Evidence of allosteric conformational changes in the antibody constant region upon antigen binding

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Abstract

We have addressed the question of whether antigen binding induces a conformational change in the heavy chain constant (CH) domain of antibodies using staphylococcal protein A or streptococcal protein G as probes, since these proteins are known to bind to IgG domains such as CH1 and CH2–CH3 domains. Biosensor assays on interactions between these proteins and mouse IgG specific to (4-hydroxy-3-nitrophenyl)acetyl (NP) or their enzymatic fragments conducted in the presence or absence of the hapten, NP-ε-aminocaproic acid (NP-Cap), showed that the binding of IgG to these proteins was inhibited by the binding of NP-Cap. The results of isothermal titration calorimetry also revealed that the association constant for the interaction of protein A with IgG2b decreased by the addition of NP-Cap. These results suggested that antigen binding induced conformational changes in binding sites for protein G or protein A located at CH1 and CH2–CH3 domains, respectively.

Introduction

It has long been debated how the effector function of an antibody is induced upon antigen binding (1,2). The slow resolution of this issue has been due to the difficulty of obtaining evidence that antigen binding to combining sites in the Fv region (VH + VL domains) induces conformational changes in CH domains. Although many crystal structures of liganded and unliganded Fab or Fv fragments have been determined, the central question about the relationship between conformational change and effector function remains unanswered (3). Some studies of antigen–antibody interactions have suggested that the binding of small monovalent antigens induced only local changes in antigen-combining sites and resulted in no significant induction of effector activity via Fc. However, it has not been possible to distinguish whether these effects are mediated solely by aggregating antibody molecules or whether antigen-induced conformational change may be required as well.

Staphylococcal protein A (SpA) is a cell wall-bound pathogenic factor which exhibits tight binding to IgG molecules at sites different from antigen-combining sites and the biological properties of SpA have been studied in detail (4,5). This protein contains a tandem repeat of five highly homologous IgG-binding domains designated E, D, A, B and C, each of which includes ~58 amino acids (6). All these domains can bind to the Fc portion of IgG of various mammalian species (4) and can also bind to the Fv of the human VH3-related family (7). A complex between a human Fc fragment and the B domain of SpA (SpA-B) has been solved by X-ray diffraction analysis which revealed two anti-parallel α helices on SpA-B interacting at the interface of CH2 and CH3 of the Fc fragment (8). Recently, the crystal structure of the D domain of SpA (SpA-D) complexed with an Fab fragment of human IgM has also been reported (9). Streptococcal protein G (SpG) contains three homologous IgG-binding domains, referred to as C1, C2 and C3, and can bind to a human Fc and a mouse Fab fragment (10-14). The crystal structure revealed that the C3 domain of SpG (SpG-C3) interacts with CH1 of mouse IgG1 by forming an anti-parallel β sheet (13,14). From these studies, it is clear that SpA-B binds to CH2–CH3 and SpG-C3 to CH1 exclusively, and that there are no direct contacts between the...
domains of SpA or SpG and the combining sites on the Fv region except for those involving the V_{H}3-related family (9).

Using SpA and SpG as probes of microenvironmental changes on the IgG surface, we have attempted to address the question of whether antigen binding to the combining sites on Fv induces a conformational change in C_{H}3 domains. In this study, interactions of a series of mouse mAb specific to (4-hydroxy-3-nitrophenyl)acetyl (NP) hapten, IgG1 and IgG2b subclasses, and the enzymatic fragments of IgG1 with SpA or SpG were analyzed in the presence or absence of NP hapten. In addition, we prepared SpA-B and the C2 domain of SpG (SpG-C2) to eliminate possible steric effects from other domains on antigen-combining sites, and compared the results with those obtained using intact SpA or SpG. We employed surface plasmon resonance (SPR) biosensors and isothermal titration calorimetry (ITC), which are widely used for analysis of protein–protein interactions (15,16). Equilibrium analysis of interactions, which is outside the scope of Biacore data analysis from the viewpoint of mass transfer considerations, showed that interactions between SpA or SpG and IgG are weakened by hapten binding to antigen-combining sites, indicating that signals resulting from hapten binding induced a conformational change in C_{H}1 and C_{H}2–C_{H}3 domains.

**Methods**

**Antigen and antibody preparation**

NP was prepared from 4-hydroxy-3-phenylacetic acid (Nakarai, Kyoto, Japan), and coupled to ε-aminocaproic acid and glycine to generate the antigens, NP-Cap and NP-Gly (16). DNP-Gly was prepared using 2,4-dinitrofluorobenzene (Wako, Osaka, Japan). Immunization and purification of anti-NP mAb were carried out as described previously (17,18). The IgG mAb used in the present study were A6, N1G9, F8, B2 and C6 (IgG1), and 6L1 and 9L2 (IgG2b). A mouse–human chimeric IgM mAb (A6–hc_{1}) which possesses mouse V_{I}4 and V_{L}1 domains, identical to those of A6, and human μ chain constant domains (hc_{μ}) was produced by transfection of a construct to J558L cells (19). The purified mAb were dialyzed against PBS. The Fab and Fc fragments of IgG1 mAb were prepared by digestion with papain, and the F(ab')_{2} fragments were prepared by digestion with pepsin. The protein concentration was determined at an OD of 280 nm, using molar absorption coefficients of 1.19 × 10^{3} M^{-1} cm^{-1} for SpG-C2 and 8.64 × 10^{3} M^{-1} cm^{-1} for SpG-C2.

**SPR measurements**

The Biacore biosensor system, Biacore 2000 (Biacore, Tokyo, Japan), was used to measure biomolecular interactions. Immobilization of SpA and SpG on a CM5 sensor chip was performed as described previously (22). In the case of the immobilization of the SpA-B and SpG-C2 fragments, we used Biacore sensor chip SA surfaces with streptavidin pre-immobilized to dextran. The biotinylated SpA-B and SpG-C2 fragments were diluted to 100 nM in PBS containing 0.005% Tween 20 (buffer A) and were applied to the sensor chip surface at a rate of 5 μl/min over a 1-min period, which resulted in the capture of ~400 RU. In order to measure the association, various concentrations of free antibodies or antigen-bound antibodies were injected at a rate of 20 μl/min over a 3- or 6-min period. At the end of each experiment, the surface was regenerated with one 15-μl injection of a solution of 3 M guanidine hydrochloride containing 1 M acetic acid. All experiments were performed at 25°C.

The background RU values resulting from a change in the bulk refractive index were estimated by passing the antibody solution over the control lanes. Immobilized BSA or the carboxymethylated dextran surface only was used as a control. Only a small variation in RU was observed in the control lanes injected with the antibody solution. The antibody interactions with SpG and SpA were then examined by injecting antibody solutions over the lanes immobilized with SpG or SpA. The sensorgrams and RU_{eq} values used for Scatchard or Sips analysis were obtained by subtracting the background RU values from those observed with SpG or SpA.

Independent measurements were made at least 3 times and equilibrium values of RU (RU_{eq}) were analyzed using Scatchard and Sips equations (see below). The association constants (K_{a}) obtained from Sips plots were averaged and SD were calculated.

**ITC measurements**

ITC experiments were carried out on a Microcal MCS calorimeter interfaced with a microcomputer. All sample solutions in PBS were carefully degassed before the titrations, using the equipment provided with the instrument. The SpA-B solution (150 μM) was titrated into the antibody solution (6 μM) in the absence or the presence of NP-Cap (30 μM) using a 250-μl syringe. Each titration consisted of a preliminary 3-μl
injection followed by 19 subsequent 13-μl additions. All experiments were performed at 25°C. The heat for each injection was subtracted from the heat of dilution of the injectant, which was measured by injecting the solution of SpA-B into the experimental buffer. Each corrected heat was divided by the moles of SpA-B injected and was analyzed with the Microcal Origin software supplied by the manufacturer.

Results

First, interactions of mouse IgG with intact SpG and SpA or their isolated domains were analyzed using SPR. We mainly focused on the second domain of SpG, SpG-C2 and the fourth domain of SpA, SpA-B. SpG-C2 and SpA-B were synthesized, and their conformations were analyzed by circular dichroism. The analysis showed that both domains folded into their proper conformations in solution (data not shown). For the purpose of immobilization on the Biacore biosensor chips, biotin was introduced at the N-terminus of SpG-C2 or the penultimate position of SpA-B, since it was expected that there would be little effect on the conformation and on interaction with IgG. Biotinylated SpG-C2 and SpA-B were immobilized via streptavidin, while the intact SpG and SpA were conjugated directly onto the chip surfaces by the amine-coupling method. As described below, there was essentially no significant difference between intact proteins and the synthesized domains in terms of specificity of the interaction with IgG. This indicates that both fragments and intact proteins can be used for studies on the conformation of IgG, although the binding affinity for SpG-C2 was lower than that for intact SpG (see below).

Binding sites of SpG or SpA on mouse IgG

In order to elucidate the conformation of C\textsubscript{H} domains using SpG and SpA, it was necessary to determine whether the binding of these proteins occurs specifically to C\textsubscript{H} domains and not to V\textsubscript{H} or V\textsubscript{L} domains. In the present experiments, we used a group of anti-NP mAb, all of which were encoded by V\textsubscript{H}186.2 and V\textsubscript{L}1 genes. We selected one of the mAb, A6, and examined whether the products of V\textsubscript{H}186.2 and V\textsubscript{L}1 genes can bind to SpG or SpA. Since it has been shown that there is no binding of SpG and SpA to the constant region of human IgM (hC\textsubscript{M}) (9,23), we measured the interaction of these proteins with a chimeric antibody (A6–hC\textsubscript{M}) encoded by a construct consisting of a rearranged V\textsubscript{H}186.2–DFL16.1–J\textsubscript{L}2 gene derived from A6 and the hC\textsubscript{M} gene. Although both antibodies bound to NP-BSA (data not shown), A6–hC\textsubscript{M} showed no binding to SpG or SpA, in spite of the significant binding of intact A6 (Fig. 1A and B). On the basis of these results, we concluded that no binding sites for SpG and SpA were located in V\textsubscript{H}, V\textsubscript{L} and C\textsubscript{L} domains of the anti-NP mAb used in the present experiments.

For estimating the location of binding sites for SpG and SpA, we prepared Fab, F(ab')\textsubscript{2} and Fc fragments from IgG1, B2 and C6, and measured the interactions similarly to measurements with intact IgG (Fig. 1C and D). Fab and F(ab')\textsubscript{2} fragments showed significant binding to SpG, but the Fc fragment did not, indicating that the C\textsubscript{H}1 domain is the only domain responsible for the interaction of SpG or SpG-C2 with IgG1. On the other hand, only Fc fragments showed binding to SpA or SpA-B. Therefore, we considered that the binding site for SpG would be located at the C\textsubscript{H}1 domain, while that for SpA would be at the C\textsubscript{H}2–C\textsubscript{H}3 domains, similar to human IgG as shown by X-ray crystallography (8). A schematic representation of the interaction sites of mouse IgG1 with SpG and SpA is shown in Fig. 2.

Since Fab or Fc fragments of IgG2b were unavailable due to high sensitivity to proteolytic enzymes, we could not measure the interaction of these fragments with SpG or SpA.

Binding affinity between IgG1 or IgG2b and SpG or SpA

Interactions of IgG1 and IgG2b with SpG and SpA were first analyzed with the Scatchard equation:

\[
\frac{RU_{eq}}{C} = K_a \times RU_{eq} - K_a \times RU_{eq}
\]

where \(C\) is the free analyte concentration, \(RU_{eq}\) is the steady-state response and \(RU_{eq}\) is the total surface binding capacity. The equilibrium association constant \(K_a\) values were provided from the slope of the plots. However, the plots appeared not to be strictly linear, suggesting that the interactions were not homogeneous (Fig. 3A and B). Therefore, we analyzed the data using the Sips equation:

\[
\log\left[\frac{RU_{eq}}{RU_{eq} - RU_{eq}}\right] = a\log K_a + a\log C
\]

where \(a\) is the Sips heterogeneity coefficient. The \(RU_{eq}\) obtained from Scatchard analysis was used for calculation of \(\log\left[RU_{eq}/(RU_{eq} - RU_{eq})\right]\). The plots generated with the Sips equation were linear, and from these \(K_a\) and \(a\) were obtained (Fig. 3C and D). Since the \(a\) values were close to 1 (0.99–1.09) for IgG1 mAb, interaction of SpG or SpA with IgG1 seemed to be essentially homogeneous and the \(K_a\) values were in good agreement between these two plots. On the other hand, the \(a\) values for IgG2b mAb were in the range of 0.68–0.84, suggesting that the interactions were heterogeneous. A possible explanation for the heterogeneity of mouse IgG2b would be that there are binding sites for SpG or SpA in both the Fab and Fc regions, as in human IgG (7). Such a multivalent interaction would result in various species of complexes between IgG2b and SpG or SpA.

The resultant binding affinities from Sips equation, including Fab and Fc fragments, are summarized in Table 1. Two IgG2b mAb showed higher affinity to SpG and SpA than did IgG1. This may be due to the high affinity of binding of their Fab or Fc region to these proteins, as with human and rabbit IgG (24). The analysis also showed that \(K_a\) values differed slightly among these IgG1 mAb.

The finding that the Fab fragments bound to SpG with a 6-fold lower affinity, compared to the binding of intact mAb or the F(ab')\textsubscript{2} fragment, indicated that the latter were strengthened by a bivalent interaction using two Fab arms (avidity) (see Fig. 2). On the other hand, a 4-fold lower affinity of the binding of the Fc fragment to SpA compared to that of the intact mAb could not be explained in terms of changes in valency during the interaction and suggests that the conformational change occurred with the deletion of the Fab fragment by proteolysis.

The comparisons of \(K_a\) values between SpG or SpA and their respective synthesized domains, SpG-C2 or SpA-B, for the interactions with IgG and the proteolytic fragments, Fab, F(ab')\textsubscript{2} and Fc, are shown in Table 1. Although slight
differences in the magnitude of the $K_a$ values were observed between SpG and SpG-C2, binding tendencies in terms of specificity in interactions with IgG fragments as well as preferential binding to IgG2b rather than to IgG1 were quite similar to each other, suggesting that isolated domains behaved the same as in intact SpG or SpA and there was little steric hindrance from other domains towards SpG-C2 or SpA-B.

Effects of haptens on the equilibrium of interactions of IgG with SpG or SpA

Selected sensorgrams of the interaction of IgG2b (9L2) or IgG1 (C6 and F8) with SpG at a concentration of 0.1 μM in the presence of various concentrations of NP-Cap are shown in Fig. 4. In the cases of 9L2 and C6 [$K_a$ values for NP-Cap were $4.7 \times 10^6$ and $3.3 \times 10^7$ M$^{-1}$ respectively (18)], the RU$_{eq}$ values decreased with an increase in NP-Cap and reached a minimum value at concentrations of 1–10 μM, indicating that interactions between IgG and SpG were weakened by the binding of NP-Cap to the combining sites (Fig. 4A and B). Such an effect of NP-Cap on interactions with SpG was observed with all IgG1 and IgG2b so far studied. This effect was, however, largely dependent on the affinity of the antibodies to NP-Cap, e.g. in the case of F8 which has a $K_a$ for NP-Cap of $2.7 \times 10^5$ M$^{-1}$ (18), little effect was observed with the addition of 0.1 μM of NP-Cap, although a change became apparent at 10 μM of NP-Cap (Fig. 4C). A similar hapten effect

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Fig. 1. Sensorgrams of the binding of a mouse IgG1, A6, a mouse–human chimeric IgM, A6–hCm, and an Fab or Fc fragment to SpG or SpA. Sensorgrams of the binding of A6 and A6–hCm to SpG are shown in (A), and of binding to SpA in (B). Sensorgrams of binding of another IgG1, C6, and its Fab and Fc fragments to SpG are shown in (C) and to SpA in (D). A 200 nM solution of mAb or fragments was passed over both the chip-immobilized SpG or SpA. (A and B) A6 (continuous line) and A6–hCm (broken line). (C and D) Intact C6 (thick continuous line), F(ab')$_2$ (thin continuous line), Fab (dotted line) and Fc (broken line).

Fig. 2. Schematic diagram of interactions between mouse IgG1 and SpG or SpA.
was observed with NP-Gly, but not with DNP-Gly. The inhibition by NP-Cap was observed in the Fab or F(ab’)_2 fragments, similar to results seen with intact IgG1 or using biosensor chips immobilized with SpG-C2. Therefore, it is likely that the binding of NP-Cap to the antigen-combining sites induced the conformational change in C_ν1.

Table 1. $K_a$ values of anti-NP mAb with $\gamma_1$ isotype (N1G9, F8, B2, and C6), $\gamma_2b$ isotype (6L1 and 9L2), and the proteolytic fragments of B2 and C6 for the binding to the immobilized SpG, SpG-C2, SpA, and SpA-B

<table>
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<tr>
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<th>SpG</th>
<th>SpG-C2</th>
<th>SpA</th>
<th>SpA-B</th>
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<td>N1G9</td>
<td>$9.1 \pm 0.7 \times 10^6$</td>
<td>$4.1 \pm 0.1 \times 10^6$</td>
<td>$1.7 \pm 0.3 \times 10^6$</td>
<td>$3.5 \pm 0.1 \times 10^6$</td>
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<td>F8</td>
<td>$1.4 \pm 0.1 \times 10^7$</td>
<td>$8.3 \pm 0.1 \times 10^6$</td>
<td>$5.0 \pm 0.3 \times 10^6$</td>
<td>$7.2 \pm 0.2 \times 10^6$</td>
</tr>
<tr>
<td>B2</td>
<td>$1.1 \pm 0.2 \times 10^7$</td>
<td>$5.4 \pm 0.4 \times 10^6$</td>
<td>$3.8 \pm 0.2 \times 10^6$</td>
<td>$7.4 \pm 0.3 \times 10^6$</td>
</tr>
<tr>
<td>C6</td>
<td>$1.4 \pm 0.2 \times 10^7$</td>
<td>$3.3 \pm 0.4 \times 10^6$</td>
<td>$4.1 \pm 0.1 \times 10^6$</td>
<td>$4.3 \pm 0.2 \times 10^6$</td>
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<tr>
<td>B2 Fab</td>
<td>$1.7 \pm 0.1 \times 10^6$</td>
<td>$4.7 \pm 0.8 \times 10^5$</td>
<td>NDa</td>
<td>NDa</td>
</tr>
<tr>
<td>C6 Fab</td>
<td>$2.2 \pm 0.1 \times 10^6$</td>
<td>$5.0 \pm 0.1 \times 10^5$</td>
<td>NDa</td>
<td>NDa</td>
</tr>
<tr>
<td>C6 F(ab’)_2</td>
<td>$9.0 \pm 0.6 \times 10^6$</td>
<td>$4.4 \pm 0.8 \times 10^5$</td>
<td>NDa</td>
<td>NDa</td>
</tr>
<tr>
<td>B2 Fc</td>
<td>NDa</td>
<td>NDa</td>
<td>$1.0 \pm 0.2 \times 10^6$</td>
<td>$2.5 \pm 0.1 \times 10^6$</td>
</tr>
<tr>
<td>C6 Fc</td>
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<td>NDa</td>
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<td>$1.8 \pm 0.2 \times 10^7$</td>
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<td>9L2</td>
<td>$4.8 \pm 0.1 \times 10^7$</td>
<td>$2.5 \pm 0.3 \times 10^7$</td>
<td>$2.3 \pm 0.2 \times 10^7$</td>
<td>$1.4 \pm 0.1 \times 10^7$</td>
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</table>

The $K_a$ values were determined by Sips analysis in at least three separate experiments.

*aNot determined from the sensorgram because no increase in the RU was observed.
Sensorgrams for the binding of IgG2b or IgG1 mAb to SpA in the presence of NP-Cap are shown in Fig. 5. Similar to the interaction with SpG, binding to SpA was weakened by the addition of NP-Cap and the extent of this inhibition was largely dependent on the $K_a$ values of interactions between the mAb and NP-Cap. Although the Fc fragment can bind to SpA, no effect of NP-Cap on this binding was observed. These results indicated that the conformation of the C$_H$2±C$_H$3 domains had changed as a result of hapten binding.

The binding of NP-Cap resulted in a decrease in affinity, but not in a complete loss of interaction between IgG and SpG or SpA. In order to estimate the binding affinity of IgG to SpG or SpA, whose combining sites were saturated with NP-Cap, interactions of mAb in the presence of a molar excess of NP-Cap or NP-Gly were measured using sensor chip-immobilized SpG-C2 or SpA-B followed by analysis according to Scatchard and Sips equations. The $K_a$ values in the presence of hapten as well as the ratios of $K_a$ values in the presence and...
absence of hapten are shown in Table 2. A decrease in the affinity of SpG-C2 and SpA-B as a result of hapten binding was observed with all mAb, irrespective of subclass. The ratio of $K_a$ indicated that binding of NP-Cap or NP-Gly brought about a 31–64% decrease in affinity with SpG-C2 and a 28–67% decrease in affinity in the interaction with SpA-B.

The effect of hapten binding on the interaction of IgG with SpA-B was also examined using ITC. As shown in Fig. 6(A), an exothermic heat pulse was observed after each injection of SpA-B into 6L1 (IgG2b) in the absence or presence of NP-Cap, and the $K_a$ values were determined to be 1.58 (± 0.23) × 10⁶ and 1.06 (± 0.19) × 10⁶ M⁻¹ respectively. Solid lines in Fig. 6(B) represent theoretical curves calculated using thermodynamic parameters summarized in Table 3. A decrease in the binding affinity of IgG2b to SpA-B in the presence of NP-Cap was also evident in the ITC measurements and indicated that antibody binding to SpA-B is weakened by hapten addition, supporting the results of the Biacore experiments. However, the $K_a$ values obtained from ITC were ~2 orders of magnitude lower than those determined by SPR (Table 2). Although we have also attempted to analyze the interaction between C6 (IgG1) and SpA-B by ITC, the affinity was too low to determine the $K_a$ values. Such a discrepancy of affinity between SPR and ITC measurements was considered to have arisen from the difference in valency of the interactions measured by the two methods. Since SpA-B was immobilized on the sensor chip and IgG contained two binding sites for SpA-B, the interaction would proceed basically as divalent in the case of SPR, and the $K_a$ value would reflect avidity, whereas that was monovalent in the case of ITC, since both IgG and SpA-B were free in the solution.

**Effect of haptens on the kinetics of the interaction between IgG and SpG or SpA**

Figure 7 shows changes in RU values accompanying association and dissociation phases in the interaction of 9L2 (IgG2b) with immobilized SpA. Since the mol. wt of NP-Gly is ~270, its binding to 9L2 was expected to result in only a slight increase in RU or to be undetectable on the sensorgram. However, an injection of NP-Gly or NP-Cap during the dissociation phase resulted in a decrease in RU, while that of an unrelated hapten, DNP-Gly, showed no effect on the sensorgram. These NP haptens had a similar effect during the dissociation phase of the interaction between 9L2 and SpG (data not shown). These results indicate that antigen binding accelerated the dissociation of mAb from the complexes, as observed in other biological interactions on the Biacore sensor chip (25). On the other hand, the dissociation of IgG1 from SpA occurs too rapidly to detect any effect.

**Discussion**

**Binding sites of SpG or SpA on mouse IgG**

Fab-mediated binding to SpA has been observed with murine antibodies of $V_H$ gene products belonging to the $V_H3$ family (26). However, the anti-NP mAb used in the present experiments were expected to be unable to bind to SpG since the $V_H$ regions of these antibodies were encoded by the $V_H186.2$ gene which belongs to the J558 $V_H$ family and is located on chromosome 12. We confirmed this fact using a mouse-human chimeric antibody as well as Fab or Fc fragments from mouse IgG1 (Fig. 1 and Table 1). Results of the Biacore experiments showed that the Fab fragments could not bind to SpA or that the binding was under the detectable limits, indicating that the major binding site exists in the C1₂-C1₃ domains. In contrast, the binding sites for SpG were found to be different from those for SpA (27). X-ray and NMR studies showed that an Fab fragment of mouse IgG1 formed a complex with the SpG domain through the C1₁ domain (10–14). The amino acid segments 212–214 and 118–121 from SpA among mAb suggested that the conformations of the CH₁ domain and C H₂–CH₃ domains of these mAb were not identical with one another (Fig. 3 and Table 1). Since only

<table>
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<tr>
<th>mAb</th>
<th>Antigen</th>
<th>$K_a$ (6)</th>
<th>SpG-C2</th>
<th>Ratio²</th>
<th>SpA-B</th>
<th>Ratio²</th>
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<td>N1G9</td>
<td>NP-Cap</td>
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<td>NP-Cap</td>
<td>3.8 ± 0.4 × 10⁶</td>
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<td>NP-Cap</td>
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<tr>
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<tr>
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<td>0.64</td>
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Table 2. $K_a$ values of anti-NP mAb for the interaction with the immobilized SpG-C2 and SpA-B in the presence of antigen

The $K_a$ values were determined by Sips analysis in at least three separate experiments.

²The ratio was calculated by dividing the $K_a$ obtained in the presence of NP hapten by that observed in the absence of NP hapten, shown in Table 1.
the amino acid sequences in the \(V_\text{H}\) and/or \(V_\text{L}\) domains would be different in these mAb (18), we considered that the \(V_\text{H}\) domain structure influenced folding or inter-domain interactions, although Ig domains have been thought to fold independently on the basis of physicochemical measurements (28). This implies that SpG and SpA were capable of distinguishing the fine conformation of their \(C_\text{H}\) domains on various mAb even though they belong to same isotype.

In the presence of saturating amounts of NP haptens, the interaction between \(C_\text{H}\) and SpG was weakened by ~31–64%, indicating that NP binding to combining sites induced the conformational change in \(C_\text{H}\) domains. The three-dimensional structure of N1G9 in unliganded and liganded states showed major structural differences between these two states in amino acid residues of their complementarity-determining regions (CDR): the root-mean-square-deviation (r.m.s.d.) of the side chain of Lys58H is 3.37 Å and that of Tyr97H is 1.11 Å (29), suggesting that an adaptation had occurred in CDR upon complex formation. Similar movements of CDR amino acid residues induced not only in side chain but also in backbone atoms were reported to occur in other antigen–antibody complexes (3,30). Therefore, conformational change around antigen-combining sites accompanying antigen binding appears to be a common feature, although its magnitude depends largely on the particular antigen–antibody system in question. In addition to changes around combining sites, relatively large differences in the r.m.s.d. upon complex formation were also observed in the second framework segment (FW2) of the \(V_\text{H}\) domain (Pro41H–Gly44H), in the \(C_\text{L}\) domain (Gly152L–Gln157L) and in the \(C_\text{H1}\) domain (Thr186H–Ser203H). Although it was not yet certain whether the changes in the r.m.s.d. were related to the conformational change upon hapten binding, the observed change in the r.m.s.d. of the segment Thr186H–Ser203H in the \(C_\text{H1}\) domain was of particular interest since this region was shown to be involved in the interaction with SpG (13). IgG2b showed higher affinity in the interaction with SpG, whose interaction was also weakened by hapten binding. Since proteolytic fragments were unavailable with this isotype, the SpG binding sites were not identified.

SpA was shown to bind to Fc fragments, but not to Fab or F(ab')\(_2\) fragments (Table 1). This suggested that SpA bound to the \(C_\text{H2}\)–\(C_\text{H3}\) domains, as with human and rabbit IgG, and was considered to be a sensitive probe for detecting conformational change in this region. The decrease in the affinity to

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<th>Table 3. Thermodynamic parameters for the interaction between SpA-B and IgG2b (6L1)</th>
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All ITC measurements were carried out in PBS at 25°C and the SD were based upon two separate experiments.

Fig. 6. Typical ITC profiles of the binding of SpA-B to 6L1 in the presence or absence of NP-Cap. (A) (Lower) The 150 \(\mu\)M SpA-B solution was injected into the 6 \(\mu\)M 6L1 solution in the presence of 30 \(\mu\)M NP-Cap. (Middle) The 150 \(\mu\)M SpA-B solution was injected into the 6 \(\mu\)M 6L1 solution. (Upper) The SpA-B solution was injected into the experimental buffer. (B) The data points were obtained by integration of the peaks in (A), corrected for the dilution heat and plotted against the molar ratio (SpA-B/6L1). The data were fitted using a non-linear least-squares method. Filled circles are for the presence of NP-Cap and open circles are for the absence of NP-Cap.

Fig. 7. Effect of NP-Gly on the dissociation of 9L2 from the 9L2–SpA complex. A 200 nM solution of 9L2 in buffer A was passed over the immobilized SpA surface and a 200 nM solution containing NP-Gly (solid line) or DNP-Gly (broken line) was then applied at 285 s during the dissociation phase, which began at 180 s.
SpA-B with the addition of haptens, observed in both SPR and ITC measurements, showed that the signals resulting from hapten binding induced a conformational change in the C_{1r}2-C_{1r}3 regions which are rather far-removed from the antigen-combining site. X-ray crystallographic models of Fab fragments and intact IgG revealed that antibody molecules are highly flexible since the fine structural features are different in terms of the relative positions of Fab and Fc, Fab elbow angles, and inter-domain interactions (31,32). This flexibility may be related to signaling by an allosteric mechanism upon antigen binding.

In the present experiments, we used anti-NP mAb from C57BL/6 mice. Similar results were obtained with anti-hen egg lysozyme mAb from BALB/c mice (data not shown). Therefore, our observations were not specific to anti-NP mAb. However, there have been controversial reports on the effect of ligation on the interaction with SpA, which showed that antigen binding brought about an increase in affinity to this protein (4). This may be due to the multivalent nature of the interaction between SpA and IgG. Immune complexes containing oligomeric IgG would have higher avidity to SpA than would unliganded IgG. Recently, Kravchuk et al. reported that the binding affinity to SpA of two mouse anti-ferritin IgG2a mAbs was influenced by antigen binding: with one antibody, binding was enhanced, and with the other, it was reduced (33). These results were difficult to explain in view of steric hindrance by these large antigens. In the present study, a decrease in \( K_a \) values in the interaction with SpA or SpG was shown using NP-Cap which is a small, monovalent molecule whose presence did not result in the aggregation of mAb by gel chromatography using a TSK G3000SW_{XL} column (data not shown).

**Thermodynamics of SpA binding to mouse IgG2b**

Thermodynamic information on the interaction between SpA-B and mouse IgG2b was obtained by ITC (Table 3) (15). To our knowledge, the calorimetric analysis for the SpA binding to mouse antibodies has not been investigated in detail. The stoichiometry of binding (\( n \)) represents the number of binding sites on IgG2b and was slightly larger than the predicted value, 2.0. This could be due to either inaccurate estimation of SpA-B concentration or the existence of additional SpA binding sites on mouse IgG2b. As mentioned in Results, the \( K_a \) value obtained by ITC for the binding of SpA-B to 6L1 was lower than that obtained by Biacore (Tables 2 and 3). This was unexplainable in terms of differences in methodology and was considered to be due to the difference in valency of SpA-B, since SpA-B was free in the ITC measurements while immobilized in the Biacore measurements. In fact, the \( K_a \) value for binding of free SpA-B to immobilized 6L1 in the Biacore measurements was 1.78 \( \times \) 10^{5} M^{−1} (unpublished results), which is similar to that determined by ITC (Table 3). The enthalpy change \( (\Delta H) \) for the binding of SpA-B to mouse IgG2b was larger than that for the binding of domain E or domain Z of SpA to the Fc fragment of human mAb (34). In order to avoid the effect of \( \Delta H \) accompanying the dissociation of NP-Cap, ITC measurements were made with a molar excess of NP-Cap. However, we cannot exclude the possibility that the dissociation \( \Delta H \) of NP-Cap as a result of SpA-B binding would be involved in the calorimetric profiles. Even though this could occur, the difference of profiles in the presence or absence NP-Cap strongly supports the notion that antigen binding induces a change in equilibrium for the interaction between SpA-B and 6L1.

It has long been a matter of controversy as to whether antigen binding induces a conformational change in C_{1r} domains (32,35). As described above, we have succeeded in detecting the fine conformational change in C_{1r} domains as a result of antigen binding using SpG and SpA as probes using SPR and ITC. Since it was shown previously that the binding of small, monovalent haptens was not accompanied by a significant conformational change in IgG (36), the conformational change would be subtle if any. Therefore, precise analyses of antigen-antibody interactions were required. Our success was largely due to the development of new methodology, such as SPR and ITC, as well as to employment of SpG and SpA as probes of conformational changes. Even though these changes were undetectable by other methods, they were certainly recognized by Fc receptor-related proteins of bacterial origin.

**Functional implications**

At present, we have no direct evidence suggesting that conformational change in C_{1r}1 and C_{1r}2-C_{1r}3 domains, detected using SpG or SpA, is related to any of the effector functions of the Fc region, such as activation of the complement cascade or IgG transport through the membrane. Since the effect of NP-Cap on the SpG or SpA interaction was observed in IgG whose interchain disulfide bonds were reduced (data not shown), it is likely that the conformational change in the C_{1r} domains would not be related to complement activation. It was previously shown that the interaction between mouse Fc receptor (Fc\( R_{II} \)) and IgG1 was not affected by binding of DNP hapten (36). However, a study on the interaction between IgG and the neonatal Fc receptor, FcRn, which is thought to transport maternal IgG to the newborn, was of particular interest since the authors showed by X-ray crystallography that the C_{1r}2-C_{1r}3 interface is involved directly in the interaction (37). The interaction of FcRn with IgG was extremely sensitive to pH, occurring at pH 6, but not at pH 7.3. It would be of interest to examine whether antigen binding affects the interaction of IgG with FcRn.

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**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>CDR</td>
<td>complementarity-determining region</td>
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<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
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<td>NP</td>
<td>(4-hydroxy-3-nitrophenyl)acetyl</td>
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<tr>
<td>NP-Cap</td>
<td>( \epsilon )-NP-aminoacaproic acid</td>
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<tr>
<td>SpA</td>
<td>staphylococcal protein A</td>
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<td>SpA-B</td>
<td>B domain of SpA</td>
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<td>SpG</td>
<td>streptococcal protein G</td>
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<td>SpG-C2</td>
<td>C2 domain of SpG</td>
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<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
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<tr>
<td>r.m.s.d.</td>
<td>root-mean-square-deviation</td>
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Conformational changes in IgG upon antigen binding

References