Intermediate State during the Crystal Transition in Aspartame, Studied with Thermal Analysis, Solid-State NMR, and Molecular Dynamics Simulation

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Aspartame (L- α -aspartyl-L-phenylalanine methyl ester) is a dipeptide sweetener about 200 times as sweet as sugar. It exists in crystal forms such as IA, IB, IIA, and IIB, which differ in crystal structure and in the degree of hydration. Among these, IIA is the most stable crystal form, and its crystal structure has been well determined (Hatada *et al.*, *J. Am. Chem. Soc.*, 107, 4279–4282 (1985)). To elucidate the structural factors of thermal stability in the IIA form of aspartame and to examine the physical process in the crystal transformation between the IIA and IIB forms, we performed a thermal analysis and solid-state NMR measurements. We found that a *quasi-stable* intermediate state exists in the transformation, and it has the same crystal lattice as the usual IIA form, despite the dehydration from 1/2 mol to 1/3 mol per 1 mol of aspartame. The results of the energy component analysis and the molecular dynamics simulation suggest that the entropic effect promotes the generation of the intermediate state, which is presumably caused by the evaporation of the water of crystallization and the increase of molecular motion in aspartame. Thus, the thermal stability of the IIA form is attributable to a structural property, *i.e.*, the crystal lattice itself is retained during the above dehydration. Moreover, the molecular dynamics simulations suggest that the aspartame molecules have two kinds of conformational flexibility in the intermediate state.

Key words aspartame; crystal transformation; thermal analysis; solid-state NMR; molecular dynamics simulation

Aspartame (L- α -aspartyl-L-phenylalanine methyl ester) is a low-calorie sweetener possessing about 200 times the sweetness of sugar, and has a sweetening quality similar to that of sugar.¹⁾ It can be digested, absorbed and metabolized in the human body in the same way as the natural peptides.²⁾ Aspartame is used in various foods and beverages and is also reported to be effective as part of a diet for diabetics using calorie control.^{3,4)} Thus, for industrial and pharmaceutical purposes, aspartame has been extensively studied; *e.g.*, the structure–taste relationships,^{5–7)} the model of the receptor site,^{8–10)} its conformation in aqueous solution,^{11,12)} and its interaction with cyclodextrin in the solid state.¹³⁾

The X-ray crystal structure of aspartame was reported by Hatada *et al.*¹⁴⁾ in 1985, and an interesting arrangement of molecules was revealed; the space group is $P4_1$, so there are four aspartame molecules with the same conformation in one unit cell, with a fourfold screw along the *c*-axis, while the water molecules are located almost at the center of the hydrophilic channel that consists of the NH_3^+ and COO^- groups of aspartame and form an infinite column along the *c*-axis. Since the four symmetry-related positions of the water molecules are too close to exist simultaneously in each position, they occupy the two sites side by side out of the four crystallographic sites. Thus, the occupancy rate of water molecule at each site is 50%.

We call the crystal form of the above structure the IIA form of aspartame, according to the classification by Sugiyama *et al.*; they first recognized the difference among the crystal forms of aspartame and defined the crystal forms as IA, IB, IIA, and IIB based on the results of powder X-ray diffraction (XRD) studies.^{15,16)} IA is the high-hydrated crystal form and converts to IB by drying. IIA is less hydrated

than are IA and IB. IIB is the anhydrous form which is obtained at high temperatures; its crystal structure has not been determined. The degree of hydration in the IIA form is 0.5 mol per 1 mol of aspartame,¹⁴⁾ and the crystal transformation proceeds along with the dehydration.^{15,16)}

The thermal stability of each crystal form has been well studied in our laboratories, and is one of the properties important to the manufacturing process. We found that the IIA form is very stable at a wide temperature range, while the IIB form easily converts to the IIA form by water adsorption, *e.g.*, at 34 °C in 78% relative humidity, in which the equilibrium moisture content of the sample is 3% (w/w)^{15,16)} and corresponds to the degree of hydration in the IIA form. However, the factors of thermal stability in the IIA form have not been identified in terms of structure, nor have the details of the structural changes produced by the crystal transformation of the IIA form.

Therefore, to elucidate the thermal stability factors and the physical process of the crystal transformation in the IIA form, thermogravimetry and differential thermal analyses (TG/DTA), powder XRD and solid-state NMR measurements were carried out in the present study. The details of the crystal transition from the IIA form to an intermediate state will be discussed together with the structural feature of the intermediate state and its relationship with the thermal stability of the IIA form.

Moreover, energy calculations and molecular dynamics (MD) simulations for the IIA form and the intermediate state were performed to elucidate the stabilization mechanism and the structural properties of the intermediate state.

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Fig. 1. The Molecular Structure of Aspartame

The carbon numbering is based on the numbering system of the IUPAC. ^{13}C -NMR signal assignments in the IIA form were shown in Table 1 and Fig. 7a.

Experimental

Materials and Crystallization The aspartame sample was the product of Ajinomoto Co., Inc. The IIA form sample was obtained by heating the IB form at 80 °C in 70% relative humidity. The IB form was obtained by drying the IA form *in vacuo*. The IA form was crystallized from the aqueous solution without stirring. The molecular structure and atom-numbering scheme are shown in Fig. 1.

Powder XRD Measurement The powder XRD patterns were obtained using an X-ray diffractometer (PW1700, Phillips) with $\text{CuK}\alpha$ radiation (40 kV, 30 mA). The sample was maintained in a constant temperature environment with a variable temperature unit, and scanned in the 2θ range from 3° to 30° with a scanning speed of 0.05°/s. The isothermal powder XRD was performed at each temperature from 30 °C to 70 °C at 5 °C increments. The crystal form identification was performed before and after the NMR measurement. Since no diffraction peak of CaCl_2 , which partially filled the NMR rotor (*vide infra*), was observed at a lower angle ($2\theta < 14$ degree), it did not disturb the crystal form identification.

TG/DTA Measurement The TG/DTA curves were obtained using a TG/DTA220 system (SEIKO Electronics). The measurement was performed on the IIA form sample (ca. 5 mg) in an open aluminum pan from room temperature (r.t.) to 90 °C at a heating rate of 0.5 °C/min. The initial relative humidity was ca. 50% at r.t.

^{13}C -Cross-Polarization Magic-Angle Spinning (CPMAS)-NMR Spectroscopy The 100.6 MHz high-resolution solid-state ^{13}C -NMR spectra of the IIA form were recorded on a DSX-400WB NMR spectrometer (Bruker) by means of CPMAS at eleven temperatures from r.t. (24 °C) to 80 °C. The IIA form sample (ca. 150 mg) was contained in a cylindrical rotor and spun at 6 kHz, sandwiched by anhydrous CaCl_2 upper and lower layers. This device was not indispensable for the dehydration at a low temperature range (r.t.–40 °C), but it was used to ensure the smooth dehydration at a higher temperature range in a closed system. The free induction decay was recorded with 4 K data points and a spectral width of 35211 Hz. The data were zero-filled to 8 K prior to Fourier transformation. The durations of the 90° pulse, contact time, and repetition time were 4.2 μs , 1.0 ms and 5 s, respectively. The ^{13}C chemical shifts were calibrated by using the carboxyl peak of glycine (176.03 ppm) and converted to the value from tetramethylsilane. The actual temperature within the spinning sample was calibrated using samarium acetate tetrahydrate.¹⁷⁾ Since it was found to be almost the same as the indicated temperature on the spectrometer, e.g., the actual temperature was 39.3 °C when the indication was 40 °C, the indicated temperatures are used in the Results and Discussion sections.

Carbon $T_{1\rho}$ Measurement To examine the conformational flexibility in the Phe moiety in aspartame, the measurements of ^{13}C spin-lattice relaxation time in the rotating frame ($T_{1\rho}$) were carried out at r.t. and 45 °C using a standard cross-polarization $T_{1\rho}$ pulse sequence. The ^{13}C spin locking periods of 2, 10, 30, 60, 80, 100, 120 ms were used in each measurement.

Deuterium NMR Measurement The 46.07 MHz static deuterium NMR spectra of the IIA form were measured on a DSX-300 NMR spectrometer (Bruker) using a standard quadrupolar echo pulse sequence at r.t. (24 °C) and 45 °C. To prepare the D_2O type IIA form, the sample (H_2O type) was placed in cold D_2O (4 °C) for a few minutes, and then filtered, and transformed to the IIA form by heating. (Since the protons in NH_3^+ group form the hydrogen bonds with COO^- groups in the crystal, the deuteration rate in NH_3^+ group is considered to be lower than that in the water molecules; the deuteration rate in water molecules itself was found to be rather low judging from the signal sensitivity in D-NMR.) The durations of the 90° pulse, and the τ value of the refocusing period were 3.0 and 30 μs , respectively. Recycle delays of 1.0 and 3.0 s were used at each temperature for checking the ef-

fect of T_1 relaxation on the signal intensity. Since the integrated areas of D-NMR signal in the different recycle delays were constant at the same temperature, the area ratio at r.t. (24 °C) and 45 °C was determined using the spectra in the recycle delay of 1.0 s.

Computer and Programs A personal computer IRIS Indigo2 (Silicon Graphics Inc.) was used for the graphical display, the energy minimization and MD simulation of the crystal structure. The energy component analysis and the conformational flexibility analysis for the IIA form and the intermediate state were performed with the Cerius2 program (Molecular Simulation Inc.), and the calculation of the time-averaged structure in the IIA form was performed with the APRICOT program.^{18,19)} The lattice parameters were fixed at the experimental values in all energy minimizations and MD simulations.

X-Ray Structure of the IIA Form and the Model Structures of the Initial IIA Form and the Intermediate State The X-ray crystal structure of the IIA form¹⁴⁾ was taken from the Cambridge data base (reference code DAWGOX). Since the coordinates of hydrogen atoms in the water molecule were not contained in DAWGOX, their coordinates were generated with the standard orientation and all hydrogen atoms were structurally optimized using the Cerius2 program so as to exclude the van der Waals short contact. The coordinates for one unit cell were made by the $P4_1$ symmetry operation for the X-ray structure data, and then the two sets of water coordinates were removed so that the occupied site and the unoccupied site were located alternately, because the occupancy rate of water molecules is 50% (as described above). When two water molecules were located side by side, the van der Waals contact between them was extensive, so such an initial position was found to be unstable and not actual, as Hatada *et al.* pointed out.¹⁴⁾ Since the degree of hydration is 1/2 mol per 1 mol of aspartame in the IIA form and 1/3 mol in the intermediate state (*vide infra*), hereafter we designate the former as IIA(1/2 H_2O) and the latter as IIA(1/3 H_2O).

In the calculation with the Cerius2 program, the model structure for IIA(1/2 H_2O) was made by stacking up three unit cells in the direction of the *c*-axis as shown in Fig. 2. The model structure for IIA(1/3 H_2O) was generated by removing each water molecule (marked by arrow in Fig. 2b) from the first and second layers in the above IIA(1/2 H_2O) model.

In generating the intermediate model, the representative permutations of water locations along the *c*-axis are $\langle \text{v o v o o o} \rangle$, $\langle \text{v o o v o o} \rangle$ and $\langle \text{o o v v o o} \rangle$ (v; vacant, o; occupied) in three unit cells, because $\langle \text{v o v o o o} \rangle$ is the same state as $\langle \text{o v o v o o} \rangle$ or $\langle \text{o o v o v o} \rangle$ under the periodic boundary conditions. The energy minimization results showed that the IIA(1/3 H_2O) model in the water location $\langle \text{v o v o o o} \rangle$ were most stable among the three locations; ΔE was ca. 3.0 and 3.5 kJ per three units for $\langle \text{v o o v o o} \rangle$ and $\langle \text{o o v v o o} \rangle$, respectively. Thus, we selected IIA(1/3 H_2O) in the water location $\langle \text{v o v o o o} \rangle$ as a starting model of the intermediate state. The trajectory in MD simulation was thought not to be affected by the selection of the initial permutation of the water location essentially, because 1) ΔE between the states was not so large, and 2) we used the doubling temperature in starting the MD simulation. Additionally, the animation of the trajectory showed that the location of water along the *c*-axis was not always fixed to a constant permutation after the equilibrium of temperature by appearances. Since a calculational unit includes twelve aspartame molecules, one course of MD simulation was thought to be enough for the structural sampling in the aspartame dynamics. In the MD simulation with the APRICOT program, the model structure for IIA(1/2 H_2O) was made by stacking up four unit cells in the direction of the *c*-axis for the detailed investigation of the hydrogen bond length. The whole procedure of the modeling and simulation was showed in Fig. 3.

Energy Component Analysis The energy component analysis for IIA(1/2 H_2O) and IIA(1/3 H_2O) was performed for the comparison of the enthalpic energy between these two states. A system of three unit cells ($17.685 \times 17.685 \times 14.757 \text{ \AA}^3$) in periodic boundary conditions was used for each model. The Ewald method²⁰⁾ was used for the calculations of the electrostatic and van der Waals interactions, and the cutoff distance was set at 10.2 Å for the former and 17.1 Å for the latter.

The electrostatic potential (ESP) charges used for the aspartame molecule were calculated by the MNDO method without the structural optimization, after the structural optimization by the PM3 method using MOPAC 6.01. In case some atoms are chemically equivalent such as protons in NH_3^+ , the ESP charges were averaged. The total of all charges was adjusted to zero.

The energy of the initial model structure was minimized by the conjugate gradient method using the Dreiding force field²¹⁾ and 1.0 of dielectric constant, before the energy component analysis was carried out. The minimization continued until the root mean square gradient became $0.3 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$.

Fig. 3. The Whole Procedure of the Model Building and the MD Simulation

Fig. 2. The Calculational Unit with Cerius2 Program for the Model Structures of IIA(1/2H₂O) and IIA(1/3H₂O)

a) The projection along the *c*-axis, b) The side view except for the position A molecules. Two water molecules (marked by closed circles) are located in the hydrophilic channel of one unit cell, since its occupancy is 50%.¹⁴⁾ The hydrogen bonds between the peptide bond sites are shown as broken lines. The model structure for IIA(1/2 H₂O) was made by stacking up three unit cells, while that for IIA(1/3 H₂O) was generated by removing each water molecule marked by arrow. *a*=*b*=17.685 Å, and *c*=14.757 Å, which show the calculational unit size.

Conformational Flexibility Analysis After the energy minimization, the sequential MD simulation of 50 ps was performed in periodic boundary conditions using the Nose–Hoover method²²⁾ with the Cerius2 program. The final simulation temperature was set at 300 K for IIA(1/2H₂O) and at 320 K for IIA(1/3H₂O). A timestep of 1 fs was used, and the coordinates were

Fig. 4. Temperature Dependence of Powder XRD Pattern from 30 °C to 60 °C

The initial pattern is that of the IIA form, and the pattern at 50 °C shows that of a mixture of the IIA and IIB forms. The powder patterns at 35 °C, 40 °C, 65 °C, and 70 °C were omitted.

stored with an interval of 0.5 ps. The time-averaged dihedral angle, χ_1 (C γ –C β –C α –N), in the Phe moiety was calculated using the sampling structures in 15.0–50.0 ps.

Molecular Dynamics Simulation of IIA(1/2H₂O) For a detailed examination of the time-averaged structure in IIA(1/2H₂O), the energy minimization by the conjugate gradient method and the sequential MD simulation of 50 ps were performed with the APRICOT program. A system of four unit cells (17.685×17.685×19.676 Å³) in periodic boundary conditions was used. The AMBER version 3.0 force field²³⁾ (all atom type) for the aspartame molecule and the TIP3P potential for water²⁴⁾ were used. The cut-off distance for nonbonded interactions was set at 8.8 Å in the minimization, and all non-bonded atom pairs in the periodic boundary were calculated in the MD simulation. The dielectric constant, the atomic charges and convergence condition were the same as in the energy component analysis. A timestep of 2 fs was used in the MD simulation using the SHAKE method.²⁵⁾ The temperature of the initial random velocity was 150 K. Velocity scaling²⁶⁾ was used to increase the temperature to 300 K with a relaxation time of 0.5 ps. The coordinates were stored with an interval of 0.5 ps. The time-averaged length of the intermolecular hydrogen bond between amide nitrogen and carbonyl oxygen atoms in the peptide bond was calculated using the sampling structures in 0.5–50.0 ps (see Fig. 2).

Results

Thermal Analysis by Powder XRD and TG/DTA The powder XRD patterns remained identical to that of the IIA form during the temperature change from 30 °C to 45 °C, and the crystal transformation to the IIB form started at 50 °C and was almost complete at 60 °C, as shown in Fig. 4.

The results of the TG/DTA are shown in Fig. 5. The de-

gree of hydration, which was 1/2 mol per 1 mol of aspartame at r.t., was gradually reduced by heating and showed a plateau of 1/3 mol per 1 mol of aspartame at 40 °C–50 °C, and the transformation to the IIB form started at around 55 °C.

The same lot sample was measured after one week to check the reproducibility of the results. The TG/DTA curve obtained was almost the same. Additionally, the stability of the plateau with 1/3 mol of H₂O was checked by holding the temperature at 46 °C for about three hours. Figure 6 shows the weight change of this intermediate state. It was observed that this state was very stable at 46 °C and returns quickly to the initial state with 1/2 mol of H₂O after switching off the heating system.

The crystal form of the intermediate state is the IIA form, because the powder XRD pattern at 45 °C shows the exact IIA form. This is the reason that we designated the intermediate state as "IIA(1/3 H₂O)" in the experimental section.

Solid-State ¹³C-CPMAS-NMR Spectroscopy a) Signal Assignments in the IIA Form: The signal assignments in the

IIA form were carried out based mainly on the comparison with the complete assignments in a solution state (Table 1, Fig. 7a). Especially for the discrimination between the amide carbonyl and the methyl ester carbonyl carbons, the assignment was based on the deuterium isotope effect (–COND– / –CONH–) observed in the recrystallized sample from D₂O solution.

b) ¹³C-CPMAS-NMR Spectra in the Crystal Transformation: Figure 7 illustrates the spectral changes of the IIA form in the heating process from r.t. to 40 °C, and Fig. 8 shows the enlarged lower magnetic field regions (120–180 ppm) of the same spectra. The powder XRD pattern of the sample showed the characteristic features of the IIA form when measured immediately after the solid-state NMR measurement at 40 °C, and the NMR spectrum of the same refilled sample showed the specific pattern to the dehydration state again even at room temperature, as shown in Fig. 7e; it corresponds to the state between those shown in Figs. 7b and 7c. Therefore, these results indicate that the crystal form at 40 °C is

Fig. 5. The TG/DTA Curve of the Aspartame Crystal from r.t. to 90 °C. The heating rate was 0.5 °C/min.

Fig. 6. The Stability Check of the Intermediate State by Thermogravimetry

The heating rate was 0.5 °C/min to 46 °C from r.t., and the temperature was held at 46 °C for about 3 h.

Fig. 7. ¹³C-CPMAS-NMR Spectra of the IIA Form at r.t. (a), 28 °C (b), 35 °C (c), 40 °C (d), and r.t. (e) (Refilled Sample)

The number at each signal in (a) corresponds to the carbon numbering in Fig. 1. The new signals due to the transition were marked by arrow. The chemical shift difference between the new and original signals was approximately 1.1, 1.0, and 1.8–2.0 ppm in the carboxyl carbon of the Asp moiety, in the γ-carbon of the Phe moiety and in each methylene carbon, respectively.

Table 1. Signal Assignments^{a)} of Aspartame in ¹³C-CPMAS-NMR and Solution NMR

	Carbon No.											
	1	2	3	4	5	6	7	8	9	10	11	12
IIA	171.5	52.8	33.3	137.0	131.3	128.3	126.6	170.0	51.5	39.6	176.2	52.8
					129.3	127.7		169.5				
Solution	174.0	55.4	37.3	137.3	130.1	129.8	128.2	170.8	51.5	38.2	177.0	53.9

a) Unit of ¹³C chemical shift in ppm.

Fig. 8. The Enlarged Lower Magnetic Field Regions in Fig. 7a—d
The new signals due to the transition were marked by arrow.

still the IIA form, and that at the same time the dehydration is induced at 40 °C to a restricted extent from 1/2 H₂O toward 1/3 H₂O, which was evaluated from the TG results; *i.e.*, the IIA form has a zeolitic property in the early stage of crystal transformation. The spectrum in Fig. 7e is thought to show a slightly humidified state produced by the cooling down to r.t. As shown in Figs. 7 and 8, it was found that a new signal (marked by arrow) gradually appeared in the field nearby and lower than each original signal as the temperature rose in the carboxyl carbon of the Asp moiety, and in the γ -carbon of the Phe moiety and in each methylene carbon. The signal intensity ratio of the new and original signals eventually reached 1 : 1 at 45 °C, as shown in Fig. 9a.

Figure 9 illustrates the spectral changes from 45 °C to 80 °C. The signal pattern turned out to be almost invariable during the 40 °C—50 °C period, and this is well in agreement with the finding by TG measurement that the intermediate state is comparatively stable at this temperature range.

c) Carbon $T_{1\rho}$ Measurement: The ^{13}C spin-lattice relaxation time in the rotating frame ($T_{1\rho}$) was measured for the conformational flexibility analysis in the Phe moiety in aspartame. As the results, $T_{1\rho}$ in the γ -carbon of the Phe moiety was 208 ms at r.t., and it decreased to 135 and 172 ms at 45 °C, in a new signal (marked by arrow in Fig. 7) and in an original position signal, respectively.

Deuterium NMR Measurement Figure 10 shows the static D-NMR spectra of the D₂O type IIA form at r.t. and 45 °C. It was found that the integrated area ratio at r.t. and 45 °C is 0.5 : 0.33, and this is well in agreement with the amount ratio of water of crystallization in IIA(1/2 H₂O) and IIA(1/3 H₂O). The splitting width at the peak top was *ca.* 7.0 kHz at r.t., and it was *ca.* 15.6 kHz at 45 °C, respectively. The result of the line shape measurements indicates that the molecular motion of some water molecules in IIA(1/3 H₂O) reduced compared with the case in IIA (1/2 H₂O). In other words, the result suggests that some water molecules, which

Fig. 9. ^{13}C -CPMAS-NMR Spectra at 45 °C (a), 50 °C (b), 55 °C (c), 60 °C (d), 65 °C (e), 70 °C (f), and 80 °C (g). (120—180 ppm)

Fig. 10. D-NMR Spectra of the D₂O Type IIA Form at r.t. (a) and 45 °C (b)

remained in IIA(1/3 H₂O) after the dehydration, moved to the more stable position in the hydrophilic channel and reduced their mobility and partly helped the crystal lattice retention in IIA(1/3 H₂O).

Energy Calculations of IIA(1/2 H₂O) and IIA(1/3 H₂O)

Table 2 shows the results of the energy component analysis in the model structures of IIA(1/2 H₂O) and IIA(1/3 H₂O). We found that IIA(1/2 H₂O) is more stable than IIA(1/3 H₂O) regarding the total potential energy ($\Delta E = \text{ca. } 138 \text{ kJ}$ per three unit cells). In the detailed comparison, IIA(1/2 H₂O) turned out to be more stable (*ca.* 294 kJ) regarding the electrostatic term including H-bonds and to have a more unstable (*ca.* 140 kJ) van der Waals term than IIA(1/3 H₂O). The two states were not so different regarding the energies of bonds, angles and torsions.

MD Simulations a) Averaged Location of Water Molecules in IIA(1/2 H₂O): We found that the time-averaged location of the water molecules has 2₁ symmetry and that the in-

Table 2. Decomposition of Potential Energy^{a)} in IIA(1/2H₂O) and IIA(1/3 H₂O)

	IIA(1/2 H ₂ O)	IIA(1/3 H ₂ O)	$\Delta E^{b)}$
Total potential energy	-4270.5	-4132.3	138.2
Internal interaction			
Bonds	204.1	218.3	14.2
Angles	504.0	468.2	-35.8
Torsions	205.9	209.1	3.2
Inversions	1.1	3.9	2.8
Total	915.1	899.5	-15.6
Nonbonded interaction			
van der Waals	820.9	681.2	-139.7
Electrostatic	-5324.8	-5149.9	174.9
H-bonds	-681.7	-563.1	118.6
Total	-5185.6	-5031.8	153.8

a) Units in kJ per three unit cells. b) $\Delta E = E_{\text{IIA}(1/3\text{H}_2\text{O})} - E_{\text{IIA}(1/2\text{H}_2\text{O})}$.

Table 3. Intermolecular Hydrogen Bond Length^{a)} in IIA(1/2 H₂O)

	Position ^{b)}			
	A	B	C	D
1st layer	2.95 (0.10)	3.07 (0.17)	2.97 (0.11)	3.06 (0.16)
2nd layer	2.95 (0.12)	3.04 (0.13)	2.95 (0.13)	3.07 (0.16)
3rd layer	2.99 (0.12)	3.06 (0.15)	2.94 (0.11)	3.07 (0.17)

a) Time-averaged values for the trajectory of 50 ps MD simulation. Units in Å. The figures in parentheses denote the standard deviations. b) Position A and C sites are face-to-face about the location of the aspartame molecules around the *c*-axis, whereas the position A and B sites are side-by-side. The four positions and three layers are shown in Fig. 2.

interval of each water molecule along the *c*-axis is about 2.46 Å, which is half of the *c*-axis length (4.92 Å). The free movement of water molecule from the first crystallographic site to the second was not observed during 50 ps.

b) Averaged Structure of Aspartame Molecules: As shown in Fig. 2, the intermolecular hydrogen bonds are formed in IIA(1/2H₂O) between the peptide bond sites such as NH $\cdots$ O=C in the direction of the *c*-axis. Table 3 shows the time-averaged length of the hydrogen bond calculated based on the MD simulation in 50 ps.

We found that there are two types of length in the time average of this hydrogen bond; they locate alternately around the hydrophilic channel, and the difference between them is about 0.1 Å, whereas the time-averaged conformation of the aspartame molecule is almost identical. It is reasonable that the fluctuation of the hydrogen bond length was smaller in positions A and C, where the hydrogen bond length is shorter.

c) Conformational Flexibility of Aspartame Molecule: Table 4 shows the comparison of the conformational flexibility in the aspartame molecule between IIA(1/2H₂O) and IIA(1/3 H₂O), using the time-averaged values and the variances of the χ_1 dihedral angle in the Phe moiety. The mean value of the time-averaged χ_1 angles was 63.4° and 63.5° in IIA(1/2H₂O) and IIA(1/3 H₂O), respectively, and the time-averaged conformation turned out to be about the same between the two states. However, the mean value of the variances was 33.2 in IIA(1/2H₂O), while it was grouped into 33.4 or 52.5 in IIA(1/3 H₂O) assuming that the ratio of rigid molecules and flexible molecules was almost 1:1. Thus, it was found that there were at least two kinds of conforma-

Table 4. Conformational Flexibility in IIA(1/2 H₂O) and IIA(1/3 H₂O)

	Position ^{a)}							
	A	B	C	D	A	B	C	D
	IIA(1/2 H ₂ O)				IIA(1/3 H ₂ O)			
a) Time-averaged values ^{b)} of χ_1 angle in Phe moiety for the trajectory of 15–50 ps MD simulation								
1st layer	64.7	62.7	64.4	63.1	62.7	63.8	62.9	62.5
2nd layer	61.9	61.5	64.2	63.9	62.8	64.5	62.9	61.3
3rd layer	63.9	63.3	63.8	63.1	64.0	68.7	62.2	63.9
Mean	63.4 ^{c)}				63.5 ^{c)}			
b) Variances ^{d)} of χ_1 angle in Phe moiety								
1st layer	36.7	31.6	32.4	40.9	42.2	<u>22.8</u>	<u>28.7</u>	43.8
2nd layer	33.3	30.7	31.9	32.7	49.2	59.4	<u>31.9</u>	<u>36.4</u>
3rd layer	30.6	32.1	33.4	31.7	<u>41.4</u>	67.3	<u>39.1</u>	53.1
Mean	33.2 ^{e)}				33.4 ^{f)} , 52.5 ^{g)}			

a) The four positions and three layers are shown in Fig. 2. b) Units in degree. c) The mean value of the time-averaged χ_1 angle in all positions. d) Variances = $\Sigma(\chi_{1i} - \chi_{1\text{mean}})^2/(n-1)$, n = number of χ_{1i} . e) The mean value of χ_1 angle variances in all positions. f, g) The mean value of χ_1 angle variances underlined (smaller half) ^{f)} and those in the other (larger half) ^{g)}, respectively.

tional flexibility in IIA(1/3 H₂O).

Discussion

Thermal Stability Factors in the IIA Form of Aspartame

In our laboratories, the IIA form of aspartame has been recognized to be very stable over a wide temperature range, whereas IIB has a strong tendency to convert to IIA by water adsorption. However, the transformation mechanism between IIA and IIB forms has not been analyzed in detail. In the present study, the temperature-dependent change of the powder XRD pattern in a IIA sample and the TG/DTA curve were observed. It was revealed that the intermediate state exists at 40 °C–50 °C in the transformation between IIA and IIB, and its crystal form does not change from the initial IIA form, despite the different hydration number. In other words, the hydration number is variable in the IIA form to some extent, as in zeolites.²⁷⁾ In addition, the intermediate state was found to be stable as long as the temperature is held at 46 °C, *i.e.*, the amounts of adsorption and desorption of water are balanced, as in an equilibrium state. The results of the static D-NMR measurements at r.t. and 45 °C also proved that the hydration number ratio of the initial IIA form and the intermediate state was 0.5:0.33, *i.e.*, they were IIA(1/2H₂O) and IIA(1/3 H₂O), respectively. The transition starting point was different by 5 degrees between the TG/DTA result (*ca.* 55 °C) and the powder XRD analysis (*ca.* 50 °C), and the former better explains the results of ¹³C-CPMAS-NMR. The sample was in the non-crushed crystal state in the TG/DTA and NMR measurements, and it was well-ground in the powder XRD analysis; this may have caused the difference, because the transition would easily proceed in a crushed sample, and the difference in the heating process, *i.e.*, isothermal (powder XRD) or nonisothermal (TG/DTA), may also be a factor.

Thus, the IIA form can retain the crystal lattice in dehydration to some extent by generating a stable intermediate state, and these physical properties are thought to underlie the thermal stability of the IIA form.

The Specific Features of the Crystal Transition In general, an organic crystal lattice is often destroyed by dehydration when it is stabilized by the hydrogen bonding net-

work in which the water molecules play an important part. In aspartame, this is the case with the whole transformation between the IIA and IIB forms, but not with the transition between the IIA form and the intermediate state. Thus, the crystal transformation of the IIA form is a very interesting phenomenon from the view point that the dehydration proceeds in a manner similar to that in zeolites at the initial stage, generates the intermediate state, and finally brings on the crystal lattice change as in an ordinary organic crystal.

As Endo *et al.* reported for an orthogonal bis(resorcinol)-anthracene compound,²⁸⁾ there is an exceptional case where an organic crystal has a function of zeolite. In addition, as Sugawara *et al.* described for disodium ATP,²⁹⁾ there is a special case where a single crystal state is retained during a humidity-controlled transition. In both cases, there is a common structural feature in that the framework is stabilized by aromatic stacking, and large supramolecular cavities for solvent molecules or columns consisting of water molecules are present in the structure, respectively. As described before and shown in Fig. 2, a similar feature exists in the crystal structure of the IIA form, *i.e.*, the water molecules form the infinite columns reaching the crystal surface, and aspartame molecules form the surrounding frame stabilized by the intermolecular hydrogen bonds and the ring stacking in the Phe moiety.

Therefore, the crystal lattice retention in the IIA form is attributable to such a structural feature. The transition type should be that of zeolites when the frame is rigid enough, and it should be topochemical as in the case of disodium ATP²⁹⁾ when the frame is not very rigid but is firm enough to maintain the single crystallinity for the gradual change of lattice parameters.

Transformation Analysis and Characterization of IIA(1/3 H₂O) by Solid-State NMR and Energy Component Analysis Solid-state NMR is useful for the investigation of temperature-dependent structural properties in crystalline materials.³⁰⁾ The transformation analysis of the aspartame crystal is a case in point, because it has a strong tendency to crystallize as very fine fibers, and it would be difficult to elucidate the process of the structural changes by X-ray crystallographic studies.

In the transition from IIA(1/2 H₂O) to IIA(1/3 H₂O) shown in Fig. 7a—d, the original state partly remains as it was, and “a new kind of state” (marked by arrow in the spectra) is generated concerning the carboxyl carbon of the Asp moiety, the γ -carbon of the Phe moiety and each methylene carbon, and finally the intensity ratio of the new and original signals reaches 1 : 1 in IIA(1/3 H₂O). Since the chemical shift difference between the new and original signals is 1.0 ppm or more in each case, there clearly exists a new physical state, not a chemical exchange. The partial loss of the hydrogen bonds within the crystal by the dehydration presumably generated a new carboxyl carbon signal of the Asp moiety; however, this is not enough to account for “a new chemical shift in the γ -carbon of the Phe moiety.” Since the intensity ratio in the powder XRD was almost the same between IIA(1/2 H₂O) and IIA(1/3 H₂O), and their intramolecular energies are not very different (Table 2), we do not think that a large conformational change occurs in the new state.

As shown in Table 4, the conformational flexibility analysis in the Phe moiety of IIA(1/2 H₂O) and IIA(1/3 H₂O) sug-

gested that the time-averaged and space-averaged conformation of aspartame molecules is almost the same between the two states, and that is consistent with the results observed in the powder XRD. Simultaneously, the above computational analysis revealed that IIA(1/3 H₂O) has at least two kinds of flexibility in the χ_1 dihedral angle in the Phe moiety, and IIA(1/2 H₂O) has the only rigid molecules. Moreover, the $T_{1\rho}$ measurements in the γ -carbon of the Phe moiety revealed that the conformational flexibility in the Phe moiety increased in IIA(1/3 H₂O) than in IIA(1/2 H₂O), especially in IIA(1/3 H₂O) the new γ -carbon was thought to be more flexible than the original position γ -carbon, since the $T_{1\rho}$ was 208 ms at r.t., and it was 135 and 172 ms at 45 °C, in the new signal and in the original position signal, respectively.

Therefore, it is suggested that “the new NMR signal in the γ -carbon of the Phe moiety” corresponds to the flexible γ -carbon newly generated in IIA(1/3 H₂O); in a crystal, the spatial anisotropy causes the non-linear correlation between the chemical shift and the dihedral angle, and it presumably gave different effects on the chemical shifts of the flexible molecule and the rigid molecule.

The energy component analysis for the models of IIA(1/2 H₂O) and IIA(1/3 H₂O) revealed that the potential energy in the latter was higher than in the former. This is a static result, and the degree of nonstabilization must be estimated after a dynamics simulation sampling for a strict discussion; however, it is an experimental finding that the IIA(1/3 H₂O) exists in a *quasi*-stable intermediate state. Therefore, the existence of IIA(1/3 H₂O) should be explained by the entropic stabilization in a whole system. The entropic stabilization is presumably caused by the evaporation of the water of crystallization and the increase of molecular motion in aspartame, because the conformational flexibility analysis showed the increase of flexibility in the intermediate state.

It is also important that in the solid-state NMR, the signal broadening of the Phe ring carbons started from around 55 °C and corresponded to the transition starting point from IIA(1/3 H₂O) to the IIB form. Though the signal broadening of the Asp carboxyl carbon and the Phe ring carbons increase over 60 °C, presumably the former means the increase of conformation, and the latter means the increase of flip-flop motion, it is interesting that the spectral pattern basically resembles that of IIA(1/3 H₂O) at 45 °C except for the line width. Such a resemblance in spectral pattern indicates that the large rearrangement of aspartame molecules does not occur in the crystal transformation from IIA to IIB.

MD Simulation of IIA(1/2 H₂O) It is of note that the space group of the IIA form is $P4_1$ and the occupancy rate of the water molecules is 50%.¹⁴⁾ This is crystallographically a correct expression, and it means that the molecules in the IIA form show $P4_1$ symmetry in the space-averaged and time-averaged state. However, from the microscopic point of view, the arrangement of the water molecules is not 4_1 but 2_1 , because its occupancy rate is 50% and its free movement between the crystallographic sites was not observed in the MD simulation. Accordingly, in each unit cell of the crystal, the microscopic state of aspartame molecules strictly may not be $P4_1$, reflecting the 2_1 symmetry of the water molecule arrangement. Thus, we interpret the MD simulation results observed in IIA(1/2 H₂O) and IIA(1/3 H₂O) as follows. In IIA(1/2 H₂O), the 2_1 symmetry of the water molecule

arrangement influences the microscopic state of aspartame molecules, and a slight difference (*ca.* 0.1 Å) was caused in the time-averaged length of the intermolecular hydrogen bond formed between the peptide bond sites. The further that the dehydration proceeds, the more differences are partially caused such as the conformational flexibility increase in IIA(1/3 H₂O).

Conclusion

A *quasi*-stable intermediate state was discovered in the transformation between the IIA form and the IIB form of aspartame. The crystal lattice in the IIA form is retained in the intermediate state, despite the dehydration from 1/2 mol to 1/3 mol. Such a specific feature in the crystal transition originally is thought to be due to the existence of the water columns reaching the crystal surface, the framework stabilized by the aromatic ring stacking and the intermolecular hydrogen bonds in its crystal structure. The intermediate state, IIA(1/3 H₂O), is stable during the 40 °C–50 °C period of the transformation. Since IIA(1/2 H₂O) is more stable than IIA(1/3 H₂O) regarding the total potential energy, the generation of IIA(1/3 H₂O) is considered to be promoted by the entropic effect, which is caused by the evaporation of the water of crystallization and the increase of molecular motion in aspartame. Thus, the thermal stability of the IIA form is attributable to a structural property, *i.e.*, the crystal lattice itself is retained during the dehydration to some extent. Moreover, it was suggested that a) the arrangement of water molecules has 2₁ symmetry in IIA(1/2 H₂O), and this influences the microscopic state of aspartame molecules; b) a slight difference is caused in the hydrogen bond length between the peptide bond sites; c) the aspartame molecules have two kinds of flexibility in IIA(1/3 H₂O), and d) clear differences are caused in the NMR chemical shift.

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