Frederick F. Caserio and co-workers.[12] In this work, to seek the evidence of isomerization **2**, **3** and **4**, the following studies were performed. The reaction of the reaction mixture (**2**, **3** and **4**) with strong base (potassium-*tert*-butoxide) at room temperature as well as high temperature was carried out in ether. The reaction of **2** with strong base was also carried out in ether. However, the evidence of isomerization was not observed. This result indicates that the products **3** and **4** were not produced from **2**.

Formation of products **3** and **4** involves abstraction of a hydrogen from tert-butanol to form dipole intermediates **III** and **III'** which lose hydrogen by the base (potassium-*tert*-butoxide) to give products **3** and **4**.

Major product **2** (**2a**: 60%, **2b**: 55%) was obtained from dipole intermediates **II** and **II'**. This product was separated by preparative GC and characterized by GC-Mass, ^{1}H NMR, ^{13}C NMR and Dept-NMR.

The ^{1}H NMR spectrum of **2a** showed a quintet of two protons at δ 5.27 corresponding to the vinyl hydrogen and a triplet at δ3.58 (4H) corresponding to the ring hydrogen. The ^{1}H NMR spectrum of **2b** has a triplet at δ 3.22 (4H) and quintet (2H) at δ 5.05. This spin-spin resonance splitting was ascribed to the long range coupling between vinyl hydrogens (2H) and ring hydrogens (4H).

Minor products **3a** (15%) and **3b** (18%) were characterized by GC-Mass, ^{1}H NMR, ^{13}C NMR and Dept-NMR. The spectral data was in agreement with the proposed structure.

A minor products **4a**, **4b** and **5** were also present. The amount of these products was too small to be isolated (<3%); only identified by GC-Mass. The mass spectrum of these minor products exhibited the correct isotopic ratio for halogen atom and a fragmentation pattern for a proposed structure. We propose that **5** may came from the reaction of [1.1.1]propellane with potassium halide generated from KOC$(CH_3)_3$ and CHX_3.

In summary, we have obtained compounds **2**, **3** and **4** through the reaction of [1.1.1]propellane with singlet dihalocarbene. In addition, we have examined isomerization of **2**, **3** and **4** in strong base (potassium-*tert*-butoxide) at room temperature as well as high temperature. No isomerization was observed. Formation of compounds **3** and **4** in the reaction of [1.1.1]propellane with dihalocarbene strongly indicates that reaction intermediate for the products is [1.1.1.1]paddlane **I**. The detailed mechanistic study is currently under investigation and the results of these studies will be reported in due course.

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Eu(III) Luminescence Probe into the Cation Binding Sites of Subtilisin Carlsberg

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Static and time-resolved Eu^{3+} luminescence spectra are investigated to understand the environments and functions of metal cation binding sites of subtilisin Carlsberg (sC). Our results show that Eu^{3+} luminescence spectroscopy is a good probe to the structures and roles of the Ca^{2+} binding sites in sC,[1-3] which is one of subtilisin family.[4] Only the Ca^{2+} ion in the weak binding site[1,2] is replaced by Eu^{3+} ion. Eu^{3+} luminescence indicates that the weak binding site has a significantly lower symmetry than an octahedral group. The Eu^{3+} ion in the weak binding site is coordinated to four wat-

er molecules and additional coordination in solutions, compared with coordination in crystals, is suggested to make the weak binding site essential in enzyme reaction.[5,6] The environment of the weak binding site is considerably less polar than water.

Subtilisin, a kind of the most studied proteases, is very interesting not only scientifically but also industrially, for subtilisin enzymes are used in such diverse applications as meat tenderizers and laundry detergents.[7-9] Furthermore, recent researches on catalytic efficiency and specificity in various organic solvents have enhanced the practical utilities of sC related to synthetic applications.[7,9,10] sC, a serine endopeptidase secreted by Bacillus licheniformis, is a single polypeptide chain of 274 amino acid residues and has two Ca^{2+} ion binding sites.[1-3,11] The binding site with a strong affinity is reported[2,3,11] with X-ray crystal structures to have approximately octahedral coordination sphere with three carbonyl groups, two carboxyamide groups, and one carboxylate group while the other with a weak affinity to be in tetragonal pyramidal structure with three peptide carbonyl groups and two water molecules. Ca^{2+} ion binding is known to slow down autolysis and to enhance thermal stability.[1,12] Recent report[13] on subtilisin BPN', which is similar to sC in structure, indicates that the weak binding site as well as the strong one enhances the protein stability dramatically as it binds to Ca^{2+} ion. It has also been reported[1] that the affinity of the strong binding site to Ca^{2+} ion is so strong that the Ca^{2+} ion bound in this site is difficult to remove. These reports together designate that the weak binding site is important in studies of metal cation binding sites.

We are applying Eu(III) luminescence spectroscopy to understand the structural adaptation and stability of sC with respect to metal cation binding and to study the environments of metal cation binding sites, since Eu(III) luminescence probe is extensively used to solve a variety of structural problems in biological molecules[14,15] and since Eu^{3+} ion is similar to Ca^{2+} in size so that Ca^{2+} ion can be substituted with Eu^{3+} ion isomorphically.[14,16]

Experimental

The preexisting metal cations and autolyzed fragments of sC, purchased from the Sigma, were deionized and separated respectively, using a column containing weakly acidic cation exchanger (Sigma, Amberlite CG-50). Then highly concentrated $EuCl_3$ aqueous solutions were added to deionized and purified sC aqueous solutions to fabricate appropriately concentrated Eu^{3+}-exchanged sC samples. The measured pHs of final samples were about 5.5. Deuterated samples were prepared by treating the above procedures in ^2H_2O.

For the measurement of static luminescence spectra, the wavelength of sample excitation beam from the 350-W xenon arc lamp (Schoeffel, LDS 255 HR) was separated using a 0.25-m monochromator (Kratos, GM 252) and the wavelength of sample luminescence was selected using a 0.25-m monochromator (Kratos, GM 252) which was attached with a PMT (Hamamatsu, R376). To measure Eu^{3+} emission kinetic profiles, sample was excited at 393 nm with a dye laser (Laser Photonics, LN102) which was pumped by a N_2 laser (Laser Photonics, LN1000). Sample luminescence was focused to a 0.25-m monochromator (Bausch & Lomb) attached with a PMT (Hamamatsu, R928) and the signal from the PMT was recorded with a digital oscilloscope (Tektronix, TDS 350).

Results and Discussion

Figure 1 shows that the shape of Eu^{3+} luminescence spectrum becomes significantly different as the environment of Eu^{3+} ion changes from water to sC cation binding site. Spectral shifts are not noticeable with our current spectral resolution. However, the relative intensity of $^5D_0 \rightarrow {}^7F_2$ transition increases dramatically with binding to the enzyme and becomes even larger than that of $^5D_0 \rightarrow {}^7F_1$ transition. The electric dipole transition of $^5D_0 \rightarrow {}^7F_2$ is forbidden by parity in a symmetry with an inversion center such as the octahedral group.[15] However, interactions with the ligand field or with vibrational states mix electronic states of different parities and the transition arising from these interactions is called forced electric dipole transition. Since the forced transition of $^5D_0 \rightarrow {}^7F_2$ is originating from interactions with neighbors, it is hypersensitive to the environment of Eu^{3+} ion and is employed to probe environmental, especially short range, effect on the centered metal cation. The magnetic dipole transition of $^5D_0 \rightarrow {}^7F_1$ is allowed and insensitive to local stark effect. For this reason the transition of $^5D_0 \rightarrow {}^7F_1$ is frequently used as an internal standard in monitoring the surrounding of Eu^{3+} ion. For an example, if RI(2), the ratio of $^5D_0 \rightarrow {}^7F_2$ transition intensity to $^5D_0 \rightarrow {}^7F_1$ transition intensity, is larger, the binding site of Eu^{3+} ion has lower symmetry and less polar and more covalent characters.[17] As the coordination environment of Eu^{3+} ion changes from water to the binding site of sC, RI(2) enlarges over four times. This indicates that the binding site has much lower symmetry and less polar and more covalent characters than water. In particular, the large value of RI(2) unquestionably designates that the Eu^{3+} binding site of sC has a significantly lower symmetry

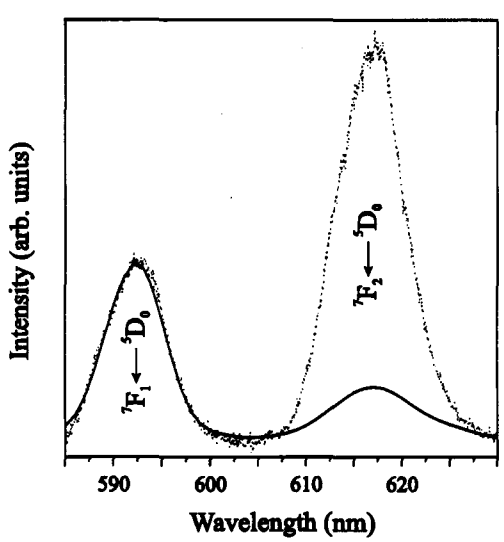

Figure 1. Eu^{3+} luminescence bands, excited at 393 nm, of $^5D_0 \rightarrow {}^7F_1$ and $^5D_0 \rightarrow {}^7F_2$ transitions for 4.0-mM sC (dotted line) and sC-free (solid line) aqueous solutions of 8.0-mM $EuCl_3$. Note that RI(2), the ratio of $^5D_0 \rightarrow {}^7F_2$ intensity to $^5D_0 \rightarrow {}^7F_1$ intensity, increases dramatically from 0.7 to 2.9 as enzyme sC is added into aqueous Eu^{3+} solution.

than an octahedral symmetry.

Figure 2 and Table 1 show that Eu^{3+} luminescence decay profiles in aqueous solutions of sC and Eu^{3+} have two decay components. The variation of $[Eu^{3+}]/[sC]$ from 0.5 to 1 and 2 changes the relative amplitude of the slow component from 59% to 39% and 24%, respectively, without modifying the number of decay components and without varying decay time constants. As seen in Figure 2(b), replacement of OH oscillators in the first coordination sphere by OD ones causes the vibronic deexcitation pathway to become exceedingly inefficient. This fact enabled[14] us to determine the numbers of water molecules coordinated to Eu^{3+} ion as 8.3 and 4.2 for the fast and slow decay components, respectively. Aqueous Eu^{3+} ion is reported to have 8 to 9 coordinated water molecules in the aspect of Eu^{3+} luminescence quenching dynamics.[14] In consequence, the slow component is attributed to emission from Eu^{3+} bound in a specific cation binding site of sC while the fast one from Eu^{3+} surrounded by water or adsorbed to a nonspecific external surface of sC. The question arising is why only one component is ascribed to emission from the Eu^{3+} of sC binding site despite the fact that X-ray crystal structures suggest two Ca^{2+} binding sites. It is considered that the Ca^{2+} ion bound in the strong binding site (K_d-10^{-8} with Ca^{2+})[13] is not deionized during our deionization process with weakly acidic cation exchanger, so that Eu^{3+} ion is exchanged into the weak binding site only. The weak binding site (K_d-10^{-2} with Ca^{2+})[13] may be strong enough to bind Eu^{3+} ion at our tried concentrations since its affinity with trivalent Eu^{3+} ion is expected to be much greater than that with divalent Ca^{2+} ion. As $[Eu^{3+}]/[sC]$ increases from 0.5, the relative amplitude of the slow component grows less with no changes in decay component's number and time constants. This suggests that the luminescence of enzyme-bound Eu^{3+} ion originates from the weak binding site and that the slow decay component is the decay profile of the Eu^{3+} ion in the weak binding site.

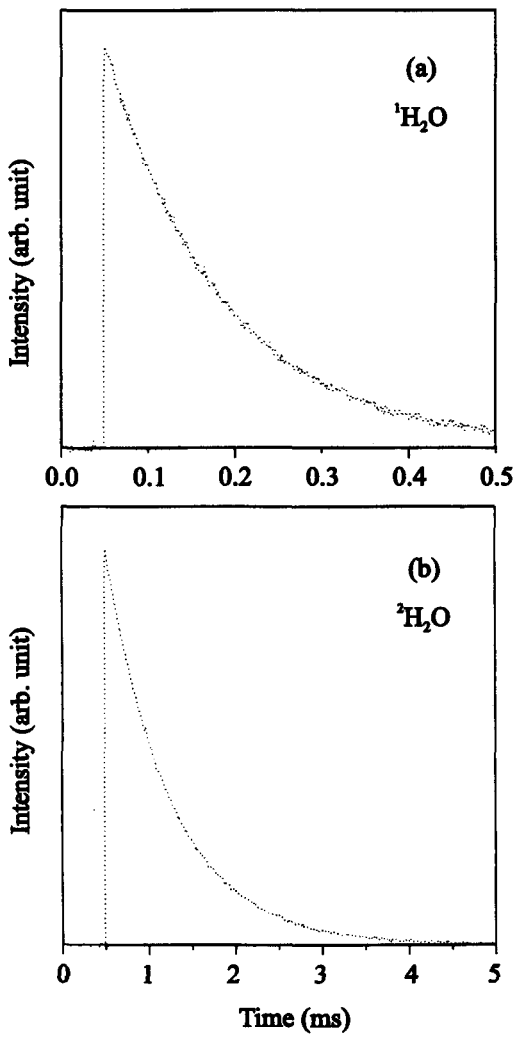

Figure 2. Eu^{3+} luminescence decay profiles, excited at 393 nm and collected at 617±12 nm, of Eu^{3+}-exchanged sC in ^1H_2O (a) and in ^2H_2O (b). The molar ratios of Eu^{3+} to sC were 2 for both profiles and the concentrations of sC were 2.6 and 4.6 mM for the profiles (a) and (b), respectively. Note that the temporal window of (b) is ten times larger than that of (a).

Table 1. Decay times with their relative amplitudes and water coordination numbers, extracted from the decay pofiles in Figure 2

Component Name	in ^1H_2O		in ^2H_2O		Water Coordination Number[a]
	τ (ms)	Amplitude	τ (ms)	Amplitude	
fast	0.11	76%	0.83	76%	8.3
slow	0.21	24%[b]	1.26	24%	4.2

[a]The numbers of coordinated water molecules were calculated following the method described in the ref. 14. [b]The relative amplitude of the slow component decreases continuously from 59% to 39% and 24% as $[Eu^{3+}]/[sC]$ increases from 0.5 to 1 and 2, respectively.

The luminescence from the Eu^{3+} ion bound in the weak binding site has a large value of RI(2) as 3 and the decay time of 0.21 ms. The Eu^{3+} ion is coordinated to four water molecules in the aspect of relaxation dynamics. These results from Eu^{3+}-exchanged sC aqueous solutions agree well with the results from crystal structures but also provide information on others such as dynamics. The weak binding site has a considerably lowered symmetry than an octahedral symmetry and the Eu^{3+} ion in this site has greatly enhanced covalent character compared with aqueous Eu^{3+} ion. However, although crystal structures have suggested five coordinated ligands, two of which are water molecules, for the weak binding site,[2,3] four water molecules are coordinated to the Eu^{3+} ion of the same site in aqueous sC solutions which would be more similar to the real active enzyme of sC than crystals. The metal cation bound in the weak site of active sC enzyme can coordinate to sixth ligand and this sixth ligand could be a portion of a substrate. Supposing that the metal cation in the strong binding site is indispensable to maintain a proper conformation of the enzyme itself, the metal cation in the weak binding site is necessary to sustain a proper conformation of the intermediate enzyme-substrate complex as well as that of enzyme itself.

In order to make sure that the substitution of Eu^{3+} for Ca^{2+} does not change the structure and function of sC notably, we have checked several aspects. The autolysis rate of Eu^{3+} binding sC is as low as that of Ca^{2+} binding sC and lower than that of deionized sC. The circular dichroism spectrum of Eu^{3+} binding sC is the same as that of Ca^{2+} binding sC and different from that of deionized sC. In addition, we could

not observe any apparent differences in static absorption and emission spectra between Eu^{3+} binding sC and Ca^{2+} one.

In summary, we have exchanged the Ca^{2+} ion in the weak binding site of sC for Eu^{3+} without changing the structure and function of the enzyme noticeably. The weak binding site is considerably less polar than water and has a significantly lower symmetry than an octahedral group. However, the exchanged Eu^{3+} ion of the weak binding site has four coordinated water molecules in aqueous sC solutions. The additionally coordinating room of the metal cation in aqueous sC solutions, compared with coordination in crystal structures, is suggested to play an important role for the metal cation of the weak binding site to bear a proper conformation of intermediate sC-substrate complex during enzyme reaction.

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Resonance Raman Scattering and Surface-Enhanced Resonance Raman Scattering of Ru(II) Complexes

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It has been recognized that surface-enhanced Raman scattering (SERS) is a very useful technique for studying the adsorption process of molecules on metal surface.[1-5] When a molecule is adsorbed on metal surface, its Raman intensities can be enhanced by five to six orders of magnitude so that the normally insensitive Raman technique is used to study adsorbed species at micromolar concentrations. For molecules which absorb light at an appropriate laser wavelength, the combination of resonance enhancement and surface enhancement (surface-enhanced resonance Raman scattering) can lead to as much as twelve orders of magnitude enhancement.[6]

Inorganic complexes have many advantages over the more studied organic molecules due to their higher symmetries, various charges, and various physicochemical properties such as hydrophobic/hydrophilic nature. In this sense, ruthenium(II) complexes containing π-conjugated ligands have become the focus of a variety of photochemical, electrochemical and spectroscopic investigations.[7-9] Resonance Raman spectra and surface-enhanced resonance Raman spectra of $[Ru(bpy)_3]^{2+}$ have also been reported.[6] Their wavelength excitation profiles in SERRS are supposed, however, to alter substantially due to the surface selection rules involved.[5,10-13]

In this paper, we have used SERS to prove the interactions of $[Ru(bpy)_2L]^{2+}$ complexes with the silver colloidal surface. Because of the luminescence of some of these compounds, this study was performed with SERRS.