Effect of pressure on interactions of anti-fluorescent probe monoclonal antibody with a ligand and inhibitors

M Nishimoto, M Goto, N Tamai, H Nagamune, S Kaneshina and H Matsuki

1Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan
2Department of Life System, Institute of Technology and Science, The University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan

E-mail: matsuki@bio.tokushima-u.ac.jp

Abstract. Interactions of anti-fluorescent probe monoclonal antibody (immunoglobulin G (IgG)-49) with a ligand (fluorescein (FL)) and three kinds of inhibitors (1-tetradecanol (C14OH), 1-tetradecanoic acid (C13COOH) and 5-aminofluorescein (5-FLNH2)) under high pressure were examined by methods of fluorescence spectroscopy. Pressure promoted the dissociation between FL and IgG-49 from the complex. The standard volume changes of the dissociation became negative, hence, the binding of FL to IgG-49 expands the volume of the complex. The volume expansion may be closely related to the large hydrophobicity around binding sites of FL in the IgG-49 molecule. Further, the standard volume changes of IgG-49 for the inhibitor binding, which were calculated from the Johnson-Eyring plots, became all negative. The volume change for 5-FLNH2 was smaller than those for C14OH and C13COOH. This means that the volume of IgG-49 shrinks by the addition of the inhibitors in contrast with the FL binding. The differences among inhibitors are attributable to the differences in interaction modes to IgG-49 among them.

1. Introduction
Interaction modes between biomolecules and ligands are different depending on the kinds of both molecules, and either specific or nonspecific interaction, or both interactions usually appears in binding of ligands to biomolecules. In order to characterize the ligand action more definitely, it is useful to show essential differences between specific and nonspecific interactions from a comparative study of interaction modes. In the previous papers [1,2], the comparative study of ligand effects on model biomolecule systems has been performed for the purpose of elucidation of anesthetic mechanism. We focused our attention on an antigen-antibody reaction as a model system containing extremely specific interactions between a protein and ligands in this study [3].

Monoclonal antibodies recognize only a singular antigenic determinant and bind specifically to the antigens in the concentration of nano-mole order. They can also recognize low molecular-weight substances bound covalently to carrier-proteins, which is called haptens, as antigens as well as cells, proteins and small peptides. Since hapten-specific monoclonal antibodies can be prepared by cell engineering techniques, they are widely used in the detection of specific interaction.

3 Author to whom correspondence should be addressed.
In the present study, we investigate the effect of pressure on an antigen-antibody reaction between a fluorescent probe (fluorescein (FL)) and an anti-fluorescent probe monoclonal antibody (immunoglobulin G (IgG)-49) in the absence and presence of specific and nonspecific inhibitors by methods of fluorescence spectroscopy. The ligand and inhibitor interaction modes to IgG-49 are discussed on the basis of the volume changes of IgG-49 induced by them.

2. Experimental
Anti-fluorescent probe monoclonal antibodies were prepared by cell engineering techniques described previously [4], and IgG-49, which binds strongly to FL, was selected by reference to experiments of the fluorescence quenching. A ligand (FL) and inhibitors (1-tetradecanol (C14OH), 1-tetradecanoic acid (C13COOH) and 5-aminofluorescein (5-FLNH₂)) were used as received. All solutions were made with Tris-HCl buffered solutions of 50 mM and pH 8.0.

The reactions between FL and IgG-49 in the absence and presence of the inhibitors under high pressure were examined by two methods of fluorescence spectroscopy, anisotropy and intensity measurements. The measurements were performed by using a Model F-2500 fluorescence spectrophotometer (Hitachi High-Technology Corp., Tokyo), which equipped with a high-pressure cell assembly PCI-400 (Syn Corporation, Kyoto). The high-pressure spectroscopic apparatus and the procedure of fluorescence measurements were described in detail elsewhere [3,5]. The excitation wavelength was 480 nm and the emission one was 520 nm. All measurements were done at 25 °C.

3. Results and Discussion

3.1. Effect of pressure on the interaction of IgG-49 with FL
The fluorescence of FL was quenched in an IgG-49 concentration-dependent manner, and the Scatchard analysis of the quenching curve proved that IgG-49 is a pure monoclonal antibody with two binding sites of FL [3,6]. We studied the effect of pressure on the reaction between FL and IgG-49 from the high-pressure fluorescence anisotropy measurements [7,8]. The fluorescence anisotropy exhibits the degree of constraint in the rotational motion for a fluorescent molecule, and the anisotropy value increases in proportion with the molecular weight of a biomolecule bound by the fluorescent molecule at constant temperature and solution viscosity. Figure 1(a) shows the pressure dependence of anisotropy values of three states of FL (free (A₀), partially bound (A) and completely bound (A₉)). At ambient pressure, the A₀ value became an appreciably small value near 0.02 due to no constraint in the rotational motion for the FL molecule. The A value increased with increasing the IgG-49 concentration, the value saturated near 0.15 in the high concentration range (the A₉ value), which means the large constraint of the motion originated from the complete binding of FL to IgG-49. All the A₀, A and A₉ values decreased by applying pressure although the pressure dependence of the A₀ value was considerably small.

Since the anisotropy of a solution is described as the sum of the anisotropies of constituents in the solution, the degree of dissociation of FL (α), the ratio of free FL molecules in the FL and IgG-49 mixed solution, can be written with the A₀, A and A₉ values in the following form,

\[ \alpha = [1 + Q(A - A₀)(A₉ - A)]^{-1} \quad (1) \]

where Q is the ratio of fluorescence quantum yields between free FL and completely bound FL and can be obtained from the areas ratio of their fluorescence spectra at an isosbestic point. The value of α is related to the dissociation constant between FL and IgG-49 from the complex (K_d) by using overall concentrations of FL ([FL]₀) and IgG-49 ([IgG-49]₀) as

\[ K_d = (n\alpha[IgG-49]₀ - \alpha(1 - \alpha)[FL]₀)/(1 - \alpha) \]  

(2)

where n is the binding number of IgG-49 and adopted the value of two. Calculating the α values by applying the equation (1) to the A₀, A and A₉ values in figure 1(a) at each pressure, and then the K_d values were evaluated by substituting the obtained α values with the [FL]₀ and [IgG-49]₀ values into the equation (2). The resulting K_d values are plotted against pressure in figure 1(b). It was found that the K_d value increased by applying pressure, that is, pressure promoted the dissociation between FL and IgG-49 from the complex.
The pressure dependence of the \(K_D\) value thermodynamically gives the standard volume changes of the dissociation (\(\Delta V_D^0\)) as
\[
\Delta V_D^0 = -RT\left(\frac{\partial \ln K_D}{\partial p}\right)_T
\]  
(3)
The \(\Delta V_D^0\) value was calculated by applying the equation (3) to the \(K_D\) vs. \(p\) curve in figure 1(b). The obtained \(\Delta V_D^0\) value was \(-5.2\) cm\(^3\) mol\(^{-1}\). The negative value of \(\Delta V_D^0\) indicates that the standard partial molar volume of the complex is larger than the sum of the standard partial molar volumes of free FL and IgG-49. The binding of FL to IgG-49 expands the volume of the complex. Taking into account that FL is an amphiphilic probe and the volume change of transfer for amphiphilic solutes from hydrophilic environments to hydrophobic ones by the dehydration is positive [9], the volume expansion may be closely related to the large hydrophobicity around binding sites of FL in the IgG-49 molecule. On the other hand, the \(\Delta V_D^0\) value decreased with increasing temperature (data not shown). The fact suggests that pressure and temperature affect the dissociation reaction of FL and IgG-49 synergistically. The temperature dependence of the \(\Delta V_D^0\) values are probably attributable to the smaller thermal expansivity for partial molar volumes of free FL and IgG-49 due to electrostriction effect of water than that of the complex [6].

### 3.2. Effect of pressure on the interaction of IgG-49 with inhibitors

Inhibitors as well as a ligand affect the volume behavior of proteins, and the effect is different depending on the kinds of inhibitors. The volume change of the protein by anesthetics gives us useful information for one of key anesthetic phenomena: the pressure reversal of anesthesia. So, the effect of pressure on the interaction of IgG-49 with inhibitors was investigated from fluorescence intensity measurements. Here we chose two long-chain amphiphiles (C14OH and C13COOH) as nonspecific inhibitors and one homolog of FL (5-FLNH\(_2\)) as a specific inhibitor [3,10]. The IC\(_{50}\) value (the concentration that inhibits 50% fluorescence quenching) is micro-molar range for the former while nano-molar range for the latter. The effect of pressure on the binding of FL to IgG-49 in the absence and presence of the inhibitors is demonstrated in figure 2(a). Here the fluorescence quenching under atmospheric pressure as 100% quenching was adopted. The fluorescence quenching by binding of FL to IgG-49 in the absence of the inhibitors (curve 1) decreased with applying pressure, which well agrees with the results obtained from the anisotropy measurements. The fluorescence quenching in the presence of the inhibitors also decreased with pressure. However, the pressure dependence of the quenching in the presence of all the inhibitors was larger than that in the absence of the inhibitors, and 5-FLNH\(_2\) showed the largest quenching inhibition among the inhibitors.

The pressure dependence of the quenching in the absence and presence of the inhibitors was analyzed by the Johnson-Eyring equations for enzyme inhibitions [11,12]. The effect of pressure on
noncompetitive binding by nonspecific inhibitors to an enzyme at constant temperature and inhibitor concentration is written by the form

\[
\ln \left( \frac{Q_1}{Q_2} - 1 \right) = -p\Delta V_{\text{nsp}}/RT + \text{(Constant)}
\]

(4)

where \(\Delta V_{\text{nsp}}\) is the standard volume change of the enzyme by adding the nonspecific inhibitor. \(K_1\) is the equilibrium constant between the unfolded inactive fraction and the native fraction for the enzyme and was assumed to be constant in this study [3]. Hence,

\[
\ln \left( \frac{Q_1}{Q_2} - 1 \right) \approx -p\Delta V_{\text{nsp}}/RT + \text{(Constant)}
\]

(5)

On the other hand, the effect of pressure on competitive binding by specific inhibitors to an enzyme at constant temperature and inhibitor concentration is expressed as the similar form to the equation (5)

\[
\ln \left( \frac{Q_1}{Q_2} - 1 \right) = -p\Delta V_{\text{sp}}/RT + \text{(Constant)}
\]

(6)

where \(\Delta V_{\text{sp}}\) is the standard volume change of the enzyme by adding the specific inhibitor.

Figure 2(b) shows the Johnson-Eyring plots for three kinds of inhibitors. By using the equations (5) and (6) with the slopes of curves, the \(\Delta V_{\text{nsp}}\) values for C14OH and C13COOH were determined as \(-11.7\) and \(-4.5\) cm\(^3\) mol\(^{-1}\) and the \(\Delta V_{\text{sp}}\) value for 5-FLNH\(_2\) as \(-1.7\) cm\(^3\) mol\(^{-1}\), respectively. The \(\Delta V_{\text{nsp}}\) and \(\Delta V_{\text{sp}}\) values became negative, and the volume change by 5-FLNH\(_2\) was the smallest. The results indicate that the volume of IgG-49 shrinks in the presence of these inhibitors, and the effect of the specific inhibitor is small as compared with that of the nonspecific inhibitors. We considered the reason of the volume shrinkage of IgG-49 by the inhibitors. For the nonspecific inhibitors (C14OH and C13COOH), the volume shrinkage may be attributable to the hyrophilicity of IgG-49 surface and the rigidity of IgG-49 structure. Because IgG is a very hydrophilic protein and has relatively rigid structure, the action to IgG-49 corresponds to the reaction between amphiphile solutes with high hydrophobicity and a rigid hydrophilic protein. In such a case, the protein volume shrinks in the presence of the inhibitors. Similar results were found for the anesthetic and bovine serum albumin (BSA) systems [2,13]. The volume shrinkage of IgG-49 by binding of the nonspecific inhibitors means that IgG-49 does not cause the pressure reversal of anesthesia by binding of these inhibitors. Therefore, we can say that IgG as well as BSA does not seem to be relevant to a model protein for the molecular mechanism of anesthesia. The volume difference between C14OH and C13COOH may result from the

**Figure 2.** (a) Effect of pressure on fluorescence quenching of 10 nM FL and 5 nM IgG-49 at 25 °C in the absence and presence of inhibitors: (1) control, (2) C14OH (24 µM), (3) C13COOH (6 µM), (4) 5-FLNH\(_2\) (10 nM). (b) Johnson-Eyring plot for inhibitors at 25 °C: (1) C14OH (24 µM), (2) C13COOH (6 µM), (3) 5-FLNH\(_2\) (10 nM).

difference in binding number between them in addition to the difference in the binding mechanism [3]. In the case of the specific inhibitor (5-FLNH\(_2\)), the volume of IgG-49 shrinks very slightly by the addition of 5-FLNH\(_2\). This is opposite to the fact that the binding of FL to IgG-49 brings about slight volume expansion of IgG as revealed above. We speculate that the slight small shrinkage of IgG-49 by 5-FLNH\(_2\) may be caused by imperfect packing of 5-FLNH\(_2\) instead of FL in the binding sites in IgG-
taking into account of that the structure of anti-fluorescyl monoclonal antibodies with a strong affinity to FL remain relatively rigid on the ligand dissociation [14,15]. Although the volume change by the addition of 5-FLNH₂ is smaller than those by the long-chain amphiphiles, the effect of the former inhibitor is markedly significant when considering the difference in the effective concentration (i.e. IC₅₀ value) among them.

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