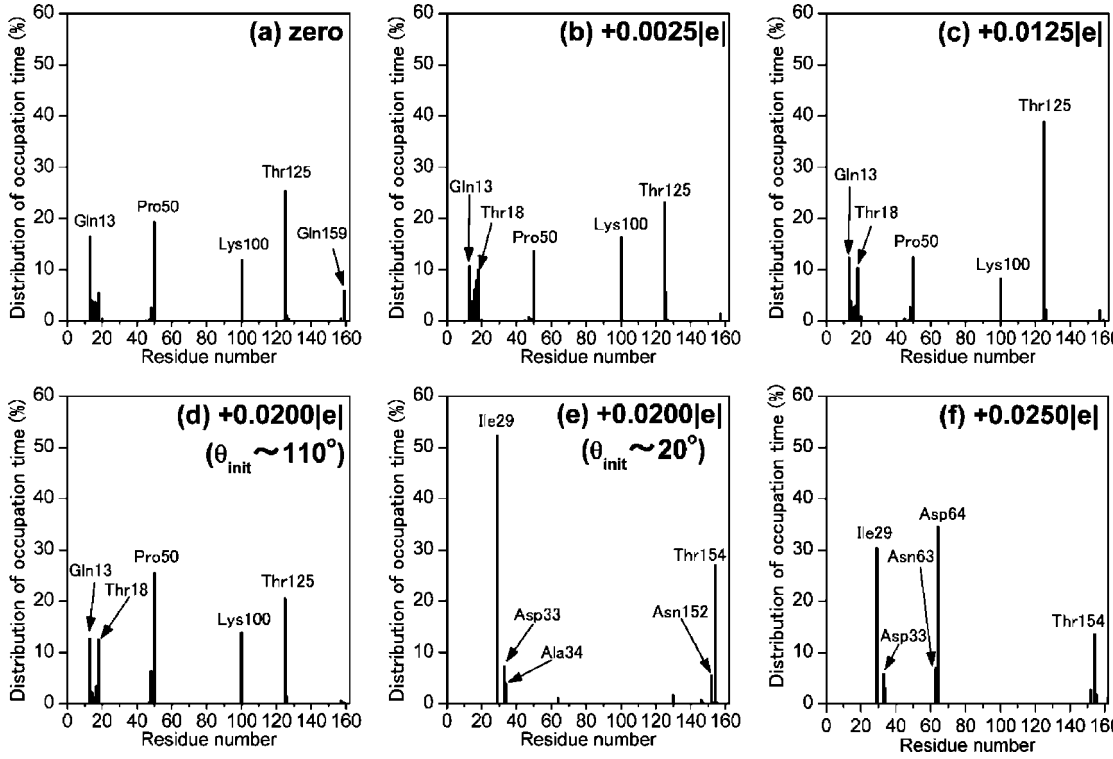

Figure 7. Time distribution of the closest amino acid residues during MD simulations. Charges assigned to individual Au atoms belonging to the (100) surface, *C*, were (a) 0, (b) +0.0025|e|, (c) +0.0125|e|, (d) +0.0200|e| with an initial θ of ca. 110°, (e) +0.0200|e| with an initial θ of ca. 20°, and (f) +0.0250|e|.

the protein surface and those on the Au surface increased so that the attractive van der Waals interactions were insufficient to hold the protein molecule close to the surface.

According to results of previous experimental studies of the adsorption of the peptides from β-Lg[14,37] and enzymatic fragmentation of β-Lg adsorbed onto stainless surfaces, the adsorption domain of β-Lg at acidic pH was estimated as the 125−135th amino acid residues. Interestingly, the simulations used for this study predicted that it was around the 125th amino residue (Thr125), which agrees well with this experimental estimation. Regarding the adsorption mechanism of β-Lg at acidic pH, the authors of previous studies proposed the following supposition: the neutral side carboxylic groups in the acidic amino acid residues such as Glu127, Asp129, Glu131, and Asp134 in

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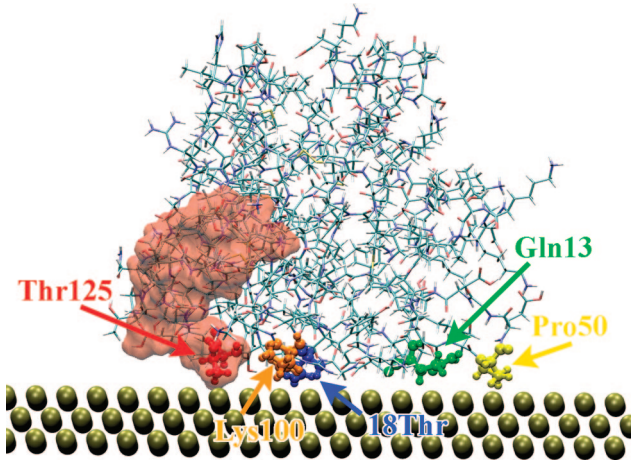

Figure 8. Typical snapshot of β-Lg near the Au surface with $C = +0.0025|e|[C]$. For clarity, descriptions of water, Na^+, and Cl^- were omitted. The tan space-filled representation is for gold. The CPK space-filling representation is for the five major residues that were closest to the surface during the MD simulations: Thr125 (red), Lys100 (orange), Pro50 (yellow), Gln13 (green), and Thr18 (blue). The Surf representation with cyan half-tone is for the adsorption domain, as predicted through previous experimental studies (125−135th amino residues). The stick representation is for other residues in β-Lg.

the 125−135th amino acid residues are induced to be deprotonated by the positively charged surface; consequently, they became negatively charged. The attractive electrostatic interactions between the negatively charged residues and the positively charged surface cause adsorption of the β-Lg molecule. This hypothesis arises from results of the FT-IR analysis of the peptide fragments from β-Lg adsorbed onto the stainless surface at acidic pH; the side carboxylic groups of the peptides adsorbed onto the stainless steel surface were induced to ionization. Several other papers describing experiments and theoretical calculations have also reported that the protonation state of a protein molecule can undergo changes attributable to the imposed electric potential when it approaches a charged surface.[38−43] However, the mechanism described above provides no clear explanation as to why only the acidic amino acid residues in the 125−135th residues contribute to the adsorption, although β-Lg has many other acidic amino acid residues. Results of this simulation might give a hint to derive the reason, although the surfaces used in these simulations were modeled using a Au (100) surface with virtually positive charges. In this study, the protonation state of dissociable amino acid residues was determined using the pK_a prediction program PROPKA. On the basis of the prediction, the Asp129 were judged to be deprotonated. Therefore, the Asp129 was deprotonated in a solution without a surface. The dissociations of other acidic amino acid residues induced by surface positive charges were not included. Nevertheless, the adsorption domain of β-Lg predicted by the simulation was near that estimated by the experimental results. Therefore, these simulation results suggest that, without the dissociation induced by the surface charge, the β-Lg molecule tends to be adsorbed spontaneously at first, with the specific part of 125−135th residues close to the surface. The electrostatic interaction between the deprotonated Asp129 and surface positive charges would not be extremely helpful in determining the initial adsorption domain of protein because the adsorption domains evaluated by the simulation without positive surface charges are almost equal. As described in the preceding paragraph, van der Waals interactions between the protein molecule and the surface would mainly contribute to the initial adsorption. After initial adsorption or simultaneously with it, dissociation of the side carboxylic groups of acidic amino acid residues near the surface, such as Glu127, Asp130, Glu131, and Glu134, might be induced, leading to firmer adsorption by attractive electrostatic interaction between the overlying residues and the surface.

Detailed chemical structure models of the stainless steel surface that are useful for molecular simulation have not been established to date. For that reason, no reports describe simulation of protein adsorption using a stainless steel surface. Stainless steel surfaces are known to include many different metal atoms and chemical groups such as Fe, Cr, and −OH. The simulation conducted here did not account for specific interaction between β-Lg and those surface materials, which can contribute to determining the adsorption domain of β-Lg molecules. For example, Schmidt and Steinemann[44] reported that amino acids tend to adsorb onto TiO_2 surfaces by their carboxylic groups, thereby replacing a basic hydroxyl on a Ti site at acidic pH. A similar reaction might occur between a stainless steel surface and side carboxylic groups of amino acid residues in a β-Lg molecule. To obtain more detailed knowledge about protein adsorption onto a stainless steel surface, a chemical model of the stainless steel surface should be established with its accompanying force-field parameters.

5. Conclusion

This study was intended to obtain more detailed molecular information related to β-Lg adsorbed onto a stainless steel surface at acidic pH, which had been difficult for previous experimental studies to assess. Although the adsorption domain of β-Lg at acidic pH was estimated as te 125−135th residues from results of previous experimental studies, no clear explanation justifies why that part tended to be close to the surface. The simulation described herein was undertaken to obtain more detailed insight into the adsorption mechanism at acidic pH. From simulation using the Au (100) surface with a virtual positive charge, we can propose a detailed mechanism by which 125−135th amino acid residues contributed to β-Lg adsorption at acidic pH as follows: β-Lg molecule is spontaneously adsorbed at first with the specific part of the 125−135th residues close to the surface through van der Waals interactions rather than electrostatic ones. Simultaneously or subsequently, dissociation of the side carboxylic groups of acidic amino acid residues is induced near the surface in the 125−135th residues. Then the adsorption becomes firmer by attractive electrostatic interaction between the residues and the surface.

It is noteworthy that the surface used in this simulation differed from stainless steel itself. Additionally, it is noteworthy that the force field used for this study was not originally designed for protein adsorption to a metal surface. As reported by many researchers, no validated current force field exists for protein adsorption to a metal surface. For additional studies, validation and development of a force field is necessary, along

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with modeling of the stainless steel surface. The system of β-Lg adsorption on stainless steel at acidic pH might be a useful simulation system for validation and development of force field because the adsorption domain has been estimated experimentally.

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