Vacuum-Ultraviolet Electronic Circular Dichroism of L-Alanine in Aqueous Solution Investigated by Time-Dependent Density Functional Theory

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The electronic circular dichoism (ECD) of L-alanine in the vacuum-ultraviolet region was calculated for various optimized structures using time-dependent density functional theory (TDDFT) to assign the CD spectrum observed experimentally in aqueous solution down to 140 nm [Matsuo, et al. *Chem. Lett.* **2002**, 826]. The structure of L-alanine in vacuo was optimized using density functional theory (DFT) at the B3LYP/6-31G* level. Its hydrated structure was optimized with nine water molecules (six and three around carboxyl and amino groups, respectively) using DFT and a continuum model (Onsager model). The dihedral angles of carboxyl and amino groups in the optimized hydrated structure differed greatly from those in the crystal and in nonhydrated structures optimized using a continuum model only. The ECD spectrum calculated for the hydrated structure had two successive positive peaks with molar ellipticities of about 2000 deg cm^2 dmol^{-1} at around 205 and 185 nm, which were close to those observed experimentally. These positive peaks were attributable to nπ* transitions of the carboxyl group, with the latter peak also influenced by the $\pi\pi$* transition of the carboxyl group that originates below 175 nm. A small negative peak observed at around 252 nm was also predicted from the hydrated structure. These results demonstrate that the hydrated water molecules around the zwitterions play a crucial role in stabilizing the conformation of L-alanine in aqueous solution and that TDDFT is useful for the ab initio assignment of ECD spectra down to the vacuum-ultraviolet region.

I. Introduction

Circular dichroism (CD) is very sensitive to the conformation of chiral molecules, which makes CD spectroscopy a useful tool for structural analyses of both biomolecules and organic materials.[1,2] The CD spectrum in the vacuum-ultraviolet region, which is hardly measurable using a conventional CD spectrophotometer, can provide more detailed and new information on the structure of biomolecules based on the higher energy transition of chromophores. Since the 1970s, there has been a great deal of effort at several facilities to extend the short-wavelength limit of CD spectrophotometer using synchrotron radiation as an intense light source.[3−6] We recently constructed a vacuum-ultraviolet CD spectrophotometer at Hiroshima Synchrotron Radiation Center that can measure CD spectra down to 140 nm in aqueous solutions due to all of the optical devices being kept under a high vacuum.[7,8] In this spectrophotometer, the optical servo-control system is newly introduced to control the photoelastic modulator accurately and to stabilize the lock-in amplifier in the vacuum-ultraviolet region, and the path-length of the optical cell is very short (from 1.3 to 50 μm) to minimize light absorption by the solvent water. A considerable body of vacuum-ultraviolet CD data has been accumulated on biomolecules such as amino acids, carbohydrates, proteins, and nucleic acid.[1,2,9−11] However, only a few limited theoretical assignments have been reported for electronic CD (ECD) spectra in the vacuum-ultraviolet region[12,13] and for vibrational CD spectra (VCD) in the infrared region,[14] because of the complicated relationship between chiroptical properties and molecular structure. The theoretical assignment of these vacuum-ultraviolet ECD spectra taking into account the electronic transition is indispensable for understanding details of the conformation of biomolecules in aqueous solution or physiological conditions.

Some theoretical approaches have been developed to estimate ECD spectra from known structures of molecules or to determine the conformation of molecules from their CD spectra. The octant rule is useful for the conformation of molecules containing ketone or aldehyde groups, with the π-SCF-CI-DV method being useful for twisted or conjugated π-electron systems, and Tinoco's method for very large molecules such as polypeptides and proteins.[15−17] On the other hand, it remains difficult to calculate ECD spectra for molecules in aqueous solution because their various equilibrium conformations are complicatedly affected by hydration. The Onsager model (continuum model) includes the effects of the solvent ab initio by assuming a dielectric medium.[18] However, density functional theory (DFT) has shown promise in evaluating the role of hydration in the structural optimization of biomolecules, because it introduces the electronic correlation effect into calculations of the conformation of molecules with high accuracy and with computational efficiency comparable with the Hartree−Fock (HF) method.[19,20] Also, time-dependent density functional theory (TDDFT) has become useful for calculating electronic excitation spectra of valence transitions and has greatly improved calculations of ECD,[21] as confirmed for helicenes by Furche et al. [22]

In the present study, we applied TDDFT to assign the CD spectrum of L-alanine in aqueous solution in the vacuum-ultraviolet region. The vacuum-ultraviolet CD spectra of L-alanine have previously been measured in film[23] and hexafluoro-2-propanol,[12] but we have recently succeeded in measuring its CD spectrum down to 140 nm in aqueous solution.[9] This

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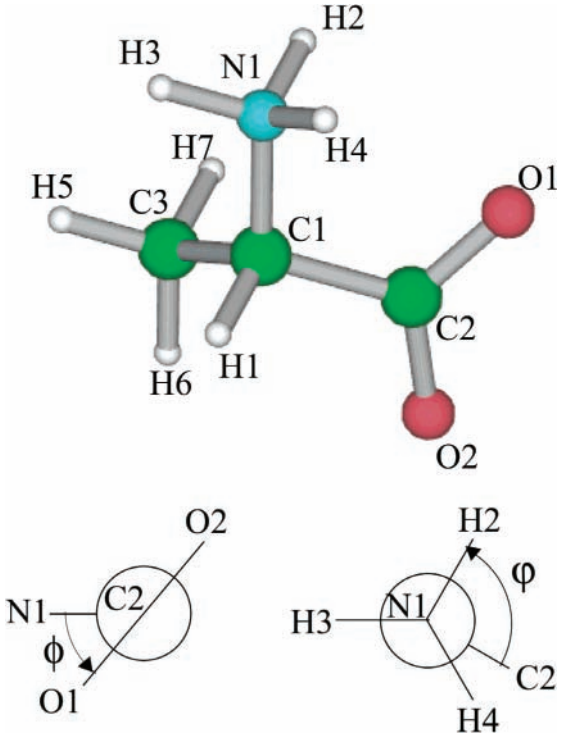

Figure 1. Model structure and definition of dihedral angles of L-alanine (zwitterion form). Atoms of O, N, H, and C are colored red, blue, white, and green, respectively. ϕ and φ indicate the dihedral angles of COO^- (N1−C1−C2−O1) and NH_3^+ (C2−C1−N1−H2) groups, respectively; these angles were varied in increments of 30° and 20°, respectively, to obtain the 36 initial structures.

spectrum exhibited two successive positive peaks at around 203 and 184 nm and a shoulder at around 170 nm, which differ considerably from the peaks in a nonaqueous system probably because L-alanine exists in a zwitterionic form with hydrated water molecules in aqueous solution. We optimized the structure of L-alanine in aqueous solution by combining DFT and the Onsager model, and we calculated the ECD spectra using the TDDFT for the crystal and the optimized structures with and without hydration to assign the spectrum observed experimentally. This is the first study to demonstrate the usefulness of TDDFT in the theoretical analysis of vacuum-ultraviolet ECD of amino acids in aqueous solution.

II. Theoretical and Experimental Methods

Initial Structures of L-Alanine. The 36 initial structures of L-alanine were constructed with the standard molecular parameters for bond lengths (C−C, 1.53 Å; C−O, 1.25 Å; C−N, 1.48 Å; C−H, 1.09 Å; and N−H, 1.03 Å) and bond angles (O−C−O, 125.00°; C−C−N, C−C−H, and C−N−H, 109.47°),[24−28] by changing the dihedral angle of the COO^- group (ϕ) from −60° to 120° in increments of 30° and that of the NH_3^+ group (φ) from −60° to 60° in increments of 20°. The model structure of L-alanine and the definition of the dihedral angles are shown in Figure 1.

Optimization of L-Alanine Structures. The optimized structure of L-alanine is closer to the experimentally measured structure when using the DFT method than when using the HF method.[29] We therefore adopted the DFT method to optimize the structure of L-alanine at the B3LYP/6-31G* level. The effect of the solvent surrounding L-alanine was calculated using a continuum model (Onsager model) with the relative permittivity of water (78.39) and a recommended cavity radius for a solute volume. Optimization was performed with the Gaussian 98 program (Gaussian)[30] on an H9000 VR360 computer (Hitachi) at the Information Media Center of Hiroshima University.

Calculation of CD Spectra. CD is induced by the interaction between electric and magnetic dipole transition moments of chromophores. As for the relationship between absorption and the dipole strength, CD is related to the rotational strength, R, which is theoretically defined by[2,31]

$$R_{0a} = \mathrm{Im}\{\langle\Psi_0|\hat{\boldsymbol{\mu}}|\Psi_a\rangle\cdot\langle\Psi_a|\hat{\boldsymbol{m}}|\Psi_0\rangle\} \quad (1)$$

where R_{0a} is the rotational strength of the electric transition from the "0" to "a" states, $\hat{\boldsymbol{\mu}}$ and $\hat{\boldsymbol{m}}$ are the electric and magnetic dipole moments, respectively, and Im means the imaginary part of a complex number. The rotational strength is expressed in cgs units (erg cm^3), which are conveniently transferred to a Debye−Bohr magnetron (1 DBM = 0.9273×10^{-38} erg cm^3 = 0.9273×10^{-51} J m^3).

The final CD spectrum can be calculated using the following equations:

$$R_i = 1.23 \times 10^{-42}\frac{[\theta]_i \Delta\lambda}{\lambda_i} \quad (2)$$

$$[\theta](\lambda) = \sum_i [\theta]_i \exp\left[-\left(\frac{\lambda - \lambda_i}{\Delta\lambda}\right)^2\right] \quad (3)$$

where $[\theta]$ is the molar ellipticity, λ_i is the wavelength of the ith transition, and $\Delta\lambda$ is the half bandwidth of a spectrum calculated assuming the Gaussian distribution. The CD spectra were calculated using Gaussian 98. The rotational strength, R_i, was first calculated using a TDDFT method at the B3LYP/6-31+G** level and a polarized continuum model (PCM)[32] to take the effect of the solvent into account. From the obtained rotational strength, CD spectra were calculated using eqs 2 and 3 with a $\Delta\lambda$ value of 12.5 nm.

Far-UV CD Measurements. L-Alanine of reagent grade was purchased from Sigma and dissolved in double-distilled water at 0.1 g cm^{-3}. The concentration of L-alanine was determined accurately by calibrating the moisture content. The far-UV CD spectrum was measured on a J-720W spectropolarimeter (Jasco) with a 10-mm-path-length quartz cell, an 8-s time constant, a 20-nm/min scan speed, and 9-times accumulation.

III. Results and Discussion

Rotational Strengths and CD Spectra of Initial Structures. To elucidate the effect of the dihedral angles of the COO^- and NH_3^+ groups on rotational strength, the rotational strengths of 36 initial structures were first calculated for their lowest energy transitions without optimization. Figure 2 shows plots of the rotational strengths (R) as functions of dihedral angle (ϕ) of the COO^- group for six dihedral angles (φ) of the NH_3^+ group (from −60° to 60°). It is evident that the rotational strength of the lowest energy transition is more sensitive to the dihedral angle of the COO^- group than that of the NH_3^+ group.

The CD spectra of L-alanine between 260 and 140 nm were calculated for typical initial structures with the following four sets of ϕ and φ values: (0°, 0°), (0°, 60°), (90°, 0°), and (90°, 60°). As shown in Figure 3, the CD spectrum for (90°, 0°) is similar to that for (90°, 60°) but differs markedly from the spectrum for (0°, 0°), indicating that the dihedral angles of the COO^- group have a large effect on the ECD of L-alanine. Therefore, it is necessary to determine accurately the dihedral angle of the COO^- group using an optimization method when

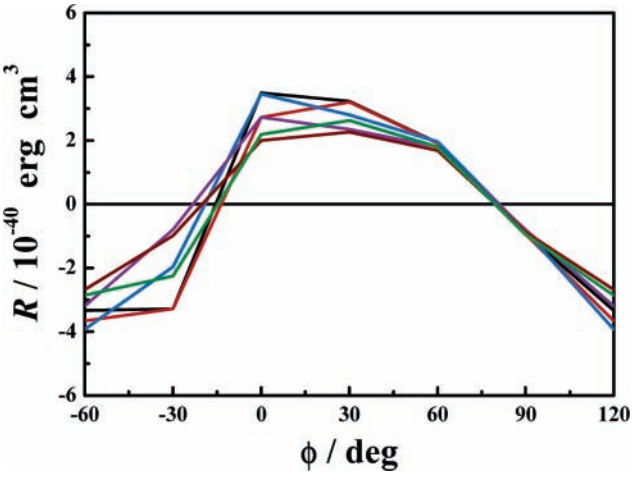

Figure 2. Rotational strength (R) as a function of the dihedral angle (ϕ) of the COO^- group at the lowest energy transition: $\varphi = -60°$ (60°), black line; −40°, red line; −20°, blue line; 0°, purple line; 20°, brown line; and 40°, green line.

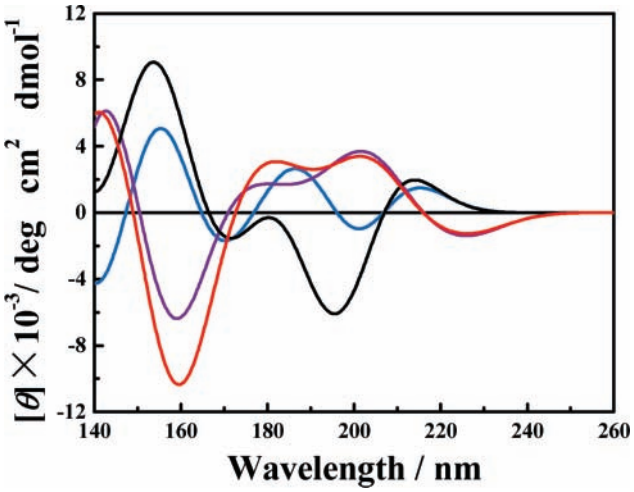

Figure 3. ECD spectra calculated for four typical initial structures of L-alanine: (ϕ, φ) = (0°, 0°), black line; (90°, 0°), red line; (0°, 60°), blue line; and (90°, 60°), purple line.

calculating the ECD spectrum of L-alanine in the vacuum-ultraviolet region.

Optimized Structures and ECD Spectra in Vacuo and in Water. The structure of L-alanine in vacuo was optimized with the four initial structures mentioned above. The averaged molecular parameters of the optimized structure (structure I) are listed in the third column of Table 1. As compared to the crystal structure (in the second column of Table 1),[33] the N1−H3 bond is significantly stretched and the bond angle of H3−N1−C1 and the dihedral angles (ϕ and φ) are remarkably changed. These results suggest that proton transfer from NH_3^+ to COO^- groups is induced in vacuo, forming the nonionized structure of L-alanine.

The structure of L-alanine in water was optimized using the Onsager model to take the effect of the solvent into account. In all of the four initial structures, the optimized structures converged on an identical structure: (ϕ, φ) = (5.6°, −5.2°). The ϕ values of these optimized structures are in agreement with those (0 ± 10°) reported in previous works.[28,34] The averaged molecular parameters of the optimized structures (structure II) are listed in the fourth column of Table 1. As compared to the crystal structure, stretching of the N1−H3 bond and a decrease in the bond angle of H3−N1−C1 still occur in the continuum model, but to lesser extents than found in vacuo.

Figure 4 shows ECD spectra calculated for the optimized structures in vacuo (structure I) and water (structure II) using the molecular parameters listed in Table 1. The spectrum for the crystal structure was also calculated for comparison using the molecular parameters in the second column of Table 1. The spectra for the optimized structures in vacuo and water exhibit positive peaks at around 230 and 210 nm, respectively, followed by two negative peaks in the vacuum-ultraviolet region. The difference between the two spectra may be mainly ascribed to the differing ionization states of zwitterions in vacuo and in a dielectric medium. The crystal structure exhibits a small positive peak at around 220 nm, a negative peak at around 200 nm, and two positive peaks in the vacuum-ultraviolet region, which are similar to those in film.[22] These three spectra of L-alanine differ markedly from that observed experimentally in aqueous solution,[9] which indicates that the optimized structure and CD spectra obtained using the continuum model only are imaginary, and therefore that the effect of hydration around zwitterions should be taken into account when determining the structure of L-alanine in aqueous solution.

Optimization of the Hydrated Structure. Some experimental and theoretical studies have evaluated the amount of hydration of L-alanine. From NMR analysis, Kuntz showed that about six and three water molecules are bound to the COO^- and NH_3^+ groups of side chains of polypeptides, respectively.[35] These values are consistent with theoretical calculations on model compounds: the COO^- group of CH_3COO^- has six hydrated water molecules,[36] and each hydrogen atom of the NH_3^+ group of $CH_3NH_3^+$ hydrates one water molecule.[37] Recently, Frimand et al. showed that nine explicit water molecules are necessary for reproducing the VCD spectrum of L-alanine in water.[14] We therefore optimized the hydrated structure of L-alanine with nine water molecules (six and three for the COO^- and NH_3^+ groups, respectively) using a continuum model for the initial structures with four sets of dihedral angles (ϕ, φ): (0°, 0°), (0°, 60°), (90°, 0°), and (90°, 60°). The following molecular parameters were used for water and its hydrogen bonds with zwitterions: O−H, 0.948 Å; H−O−H, 106.6°; COO^-- - -H−O, 2.0 Å; and NH_3^+- - -O−H, 1.8 Å. The initial structure having only (ϕ, φ) = (90°, 60°) could be optimized, and the others could not retain the hydrated water molecules around the zwitterions. The optimized structure (structure III) is shown stereographically in Figure 5, and its molecular parameters are listed in the fifth column of Table 1. Six water molecules around the COO^- group form a hydrogen-bond network with each other to restrict the rotation of this group. Each hydrogen atom of the NH_3^+ group forms a hydrogen bond with a water molecule, and two hydrated water molecules around the NH_3^+ group form a hydrogen-bond network with those around the COO^- group. The lengths of the hydrogen bonds are between 1.75 and 1.95 Å, which are consistent with the result (1.8 Å) of a Monte Carlo simulation for the hydration of $CH_3NH_3^+$.[37] The bond lengths and bond angles of the optimized structure are close to those of the crystal structure, and stretching of the N1−H3 bond (as found for structure II) does not occur. On the other hand, the dihedral angles differ markedly from those for the crystal structure and structure II, probably due to the hydrogen-bond network around the zwitterions. To confirm the propriety of the nine water molecules in the optimized structure, an additional water molecule was added around the CH_3 group of L-alanine. As listed in the last column of Table 1, the molecular parameters of the structure thus optimized (structure IV) are very close to those of structure III. This result indicates that the additional

TABLE 1: Bond Lengths, Bond Angles, and Dihedral Angles of the Structures of L-Alanine Used in Calculation of CD Spectra

	crystal structure[a]	optimized structures			
		structure I[b]	structure II[c]	structure III[d]	structure IV[e]
		Bond Length (Å)			
C1−C2	1.537	1.546	1.571	1.547	1.546
C1−N1	1.497	1.478	1.505	1.507	1.505
C1−C3	1.534	1.532	1.525	1.529	1.529
C1−H1	1.091	1.098	1.095	1.092	1.092
C2−O1	1.266	1.342	1.295	1.255	1.256
C2−O2	1.249	1.209	1.230	1.275	1.275
N1−H2	1.032	1.019	1.022	1.048	1.047
N1−H3	1.050	1.885	1.338	1.050	1.047
N1−H4	1.034	1.017	1.021	1.045	1.056
C3−H5	1.096	1.096	1.095	1.094	1.094
C3−H6	1.097	1.093	1.093	1.094	1.094
C3−H7	1.096	1.097	1.097	1.095	1.095
		Bond Angle (deg)			
N1−C1−C2	110.0	108.8	105.4	110.6	110.9
C3−C1−C2	111.2	109.3	112.9	112.4	112.4
H1−C1−C2	108.4	105.7	107.6	107.3	107.3
O1−C2−C1	116.0	113.7	113.0	117.0	117.1
O2−C2−C1	118.3	122.8	119.2	117.7	117.8
H2−N1−C1	111.2	110.8	112.6	112.6	112.6
H3−N1−C1	109.3	83.59	92.82	111.1	111.0
H4−N1−C1	108.9	111.2	113.7	108.6	108.0
H5−C3−C1	110.1	110.8	111.9	110.2	109.9
H6−C3−C1	110.3	109.9	108.6	110.0	110.1
H7−C3−C1	110.1	110.6	111.0	110.6	110.5
		Dihedral Angle (deg)			
O1−C2−C1−N1	161.5	1.121	5.595	121.5	120.9
O2−C2−C1−N1	−18.60	−178.9	−175.1	−58.97	−59.67
H2−N1−C1−C2	58.30	119.8	109.8	67.63	68.77
H3−N1−C1−C2	177.8	−0.837	−5.153	−54.50	−53.14
H4−N1−C1−C2	−64.00	−121.4	−127.7	−173.4	−172.0
H5−C3−C1−N1	−62.25	−64.10	−65.34	−57.79	−57.30
H6−C3−C1−N1	177.3	175.5	174.4	−177.0	−176.7
H7−C3−C1−N1	57.60	56.26	56.11	62.99	63.34

[a] See ref 33. [b] B3LYP/6-31G* in vacuo (average of four initial structures). [c] B3LYP/6-31G* + Onsager (average of four initial structures). [d] B3LYP/6-31G* + nine water molecules + Onsager (initial structure: $(\phi, \varphi) = (90°, 60°)$). [e] B3LYP/6-31G* + 10 water molecules + Onsager (initial structure: $(\phi, \varphi) = (90°, 60°)$).

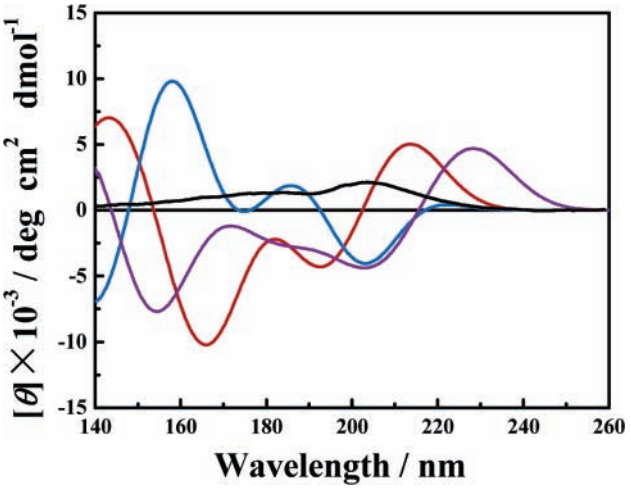

Figure 4. ECD spectra calculated for the following L-alanine structures: crystal (not optimized), blue line; optimized in vacuo, purple line; and optimized in aqueous solution, red line. The black line indicates the experimentally observed vacuum-ultraviolet CD spectrum of L-alanine in aqueous solution.[9] The spectrum for each optimized structure is calculated as the average of the converged structures.

water molecule is removed from the initial structure and that the conformation of L-alanine in water is mainly determined by nine hydrated water molecules.

ECD Spectrum of the Hydrated Structure. Figure 6 shows the rotational strength and ECD spectrum calculated for the optimized structure of L-alanine with nine hydrated water molecules (structure III). These hydrated water molecules were ignored in the calculation due to the computational limitations. Evidently, there exist many rotational strengths with positive and negative signs in the vacuum-ultraviolet region. The obtained ECD spectrum shows positive peaks at around 203 and 185 nm, negative peaks at 225 and 160 nm, and a small shoulder at around 170 nm. This spectrum is very different from that for structure II calculated using a continuum model (Figure 4), and both the wavelengths and the intensities of its peaks are similar to those observed experimentally except for a large negative peak at around 225 nm. This indicates that the continuum model is inadequate, and so its combination with the hydrated water molecules is indispensable for evaluating the effect of the solvent on ECD.

At present, we cannot explicitly explain the large negative peak at around 225 nm that is not observed experimentally in the ordinal condition. To confirm the existence of this peak, we measured the CD spectrum of L-alanine in concentrated aqueous solution. As shown in the inset of Figure 6, a very small and broad negative peak was observed at around 252 nm. A similar negative peak was also found at 241 nm by Anand and Hargreaves.[38] This indicates that the theoretically predicted rotational strength at 225 nm does actually exist, although it is overestimated and blue-shifted as compared to the experimental observation. One of the possible reasons for the large discrepancy between theory and experiment for this lowest energy transition may be attributable to the nine hydrated water molecules not being included in the TDDFT calculation, although they were taken into consideration in optimization of the hydrated structure (structure III). The network of hydrated

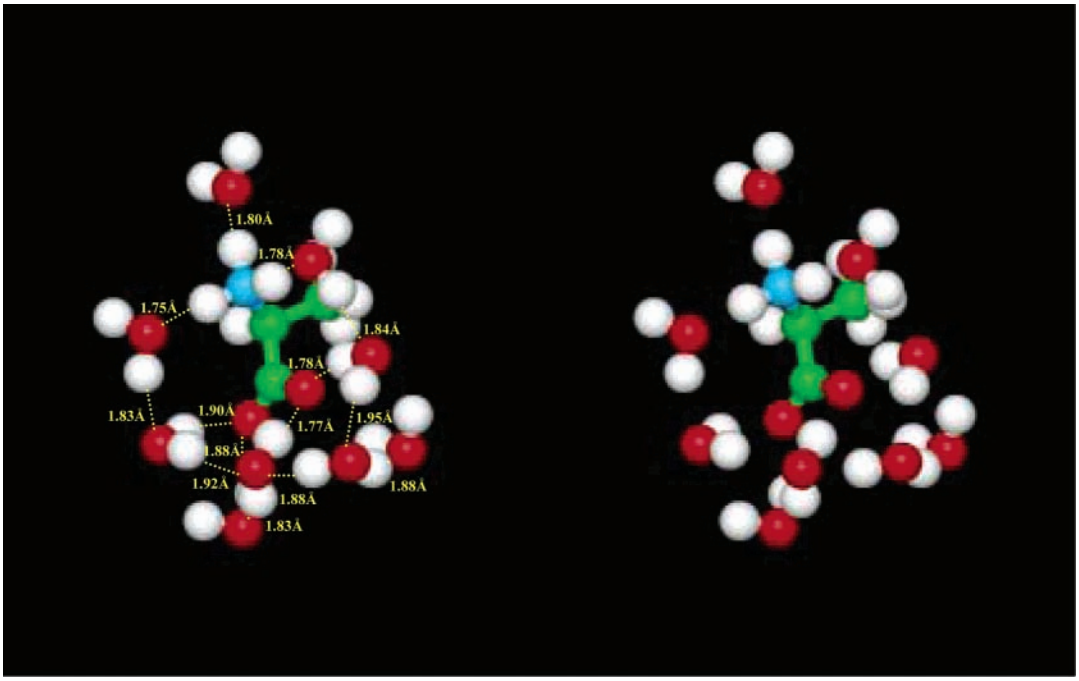

Figure 5. Stereograph of the optimized structure (structure III) of L-alanine with nine hydrated water molecules. Atoms of O, N, H, and C are colored red, blue, white, and green, respectively. The hydrogen-bond distances between O and H atoms (broken lines) are optimized within 2 Å.

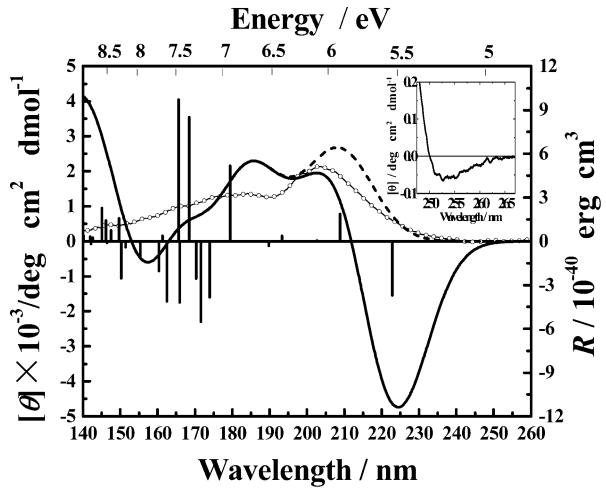

Figure 6. Rotational strengths (solid bars) and ECD spectra (solid line) of L-alanine optimized with nine hydrated water molecules (structure III). The rotational strengths and CD spectra were calculated by ignoring the hydrated water molecules. The dotted line shows the spectrum calculated by ignoring the rotational strength at around 225 nm, and the circles indicate the experimentally observed spectrum obtained by Matsuo et al.[9] The inset shows the far-UV CD spectrum of L-alanine observed experimentally in aqueous solution.

water molecules may restrict the dihedral angles of the COO^- and NH_3^+ groups of L-alanine to reduce the magnetic and/or electric dipole moments of the low energy band. It is also probable that L-alanine takes many equilibrium conformations including the optimized structure (structure III) in aqueous solution, whose rotational strengths are compensated with each other. If this is the case, it is not unreasonable to assume that the rotational strength at 225 nm was overestimated and should have been made negligible in the TDDFT calculation.

As shown in Figure 6, the ECD spectrum calculated by neglecting this contribution is in fact similar to the experimentally observed one: the positive peak at around 210 nm can be more consistently reproduced without affecting the spectrum below 195 nm. Although the intensity of the rotational strength at 225 nm must be confirmed by more detailed calculations that take into account hydrated water molecules, these results demonstrate that the hydrated water molecules play a crucial role in stabilizing the conformation of L-alanine in aqueous solution and that the TDDFT is useful for the ab initio assignment of ECD spectrum.

Assignments of the ECD Spectrum. As shown above, the experimentally observed CD spectrum of L-alanine is successfully reproduced by the TDDFT calculation using the optimized structure with nine hydrated water molecules. Therefore, for assigning the CD peaks, we calculated the molecular orbitals participating in the electronic transitions at 260−140 nm using a TDDFT method (B3LYP/6-31+G** level) and PCM. The ground states of the transitions at 220−185 nm are mainly attributable to the lone-pair orbital (n-orbital) of two oxygen atoms of the COO^- group. The excited states of the transitions in this region are mainly attributable to the π^*-orbital of the COO^- group, and the transitions below 180 nm come from the excitation of the π-orbital of the COO^- group. Thus, the positive peak at around 200 nm can be attributed to nπ^* transition of the COO^- group. The positive peak at around 180 nm is also mainly ascribed to nπ^* transition of the COO^- group, but it also is influenced by a contribution from $\pi\pi^*$ transitions of the COO^- group that originate below 175 nm. The negative peak at around 252 nm would be ascribed to nπ^* transitions of the COO^- group, although the energy of its ground and/or excited states may be sensitively perturbed by hydration effects.

These assignments for the peaks at around and above 200 nm are consistent with the results obtained previously by optical rotation dispersion and far-UV CD studies,[40,41] whereas the peaks below 200 nm have not been assigned definitively because of limited CD data for amino acids in the vacuum-ultraviolet region. Snyder et al.[12] measured the CD spectra of some amino acids in hexafluoro-2-propanol and assigned two peaks at around 200 and 170 nm to the nπ^* and $\pi\pi^*$ transitions of the COO^- group, respectively. The CD spectrum of L-alanine in this solvent is similar to that calculated using a continuum model (structure II) because hydration has no effect on the conformation of

COO^- and NH_3^+ groups, although L-alanine exists as zwitterions as in water. Despite these different conditions, their results support our assignments ($\pi\pi^*$ transitions of the COO^- group) for the peak at around 180 nm. Thus, molecular orbital calculation can be used to reveal the characteristic features of electric transitions of L-alanine in the vacuum-ultraviolet region.

IV. Conclusions

The present study is the first to apply the TDDFT to the assignment of the CD spectrum of L-alanine in aqueous solution in the vacuum-ultraviolet region. Despite some limitations, the ECD spectrum calculated using the optimized structure with nine hydrated water molecules successfully reproduced both the intensity and the wavelength of the experimentally observed CD peaks in the vacuum-ultraviolet region. This demonstrates that the water molecules hydrated around zwitterions play a crucial role in stabilizing the conformation of L-alanine in aqueous solution and that the TDDFT is a useful tool for the ab initio assignment of ECD spectra of amino acids. This theory would be especially useful when sufficient computation resources are available to allow calculations involving hydrated water molecules. Improvements on the theoretical analysis and the accumulation of further ECD spectra in the vacuum-ultraviolet region should open a new field in the structural analysis of biomolecules based on the higher energy transitions of chromophores, which thus far remain unsolved for aqueous solutions.

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