Molecular Dynamics of Hinge-Bending Motion of IgG Vanishing with Hydrolysis by Papain

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ABSTRACT We have performed dielectric relaxation measurements via a time domain reflectometry (TDR) method to study dynamic behaviors of the segmental flexibility of immunoglobulin G (IgG) in aqueous solution without antigen binding. In general, an intermediate relaxation process due to bound water is observed around 100 MHz at 25°C for common proteins between two relaxation processes due to overall rotation and reorientation of free water. However, the intermediate process observed around 6 MHz for IgG was due to both bound water and hinge-bending motion. The apparent activation energy of 33 kJ/mol was larger than 27 kJ/mol for only bound water, and the relaxation strength was about five times as large as expected for bound water. The shape of the relaxation curve was very broad and asymmetric. These characteristic differences arising from the hinge-bending motion of IgG disappeared for fragments decomposed from IgG hydrolyzed by papain, since the hinge-bending motion did not exist in this case. We have separated the relaxation processes due to hinge-bending motion and bound water for IgG and obtained the Fab-Fab angle of IgG as about 130° by Kirkwood's correlation parameter and the activation energy of 34 kJ/mol for hinge-bending motion.

INTRODUCTION

The three-dimensional structure of globular protein fluctuates incessantly and the fluctuation is closely related to the function of the protein. This fluctuation is observed by x-ray diffraction analysis (Frauenfelder et al., 1979; Artymiuk et al., 1979), Mössbauer measurements (Keller and Debrunner, 1980; Parak et al., 1981), NMR measurements (Nicholson et al., 1992), and computer simulations (Karplus and McCammon, 1986; Karplus and Petsko, 1990). Some species of enzymes such as serine protease with a ditch between two globular domains fluctuate to open and to close the domains. This intramolecular motion is called "hinge-bending deformation" (Karplus and McCammon, 1986). The hinge-bending deformation is investigated by x-ray diffraction (Dobson, 1990; Bernstein et al., 1997), NMR measurements (Li and Montelione, 1995), electron paramagnetic resonance (EPR) spectroscopy (McHaourab et al., 1997), fluorescence spectroscopy (Miki and Kouyama, 1994), computer simulation (Arnold and Ornstein, 1997). However, dynamical behavior of the hinge-bending deformation has not been clarified enough.

The immunoglobulin G (IgG) molecule consists of three globular domains of two Fab segments and one Fc segment, which are mutually connected by two flexible polypeptide chains called hinge held together by a disulfide bond (Silverton et al., 1977; Metzger, 1974; Arata et al., 1980), as shown in Fig. 1 *A*. Papain hydrolyzes IgG molecules on the hinge, and cysteine or mercaptoethanol reduces the disulfide bond of IgG molecules (Utsumi, 1969). IgG molecules are separated into the two Fab and one Fc fragments by this treatment, as shown in Fig. 1 *B*. On the other hand, Feinstein and Rowe (1965), and Valentine and Green (1967) performed electron microscopy studies, and they suggested that the angle between Fab segments of IgG molecules vary widely. Their suggestion is supported by many studies such as NMR (Arata et al., 1980), EPR spectroscopy (Käivärainen and Nezlin, 1976), electron microscopy (Roux et al., 1997), and theoretical studies (McCammon and Karplus, 1977). Especially, Hanson et al. (1981) performed a dynamical study using nanosecond fluorescence spectroscopy, and reported that wagging or cone-like wobbling motions of the Fab fragments are possible on the hinge region. Though such a dynamical study is important to understand the biological function, accurate analysis of fluorescence data is difficult and the estimation is necessarily crude.

Dielectric measurement is one of the most useful methods for dynamical study. Dielectric measurements have been performed for globular protein in aqueous solutions, and subsidiary dispersion due to bound water was found at an intermediate frequency region between two principal dispersions due to molecular motions of protein and free water molecules, respectively (Grant, 1966; Pethig, 1979; Takashima, 1989). Furthermore hydration studies on proteins were also preformed by microwave dielectric measurements (Bone and Zaba, 1992; Suzuki et al., 1996, 1997). In our previous work (Miura et al., 1996), hinge-bending deformation of trypsin was observed for the first time by microwave dielectric measurements using time domain reflectometry (TDR) method (Cole, 1975a,b; Cole et al., 1980). The dielectric measurements were performed for the trypsin aqueous solution in a temperature range between −35 and 25°C over a frequency range of 50 kHz to 4 GHz.

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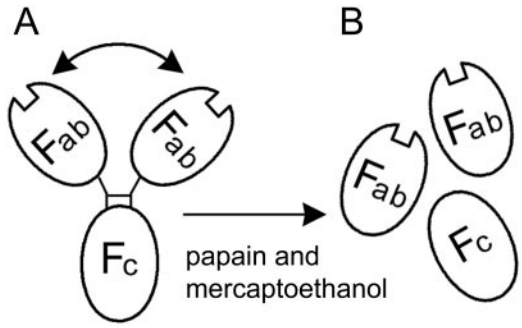

FIGURE 1 Model of the IgG molecule (*A*) and Fab and Fc fragments obtained by papain hydrolysis (*B*). (*A*) IgG is made up of two Fab fragments and one Fc fragment connecting on the hinge, and hinge-bending motion exists. (*B*) The hinge-bending motion does not exist in the system of Fab and Fc fragment mixture.

Above the freezing temperature of −7°C for free water, three relaxation processes due to reorientations of free water, reorientations of bound water, and overall rotations are observed. Because of large relaxation strengths for processes due to free water and the overall rotation, we could not distinguish the hinge-bending motion. Three relaxation processes are also observed below the freezing temperature. The high frequency relaxation process observed around 3 GHz at −10°C is due to reorientation of unfreezable water constructing a shell layer around the protein molecule, and the intermediate frequency process observed around 20 MHz is due to bound water molecules, which attach directly on the globular protein surface via hydrogen bonding. Bound water and unfreezable water have also been observed in other globular proteins (Miura et al., 1995). The low frequency relaxation process observed around 600 kHz at −10°C is due to the hinge-bending deformation of trypsin. This relaxation process vanishes, when trypsin inhibitor is caught in the ditch between two globular domains of the trypsin. This result suggests that the trypsin inhibitor prohibits not only the biological function of the trypsin but also its movement of hinge-bending deformation of the trypsin, since the trypsin inhibitor gets caught in the ditch of the trypsin as reported for x-ray analysis on the complex of trypsin with trypsin inhibitor (Sweet et al., 1974). However, the behavior of the hinge-bending deformation above the freezing temperature of free water has not yet been clarified.

In this work, we observed directly the hinge-bending motion of IgG molecules by dielectric measurements using the TDR method at various temperatures above the freezing temperature of free water. Since the wide-angle motion of Fab segments should bring a larger dielectric relaxation process than the trypsin, the hinge-bending motion is expected to be observed. We also confirm that the hinge-bending motion vanished with the hydrolysis on the hinge region of the IgG molecules by papain. Dielectric measurements for bovine serum albumin (BSA) in aqueous solution were performed to compare the globular protein consisting of a single globular domain.

MATERIALS AND METHODS

Samples

Bovine IgG, papain, and BSA were purchased from Sigma Chemical Co. (St. Louis, MO). Salts contained in IgG aqueous solution were removed by the use of a PD-10 column provided by Amersham Pharmacia Biotech AB (Uppsala, Sweden). After removing the salts, we lyophilized it, and prepared solutions with a protein concentration 50 mg/ml and pH 9.0 for TDR measurements at temperatures between −7 and 25°C over a frequency range 300 kHz to 10 GHz. Aqueous solutions of BSA 50 mg/ml were prepared for TDR measurements at temperatures between −5 and 15°C over a frequency range of 300 kHz to 10 GHz.

We also prepared Fc and Fab fragment mixtures from IgG. The digestion with papain at pH 7.5 was performed according to the method of Utsumi (Utsumi, 1969) except that the digestion was carried out for 18 h. Then alkylation with iodoacetamide provided by Wako Pure Chemical Industries, Ltd. (Osaka, Japan) was carried out for 2 h at 25°C pH 9.0 in the dark in order to stop the digestion by inactivation of papain and prevent producing Fab_2 fragment with re-bonding disulfide bond. Each IgG molecule was separated into two Fab fragments and one Fc fragment by this method. Fragments thus obtained were purified by passing through a 15 × 300 mm column of Superdex 200 provided by Amersham Pharmacia Biotech AB (Uppsala, Sweden). We confirmed that the molecular weight of fragments was about 50,000 and that of the original IgG was about 150,000 by gel electrophoresis. We removed salts contained in the solution by the PD-10 column and lyophilized the solution. Solutions were prepared at a total concentration of 50 mg/ml and pH 9.0 of Fab and Fc for TDR measurements at temperatures between −5 and 25°C over a frequency range of 500 kHz to 5 GHz.

Dielectric measurements by TDR method

Details of the TDR apparatus have already been reported in the previous papers (Cole, 1975a,b; Cole et al., 1980). By using a dielectric material with known permittivity ε^*_S as a reference sample, the permittivity of the unknown sample ε^*_X is given as

$$\varepsilon^*_X(\omega) = \varepsilon^*_S(\omega)\frac{1 + \{(cf_S)/[j\omega(\gamma d)\varepsilon^*_S(\omega)]\}\rho f_X}{1 + \{[j\omega(\gamma d)\varepsilon^*_S(\omega)]/(cf_S)\}\rho f_S}, \qquad (1)$$

where

$$\rho = (r_S - r_X)/(r_S + r_X),$$

and

$$f_X = Z_X \cot Z_X, \qquad Z_X = (\omega d/c)\varepsilon^*_X(\omega)^{1/2},$$

$$f_S = Z_S \cot Z_S, \qquad Z_S = (\omega d/c)\varepsilon^*_S(\omega)^{1/2},$$

where d is the geometric cell length, γd is the effective cell length, r_S and r_X are Fourier transforms of reflected pulses from the reference sample $R_S(t)$ and from the unknown sample $R_X(t)$, respectively, j is the imaginary unit, ω is the angular frequency, and c is the speed of propagation in vacuum. We chose the aqueous solution of sodium chloride as a reference sample, and the effective electric cell lengths γd employed were 0.161, 0.67, 2.84, and 6.1 mm. The time domain dielectric method has also been applied for biomaterials by other investigators (Feldman and Fedotov, 1988; Bone and Zaba, 1992) as an effective tool.

RESULTS AND DISCUSSION

Typical results of dielectric absorption curves observed at 25°C for the IgG aqueous solution and that of the mixture of Fab and Fc fragments obtained by hydrolysis with papain are shown in Fig. 2. Three relaxation processes were isolated independently for all samples by employing a fitting procedure, which has been already employed for aqueous solutions of other globular proteins (Miura et al., 1994). The permittivity is thus described by

$$\varepsilon^*(\omega) - \varepsilon_\infty = \frac{\Delta\varepsilon_l}{1 + j\omega\tau_l} + \frac{\Delta\varepsilon_m}{\{1 + (j\omega\tau_m)^{\beta_m}\}^{\alpha_m}} + \frac{\Delta\varepsilon_h}{1 + (j\omega\tau_h)^{\beta_h}}, \quad (2)$$

where ε_∞ is the dielectric constant extrapolated to $\omega = \infty$, $\Delta\varepsilon$ is the relaxation strength, τ is the relaxation time, α and β are parameters describing the distribution of the relaxation time, and subscripts l, m, and h indicate the low, intermediate, and high frequency relaxation process, respectively.

It is concluded that the high frequency relaxation process h is due to reorientation of free water molecules, judging from its relaxation time τ_h and relaxation strength $\Delta\varepsilon_h$ (Miura et al., 1994).

For the low frequency relaxation process l, generally overall rotation of protein molecules is observed in this frequency region (Grant, 1966; Pethig, 1979; Takashima, 1989). Fig. 3 shows that logarithm of the relaxation time τ_l observed in this work against the molecular weight M lies on the same straight line with a slope of unity obtained for the overall rotation of ten globular proteins, adenosine triphosphate, adenosine diphosphate, and adenosine monophosphate, which were measured previously (Miura et al., 1994; Mashimo et al., 1992). Therefore the relaxation process l observed in this work is also due to overall rotations of IgG, Fab and Fc fragments, or BSA. Furthermore, this result also implies that protein-protein interaction, such as forming dimer, trimer, or aggregates, was negligible. If protein molecules form dimer or larger aggregates, the plot must deviate from the line in Fig. 3.

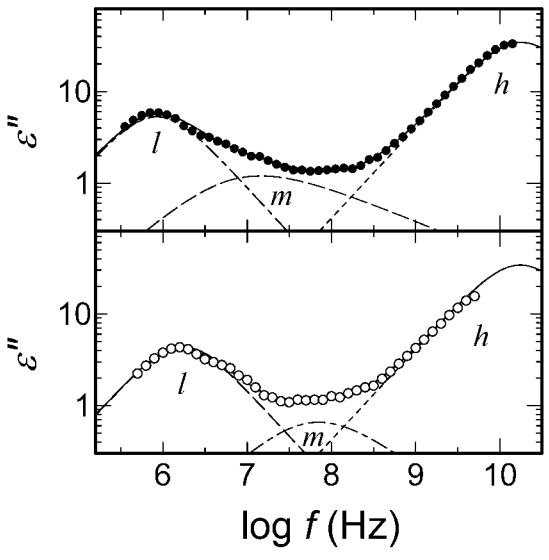

FIGURE 2 Dielectric absolute curves. (*A*) 5 wt % aqueous solution of IgG at 25°C. (*B*) 5 wt % aqueous solution of Fab and Fc fragment mixture at 25°C.

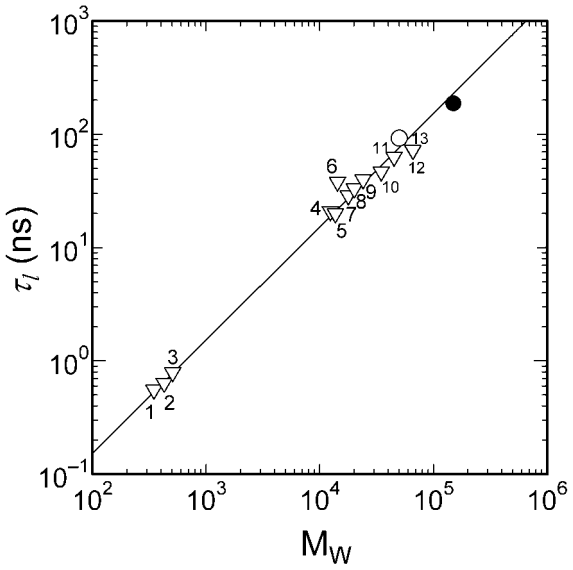

FIGURE 3 Double-logarithmic plots of relaxation time τ_l against molecular weight M_W at 25°C (1: adenosine monophosphate; 2: adenosine diphosphate; 3: adenosine triphosphate; 4: cytochrome c; 5: ribonuclease A; 6: lysozyme; 7: myoglobin; 8: trypsin inhibitor; 9: trypsin; 10: pepsin; 11: ovalbumin; 12: hemoglobin; 13: BSA; ●: IgG; and ○: Fab and Fc fragment mixture.) The numbered plots are reported in a previous work (Miura et al., 1994).

It has been reported that bound water of globular proteins in aqueous solutions was observed as the intermediate frequency relaxation process m around 100 MHz at 25°C (Grant, 1966; Pethig, 1979; Takashima, 1989; Miura et al., 1994). Therefore, the process m observed in this work is easily considered to be due to bound water. However, only in the case of IgG, the relaxation time τ_m was more than ten times as large as that of bound water observed for common aqueous solutions of globular proteins (Miura et al., 1994), helical DNA (Kuwabara et al., 1988), and moist tropocollagen (Shinyashiki et al., 1990). In another case of the Fab and Fc fragment mixture, the τ_m was almost the same as that of common cases of bound water as shown in Fig. 4. The similar tendency was also shown for the apparent activation energy. Though the apparent activation energy for the process m of the IgG (33 kJ/mol) was considerably larger than that for bound water (28 kJ/mol) observed for common proteins, the value of 27 kJ/mol obtained for the Fab and Fc fragment mixture is almost equal, as shown in Table 1. The value of 33 kJ/mol for the IgG is similar to that of 32 kJ/mol

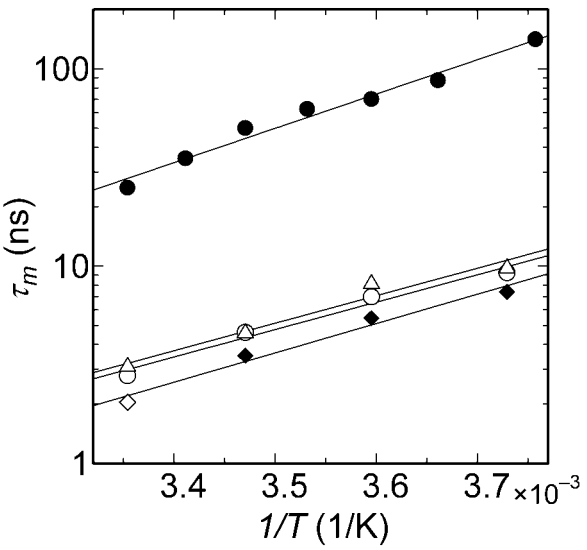

FIGURE 4 Plots of the logarithm of relaxation time τ_m against reciprocal absolute temperature for 5 wt % aqueous solution of trypsin (△) (Miura et al., 1996), BSA (◇) reported in a previous work (Miura et al., 1994), BSA (◆) (present work), IgG (●), and Fab and Fc mixture (○).

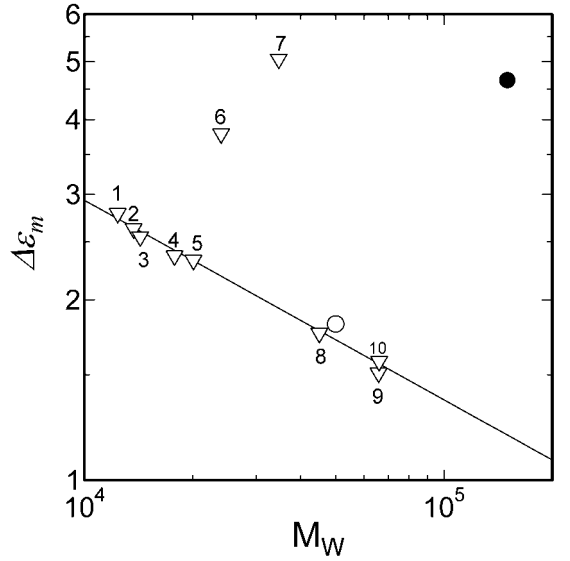

FIGURE 5 Double-logarithmic plots of relaxation strength $\Delta\varepsilon_m$ against molecular weight M_W at 25°C. 1, cytochrome c; 2, ribonuclease A; 3, lysozyme; 4, myoglobin; 5, trypsin inhibitor; 6, trypsin; 7, pepsin; 8, ovalbumin; 9, hemoglobin; 10, BSA; ●, IgG; ○, Fab and Fc fragment mixture.) The numbered plots are reported in a previous work (Miura et al., 1994).

reported for the hinge-bending motion of the trypsin observed below freezing temperature of free water (Miura et al., 1996) rather than 27–28 kJ/mol obtained for bound water. We examined the *t*-test to establish whether the apparent activation energy between IgG and papain-digested IgG was significantly different, and the obtained result ($p < 0.13$) would indicated a significant difference between them.

These results suggest that the hinge-bending motion of the IgG caused the differences in those values of τ_m and the apparent activation energy for IgG and other proteins. It means that the process *m* observed first for the IgG was an overlap of two relaxation processes due to reorientation of bound water and the hinge-bending motion. Since the relaxation time due to the hinge-bending motion was larger than that due to the bound water, the τ_m also became large.

Fig. 5 shows double-logarithmic plots of the relaxation strength $\Delta\varepsilon_m$ vs. molecular weight *M*, where the protein concentration is 5 wt % for all aqueous solutions. The plots lie on the same straight line with the slope, −1/3, except for the plots of trypsin, pepsin, and IgG. If $\Delta\varepsilon_m$ normalized by the number of protein molecules per unit volume, *N*, is proportional to the surface area of the protein spherical in shape, we obtain

$$\Delta\varepsilon_m/N \propto M\Delta\varepsilon_m \propto M^{2/3}, \quad \therefore \Delta\varepsilon_m \propto M^{-1/3}. \qquad (3)$$

Therefore, the slope of −1/3 in Fig. 5 means that the amount of bound water is proportional to the surface area of the protein. The reason why the plots of trypsin and pepsin deviate from the line is that both have active sites in cracks and bound water forms a network structure there as reported by x-ray analysis (Bode and Schwager, 1975). Thus the trypsin and pepsin should have more bound water than the common globular proteins and deviations are shown in Fig. 5. However, IgG does not have such a large crack. Although the surface area of the IgG with Y-shape is larger than the sphere, the total surface area of the IgG is not larger than that of the Fab and Fc fragment mixture. The hinge-bending motion would produce the large value of $\Delta\varepsilon_m$ for IgG.

Fig. 6 shows the temperature dependency of parameter, α_m, describing the distribution of relaxation times. The α_m value is small for broad and asymmetric shape of relaxation curve. The value of α_m for IgG which is apparently smaller than that for trypsin, BSA, Fab and Fc fragments, and other globular proteins (Miura et al., 1994) supports the suggestion for the overlap of relaxation processes due to bound

TABLE 1 Apparent activation energy ΔH

Protein	*C* (wt %)	*T* (°C)	ΔH (kJ/mol)	Reference
Trypsin (bound water)	5	−35 to 25	28	Miura et al., 1996
BSA (bound water)	20	−50 to 30	28	Miura et al., 1995
Fab and Fc fragments (bound water)*	5	−5 to 25	27	Present work
Trypsin (hinge-bending motion)	5	−35 to −10	32	Miura et al., 1996
IgG (process *m*)*	5	−7 to 25	33	Present work
IgG (process *m1*)†	5	−7 to 25	34	Present work

*Calculated by Eq. 2.
†Calculated by Eq. 4.

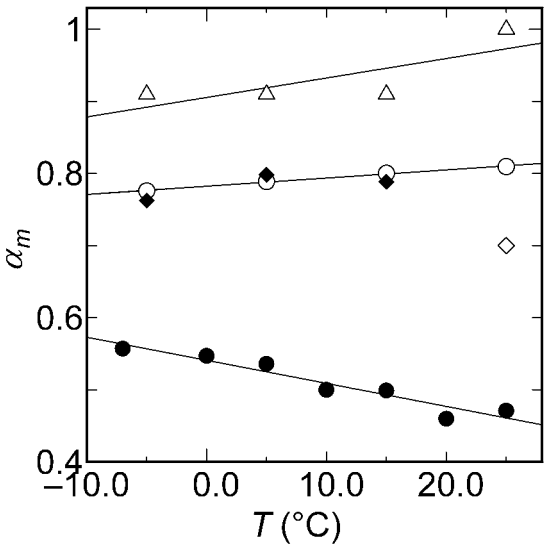

FIGURE 6 Plots of the parameter α_m for distribution of relaxation time against temperature for 5 wt % aqueous solution of trypsin (△) (Miura et al., 1996), BSA (◇) reported in the previous work (Miura et al., 1994), BSA (◆) (present work), IgG (●), and Fab and Fc mixture (○).

water and hinge-bending motion. We note that β_m did not exhibit the similar behavior with α_m.

On the other hand, a dielectric dispersion due to Maxwell-Wagner effect is generally observed for an emulsion, e.g., oil in water (O/W) system. However, Takashima (1963) performed dielectric measurements for protein solutions with various ions at the similar protein concentration with the present work, and suggested that the Maxwell-Wagner effect is not a major dielectric polarization mechanism. This suggestion was also supported by a linear relationship of relaxation time, τ_l, for the overall rotation against the molecular weight as shown in Fig. 3, since there was no indication of the Maxwell-Wagner effect. Therefore we considered that the Maxwell-Wagner effect was negligible in the present case. Wei et al. (1994) and Suzuki et al. (1996) treated Wagner or Hanai equation to study the protein hydration. In the present work, however, we did not use their analysis, because they applied the Wagner or Hanai equation to dielectric spectra above 1 GHz that is too high for the hinge-bending motion.

From all of our experimental results, we concluded that the relaxation process *m* for IgG was caused by overlapping of relaxation processes due to hinge-bending motion and reorientation of bound water. When the hinge-bending motion was vanished with the hydrolysis by papain, the relaxation process *m* for Fab and Fc fragment mixture was only caused by bound water. Then we tentatively separated the relaxation processes due to the hinge-bending motion and the reorientation of bound water for IgG in aqueous solution by the fitting procedure as shown in Fig. 7. In this analysis,

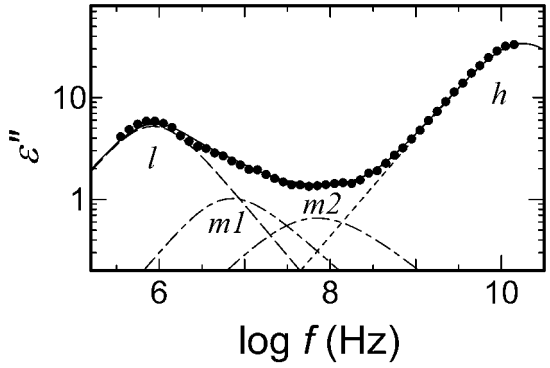

FIGURE 7 Dielectric absorption curve for 5 wt % aqueous solution of IgG at 25°C. Solid lines are calculated from Eq. 4.

the permittivity is described by

$$\varepsilon^*(\omega) - \varepsilon_\infty = \frac{\Delta\varepsilon_l}{1 + j\omega\tau_l} + \frac{\Delta\varepsilon_{m1}}{(1 + j\omega\tau_{m1})^{\alpha_{m1}}} + \frac{\Delta\varepsilon_{m2}}{\{1 + (j\omega\tau_{m2})^{\beta_{m2}}\}^{\alpha_{m2}}} + \frac{\Delta\varepsilon_h}{1 + (j\omega\tau_h)^{\beta_h}}, \quad (4)$$

where subscripts *m1* and *m2* indicate the relaxation processes *m1* and *m2*, respectively. We consider that the relaxation process *m1* was due to the hinge-bending motion of IgG and the relaxation process *m2* was due to the reorientation of bound water. Values of τ_{m2}, $\Delta\varepsilon_{m2}$, α_{m2}, and β_{m2} were chosen to be the same as those for bound water observed on the Fab and Fc fragment mixture, as shown in Fig. 8. The apparent activation energy of 34 kJ/mol for the *m1* process is reasonable for the hinge-bending motion, and this value was clearly larger than 27 kJ/mol for bound water for Fab and Fc fragment mixture ($p < 0.03$).

The relaxation strength $\Delta\varepsilon_{m1}$ for the hinge-bending motion was considerably smaller than the relaxation strength $\Delta\varepsilon_l$ for overall rotation of Fab and Fc fragments as shown in Table 2. This smaller value arises from antiparallel component of dipole moments of Fab segments in IgG molecules. Thus, Fab-Fab angle for IgG molecule can be calculated from Kirkwood's correlation parameter *g*. Relaxation strength is generally described as

$$\Delta\varepsilon = \frac{4\pi N_P \mu^2 g}{3k_B T} F, \quad (5)$$

where

$$g = 1 + Z\langle\cos\gamma\rangle,$$

where F is an internal field factor, K_B is the Boltzmann constant, N_P is the number of dipoles per unit volume, μ is the dipole moment, Z is the number of nearest neighbors surrounding the dipole moment, and $\langle\cos\gamma\rangle$ is the mean value of the cosine angle between the dipole moments, respectively (Takashima, 1989). In order to compare with

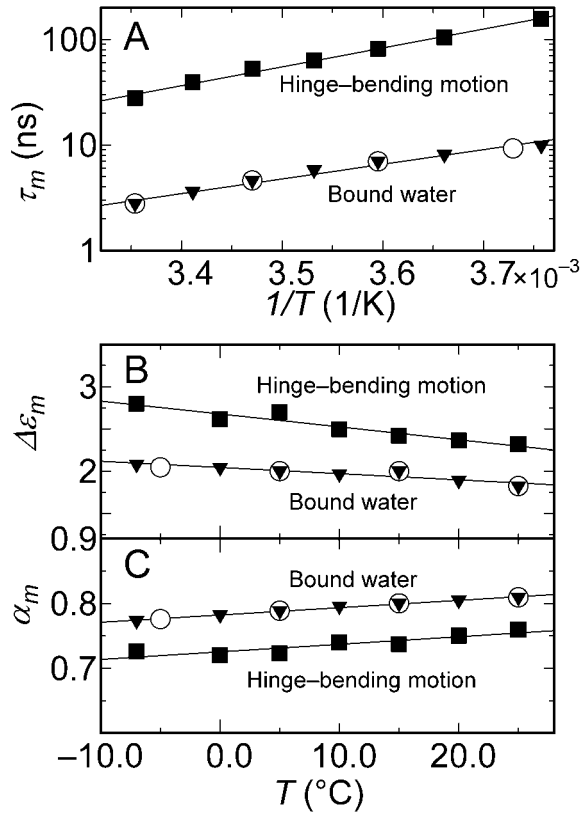

FIGURE 8 Temperature dependencies of the relaxation parameters for the relaxation processes of *m1* (■) and *m2* (▼) calculated from Eq. 4 for the IgG aqueous solution, and the relaxation process *m* (O) calculated from Eq. 2 for the aqueous solution of the Fab and Fc fragment mixture: (*A*) relaxation time; (*B*) relaxation strength; (*C*) parameter for distribution of relaxation time.

the hinge-bending motion of Fab segments and overall rotation of Fab and Fc fragments when applying Eq. 5, we supposed that: 1) dipole moment for Fab segment on IgG is not changed with hydrolysis by papain; 2) dipole moment of Fc fragment is the same as that of Fab fragment; 3) the internal field F is the same between the solutions of IgG and the solutions of Fab and Fc fragment mixture; 4) the relaxation process *m1* due to hinge-bending motion is only caused by Fab segments rather than Fc segment. The supposition 2 is reasonable, since structure of Fc fragment is not very different from Fab fragment, i.e., both Fc and Fab fragments consist of two 25,000-mol wt peptide chains extended in the same direction. We employ N_{Ph}/N_{Po} as 2/3 from the supposition 4, where N_{Ph} is the number of dipoles of Fab segments per unit volume and N_{Po} is that of Fab and Fc fragments, respectively. N_{Ph} and N_{Po} correspond to N_P for hinge-bending motion and overall rotation, respectively. Similarly, Z_h is 1, since Fab segments always exist in pair, and Z_o is 0 because of the dilute concentration of 5 wt % (Ermolina et al., 1998). Thus we employ $g = 1$ for Fab and Fc fragment mixture, and the cosine angle of Fab-Fab of IgG is obtained from Eq. 5 as

$$\langle \cos \gamma \rangle = 3\Delta\varepsilon_{m1}/2\Delta\varepsilon_l - 1. \quad (6)$$

The angles calculated from Eq. 6 are presented in Table 2. These values completely agree with the value of 117–136° reported for human IgG in the immunoelectron microscopy studies (Roux et al., 1997). This is the first time to obtain the Fab-Fab angle from dynamical measurements.

TABLE 2 Fab-Fab angle calculated by Kirkwood's correlation parameter

T(°C)	$\Delta\varepsilon_l$*	$\Delta\varepsilon_{m1}$†	γ
25	8.54	2.32	126°
15	10.24	2.42	130°
5	11.22	2.70	130°

*Overall rotation of Fab and Fc fragments.
†Hinge-bending motion of IgG.

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