Volume 328, number 1,2, 49–54 FEBS 12795 August 1993

^{13}C NMR study of the mode of interaction in solution of the B fragment of staphylococcal protein A and the Fc fragments of mouse immunoglobulin G

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Received 31 May 1993

The mode of interaction of the B domain (FB) of staphylococcal protein A and the Fc fragments of mouse immunoglobulin G (IgG) has been investigated by ^{13}C NMR spectroscopy. Mouse IgG1, IgG2a, and IgG2b proteins have been selectively labeled with ^{13}C at the carbonyl carbon of His, Met, Trp or Tyr residue and used to prepare the corresponding Fc fragments by limited proteolysis. Site-specific resonance assignments have been made for each of these Fc analogues. FB was reported to form two contacts (contact 1 and contact 2) with human Fc in the crystal [Biochemistry 20 (1981) 2361–2370]. Comparisons of the chemical shift data of the Fc fragments observed in the absence and presence of FB have led us to conclude that in solution contact 1 is responsible for the formation of the Fc–FB complexes.

Immunoglobulin G (mouse); Fc; Protein A (*Staphylococcus aureus*); ^{13}C NMR; Protein–protein interaction

1. INTRODUCTION

The Fc portion of immunoglobulin G (IgG), which is composed of two identical C_H2 domains and two identical C_H3 domains, promotes effector functions through interactions with complements or cellular receptors. The Fc portion is also known to interact with a variety of bacterial immunoglobulin-binding proteins. Protein A, which is a cell wall component of *Staphylococcus aureus*, is the most extensively studied bacterial IgG-binding protein.

The extracellular part of protein A contains a tandem of five highly homologous Fc-binding domains designated as E, D, A, B, and C. Each domain, which comprises about 60 amino acid residues, is known to bind to the Fc region of IgG molecules of various species and subclasses with varying affinities and pH dependencies [1–3]. The C terminal part is a cell wall binding domain designated as X, which does not bind to the Fc portion and comprises approximately 180 amino acid residues.

The three-dimensional structure of the B domain (FB) bound to the Fc fragment of pooled human IgG has been solved by an X-ray crystallographic analysis at 2.8 Å resolution [4]. It has been shown that FB makes two contacts with the Fc fragment in the crystal. In the Fc fragment, residues located at the interface between C_H2 and C_H3 domains contribute to contact 1, whereas contact 2 is formed by residues only from the C_H3 domain. Strong arguments have been presented for the assumption that contact 2, which involves a sulfate ion, is a crystal contact and in solution under physiological conditions only contact 1 is formed in the Fc–FB complex [4].

In a previous paper, we have shown that in solution (1) FB is composed of a bundle of three α-helices (Gln-10 – His-19, Glu-25 – Asp-37, and Ser-42 – Ala-55), and (2) all three helices are retained in the complex between FB and human IgG Fc [5]. This result is in marked contrast to that of the X-ray crystallographic study of the Fc–FB complex, which shows that the third helix does not exist [4]. We have therefore concluded that the mode of interaction in solution between FB and Fc is different from that in the crystal.

In order to obtain further information about the mode of interactions between protein A and a variety of Fc fragments, it is obviously necessary to obtain structural information from Fc–FB complexes *in solution*. A circular dichroism study by Sjöholm [6] indicated that aromatic amino acid residues including Trp are involved in the human Fc-protein A interaction. Fluorescence-quenching studies using rabbit IgG showed that tryptophan residue(s) on the Fc fragment is (are) located near the protein A-binding site [7,8]. An early ^1H NMR study detected no significant microenvi-

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Abbreviations: C_H2, C_H3, constant regions of the C-terminal half of the heavy chain of immunoglobulin; FB, B domain of staphyloccocal protein A; Fc, C-terminal half of the heavy chain of immunoglobulin; [M]Fc, Fc labeled with [1-^{13}C]Met; IgG, immunoglobulin G; NMR, nuclear magnetic resonance.

ronmental change surrounding aromatic residues of the Fc fragment of rabbit IgG upon protein A binding [7].

We have recently developed stable isotope aided NMR methods for the molecular structural study of IgGs. In a series of ^{13}C NMR studies, we have demonstrated that carbonyl ^{13}C resonances from the main chain can be useful probes for structural analyses of intact IgGs and their proteolytic fragments [9–13]. In the present paper, we report a ^{13}C NMR study of the interactions between FB and the Fc fragments of mouse IgG1, IgG2a, and IgG2b that are selectively labeled with ^{13}C at the carbonyl carbon of His, Met, Trp or Tyr residues (Table I). Each of these four residues is distributed in the regions corresponding to contact 1 and contact 2 in the crystal structure of the human IgG Fc–FB complex [4]. In addition, it is quite likely that His residues play a key role in the pH-dependent formation and dissociation of the Fc–FB complex. ^{13}C chemical shift data will be used to discuss the microenvironment surrounding the FB binding site of Fc in solution.

2. MATERIALS AND METHODS

2.1. *Materials*

L-[1-^{13}C]Tyr and L-[1-^{13}C, ^{15}N]Tyr were prepared as described previously [11,14]. DL-[α-^{15}N]His·2HCl, L-[^{15}N]Leu, L-[α-^{15}N]Lys·2HCl, and L-[^{15}N]Val were purchased from Isocommerz GmbH, Germany. L-[1-^{13}C]Met and [1-^{13}C]sodium pyruvate were purchased from Isotec, Inc $^{15}NH_4Cl$ was purchased from Shoko Co., Ltd. All other ^{13}C- and ^{15}N-labeled amino acids were purchased from ICON Service Inc., USA. The isotope enrichment is 95% or higher for each of these amino acids. β-Chloro-L-alanine, clostripain and papain were from Sigma. All other chemicals were of reagent grade and were used without further purification

2.2. *Preparation of FB*

A chemically synthesized gene for FB has been cloned and expressed in *Escherichia coli* by Saito et al. [15] FB was purified as described previously [16].

2.3. *Cell lines and stable-isotope labeling*

Switch variant cell lines 27–44 (IgG1), 27–13.6 (IgG2a), 27–1B10.7 (IgG2a(s)*), and 27–35.8 (IgG2b) [17,18] were generously provided by Professor L.A. Herzenberg and Dr. V.T. Oi. Hybridoma cells adapted to a serum-free medium (Nissui NYSF 404) were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Protocols for the preparation of antibodies selectively labeled with stable isotope(s) have been described previously [9–11]. For brevity, an IgG labeled with [1-^{13}C]Met will be designated as [M]IgG. Similar notations will be used for all labeled antibodies and their Fc fragments. Hereafter, the Fc fragments obtained from the switch variant IgG1, IgG2a [IgG2a(s)], and IgG2b will simply be referred to as Fc(γ1), Fc(γ2a), and Fc(γ2b), respectively*,**. For the assignment of the resonance originating from Tyr-435 of Fc(γ2b), which is followed by Tyr-436, an Fc(γ2b) analogue labeled with L-[1-^{13}C, ^{15}N]Tyr was prepared. For the assignments of resonances from His residue that is directly followed by another His residue (for example, His-435 of Fc(γ2a) that is directly linked by His-436), Fc analogues were prepared using with the medium containing a mixture of L-[1-^{13}C]His and L-[α-^{15}N]His at a ratio of 4.6 in place of unlabeled His. After cell growth, the cell supernatant was concentrated with a Millipore Minitan ultrafiltration system and then applied to an Affi-Gel protein A column (Bio-Rad). A typical yield was 10–40 mg of purified antibody/liter of the cell culture.

2.4. *Limited proteolysis of IgG proteins*

Fc fragments of the switch variant IgG1, IgG2a, IgG2a(s) and IgG2b antibodies were prepared by limited proteolyses as described previously [10]

2.5. *NMR measurements*

For the NMR measurements, the protein solutions were concentrated to a final volume of 2 ml in 5 mM phosphate buffer, pH 7.4, containing 0.2 M NaCl and 3 mM NaN_3 in 90% H_2O/10% D_2O unless otherwise stated. A 10-mm NMR sample tube was used with a final protein concentration of 0.2–0.4 mM. The pH was adjusted with HCl or NaOH. All pH values reported in this paper are uncorrected meter readings of NMR sample solutions made at 25°C with an electrode standardized by using H_2O buffers. NMR measurements were made on a Bruker AM 400 spectrometer. ^{13}C NMR spectra were recorded at 100 MHz with use of a WALTZ 16 composite pulse decoupling sequence. The free induction decay was recorded with 32K data points and a spectral width of 24,000 Hz. For resolution enhancement, the free induction decay was multiplied by the Gaussian window function prior to Fourier transformation. Chemical shifts are given in parts per million (ppm) from internal dioxane The probe temperature was 30°C.

Table I

Distributions of His, Met, Trp, and Tyr residues in Fc(γ1), Fc(γ2a), and Fc(γ2b)[a]

Fc(γ1)	Fc(γ2a)	Fc(γ2b)
His-285	His-285	His-285
His-310	His-292	His-292
His-429	His-310	His-310
His-433	His-429	His-386
His-435	His-433	His-429
His-436	His-435	Met-252
His-443	His-436	Met-314
Met-309	Met-252	Met-412
Met-358	Met-314	Trp-277
Met-368	Met-358	Trp-313
Met-398	Met-368	Trp-381
Trp-277	Met-373	Trp-417
Trp-313	Met-406	Tyr-296
Trp-381	Trp-277	Tyr-349
Trp-383	Trp-313	Tyr-391
Trp-417	Trp-381	Tyr-404
Tyr-349	Trp-417	Tyr-407
Tyr-391	Tyr-296	Tyr-435
Tyr-404	Tyr-349	Tyr-436
Tyr-407	Tyr-378	
	Tyr-391	
	Tyr-404	
	Tyr-407	
	Tyr-423	

[a] The numbering system used is based on human myeloma protein Eu [23].

* IgG2a(s) is a short-chain IgG2a variant antibody that lacks the entire C_H1 domain [7,25].

** It was confirmed by using carbonyl carbon resonances for His Met, Trp and Tyr as spectroscopic probes that no significant difference exists between the ^{13}C NMR spectra of the Fc fragments of IgG2a and IgG2a(s) ([10] and Kato et al., unpublished data). In the present work, the Fc fragment obtained from IgG2a(s) was used for all experiments.

3. RESULTS AND DISCUSSION

Table I shows the distributions of His, Met, Trp, and Tyr in Fc(γ1), Fc(γ2a), and Fc(γ2b) used in the present study as ^{13}C spectroscopic probes. The assignments of the Met and Trp resonances for Fc(γ1), Fc(γ2a), and Fc(γ2b) have already been reported in the previous papers [10,11]. In a similar way, on the basis of the results obtained by using a ^{13}C-^{15}N double labeling method [19], most of the His and Tyr resonances for Fc(γ1), Fc(γ2a), and Fc(γ2b) were assigned. Table II summarizes the assignments of the His and Tyr resonances of Fc(γ1), Fc(γ2a), and Fc(γ2b).

Deisenhofer [4] reported that Fc of human IgG makes two contacts (contact 1 and contact 2) with FB in the crystal. Contact 1 is contributed by the residues located on the interface between the C_H2 and C_H3 domains (Table III). On the other hand, contact 2 is formed by residues only from the C_H3 domain, i.e. 355, 362, 383, 384, 386–390, 411–413, 416, 419, and 421. In contact 2, a sulfate ion is involved, and Glu-388 of Fc and Asp-37 of FB are in close spatial proximity. Strong arguments have been presented for the assumption that contact 2 is a crystal contact and does not exist in solution under physiological conditions [4]. By contrast, a possibility has been suggested that FB is multivalent for human IgG and therefore contact 2 plays some role even under physiological conditions [20]. In the case of mouse IgGs, virtually no direct evidence has been presented that the C_H2-C_H3 interface region corresponding to contact 1 is responsible for the FB binding.

Table II

Carbonyl ^{13}C resonance assignments for His and Tyr residues in Fc(γ1), Fc(γ2a), and Fc(γ2b)[a]

Residue no.	Fc(γ1)	Fc(γ2a)	Fc(γ2b)
His-285	175.1	175.1	175.2
His-292	–[b]	172.6	172.6
His-310	176.6	176.5	176.6
His-386	–	–	173.9
His-429	173.8	173.8	173.9
His-433	175.5	175.7	–
His-435	171.8	171.5	–
His-436	172.4	172.2	–
His-443	173.5	–	–
Tyr-296	–	174.7	174.7
Tyr-349	173.5	172.9[c]	172.4
Tyr-378	–	171.4[c]	–
Tyr-391	172.7	172.5	172.6
Tyr-404	172.2	172.2	173.0
Tyr-407	173.4	173.4[d]	173.8
Tyr-423	–	175.5[d]	–
Tyr-435	–	–	174.5
Tyr-436	–	–	171.5

[a] Chemical shifts are given in ppm from internal dioxane at pH 7.3.
[b] Replaced by other amino acids.
[c] Assignments may be reversed for Tyr-349 and Tyr-378 of Fc(γ2a).
[d] Assignments may be reversed for Tyr-407 and Tyr-423 of Fc(γ2a).

Table III

Residues involved in human Fc contact 1 and corresponding residues in the mouse Fc examined in the present study[a]

IgG	252–254	308–312	433–436
Human IgG1	MIS	VLHQN	HNHY
Mouse IgG1	TIT	IMHQD	HNHH
Mouse IgG2a	MIS	IQHQD	HNHH
Mouse IgG2b	MIS	IQHQD	KNYY

[a] References: Deisenhofer [4]; Kabat et al. [24]. Residues are identified by the standard one-letter code.

In the previous papers, we have demonstrated that chemical shift data of carbonyl ^{13}C resonances are useful in mapping the antigen binding site [10,11]. We applied this method to the analysis of the Fc–FB interaction in solution.

Fig. 1 shows that the presence of FB significantly affects the titration curves of His-310 of Fc(γ1), Fc(γ2a), and Fc(γ2b), and His-433, His-435, and His-436 of Fc(γ1) and Fc(γ2a). Small but significant chemical shift changes are also observed for the resonances of His-429 of Fc(γ1), Fc(γ2a) and Fc(γ2b). The Fc-protein A complexes are known to dissociate by lowering pH. Ey et al. [21] reported that the pH ranges for elution from a protein A-Sepharose 4B column were 6.0–7.0, 4.5–5.0, and 3.5–4.0 for mouse IgG1, IgG2a and IgG2b, respectively. Fig. 1 also shows that the chemical shift differences observed in the presence and absence of FB disappear below these pH ranges. On the basis of these results we conclude that chemical shift changes induced by the addition of FB are the results of specific interactions between FB and Fc.

Chemical shift changes induced by the addition of FB were also examined by using carbonyl ^{13}C resonances for Met, Trp, and Tyr of the three kinds of Fc. Fig. 2 compares the ^{13}C NMR spectra of [M]Fc(γ2a) in the presence and absence of FB. Resonances originating from Met-252 and Met-314 were shifted up-field upon FB binding. No significant chemical shift change was observed for the other Met resonances.

The chemical shift changes observed for Fc(γ1), Fc(γ2a), and Fc(γ2b) by using His, Met, Trp, and Tyr resonances as spectroscopic probes are summarized in Fig. 3. Resonances showing large chemical shift changes (more than 0.1 ppm) by the formation of Fc–FB complex are concentrated in the interface region between the C_H2 and C_H3 domains for each of the Fc, i.e. positions 252, 310, 313, 314, 429, 433, 435, and/or 436 (Fig. 3). On the basis of these results, we conclude that in solution the region corresponding to contact 1 of human Fc–FB complex (Table III) is primarily responsible for the FB binding of the mouse Fc fragments examined.

The X-ray crystallographic study of human Fc has

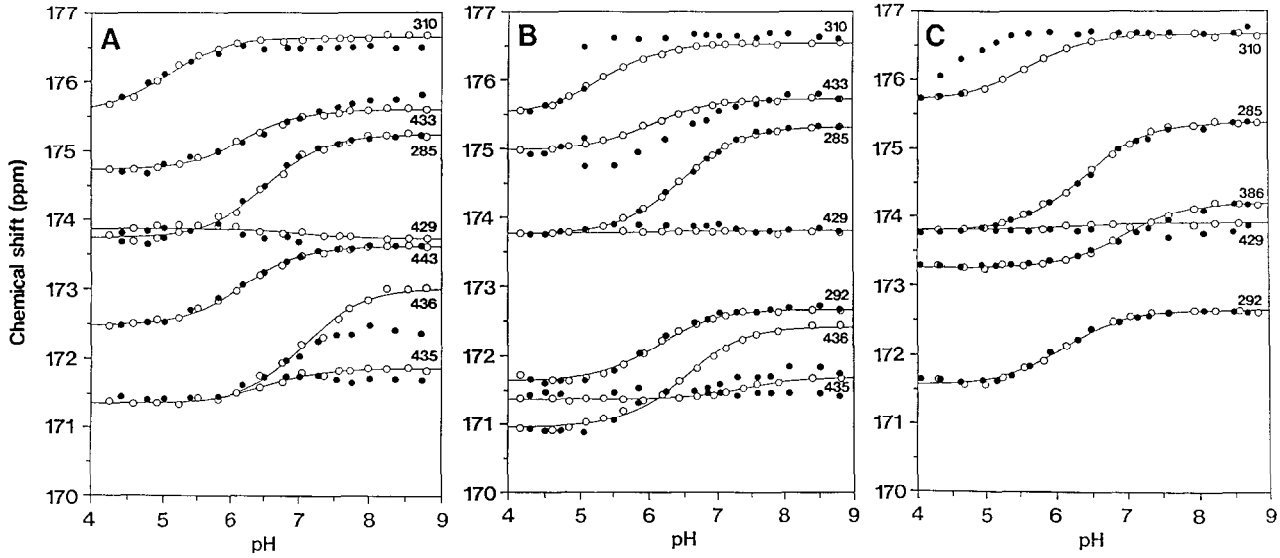

Fig. 1. The pH dependence of the chemical shift of the histidyl carbonyl ^{13}C resonances of (A) Fc(γ1), (B) Fc(γ2a), and (C) Fc(γ2b) in the absence (open circles) and presence (closed circles) of FB. The molar ratio of Fc/FB was 1.2.

shown that in contact 1, the imidazole ring of His-310 is buried whereas that of His-433 is still exposed to solvent [4]. This is consistent with the present NMR data, which show that in the case of Fc(γ2a), for example, the pK_a of His-310 was significantly lowered by FB-binding whereas that of His-433 was little affected (Fig. 1). We therefore suggest that the binding mode of Fc(γ2a) and FB is virtually identical to that in contact 1 in the crystal of the human Fc–FB complex. By contrast, resonances originating from Met-412 and His-386 of Fc(γ2b), both of which are located at the positions where contact 2 is formed in the human Fc–FB complex, did not show any significant chemical shift difference in the presence and absence of FB (Figs. 1 and 3). In addition, FB-binding induced no significant chemical shift changes for the resonances originating from Trp-381, Trp-383, and Trp-417 of Fc(γ1); these Trp residues are located in or near the region corresponding to contact 2 (Fig. 3). These results indicate that contact 2 does not exist in solution under physiological conditions. This is consistent with the arguments presented by Deisenhofer [4] on the basis of the crystal data of the human Fc–FB complex.

In the crystal, contact 2 is formed at the C-terminal segment of the second helix of FB and the residues from Asp-38 to Gln-41 that follow. We have performed the amide proton exchange experiment for the Fc(γ2a)–FB complex according to the procedure described in the previous paper [5]. On the basis of this result, we have concluded that in solution the structure of the third helix of FB, which does not exist in the crystal of the human Fc–FB complex, is also retained in solution in the Fc(γ2a)–FB complex as in the case of the human IgG Fc–FB complex (Gouda et al., manuscript in preparation). The present NMR data demonstrating the nonexistence of contact 2 in solution strongly suggest that the formation of contact 2 is responsible for the destruction of the third helix in the crystal.

Our conclusions from this work are summarized as follows: (1) in solution, mouse Fc(γ1), Fc(γ2a), and

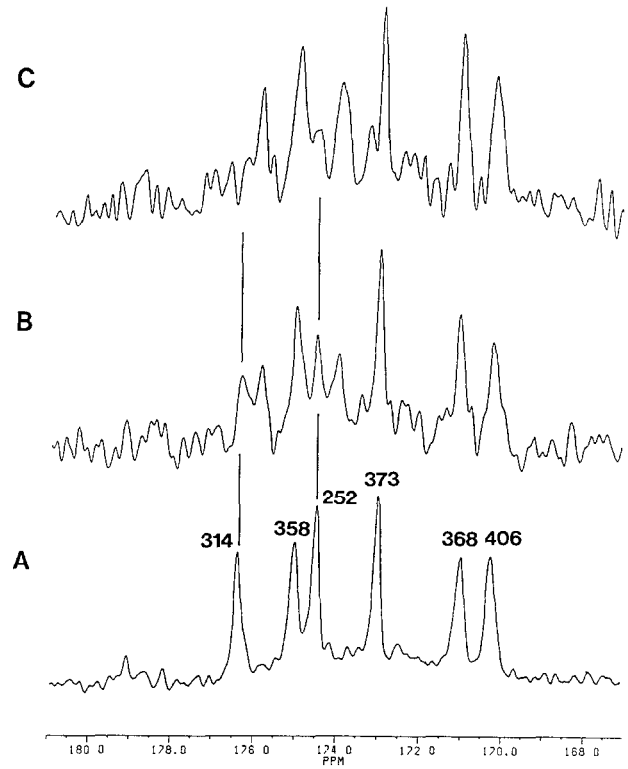

Fig. 2 100-MHz ^{13}C NMR spectra of [M]Fc(γ2a) in the presence of varying concentrations of FB The molar ratios of Fc/FB were (A) 10:0, (B) 10:8, and (C) 10:20. The concentration of the Fc protein and the pH of the sample solutions were 0.2 mM and 7.0, respectively.

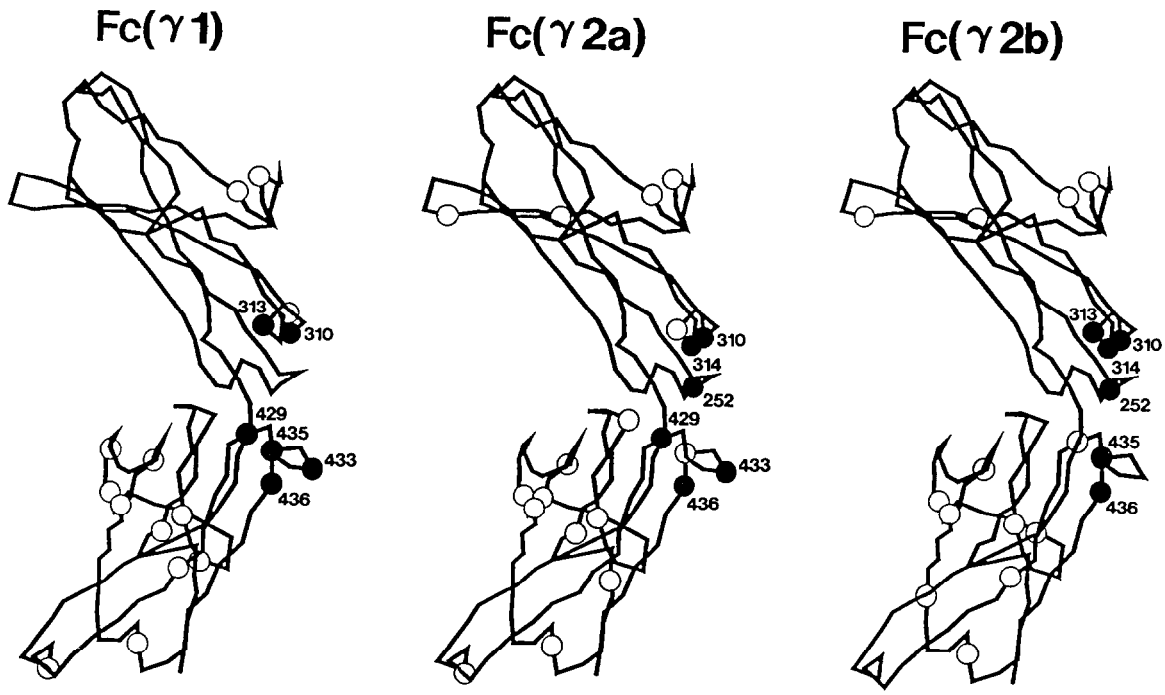

Fig. 3. Mapping of the FB binding site on Fc(γ1), Fc(γ2a), and Fc(γ2b) by use of chemical shift changes of the carbonyl ^{13}C resonances of His, Met, Trp, and Tyr induced by addition of FB as spectroscopic probes. Chemical shift changes were observed at pH 8.8, 7.0, and 6.5 for Fc(γ1), Fc(γ2a), and Fc(γ2b), respectively. Positions of the residues showing chemical shift changes larger (closed circles) and less (open circles) than 0.1 ppm are presented on the crystal structure of the human IgG Fc fragment deposited in Brookhaven Protein Data Bank [4]. Although four Tyr resonances have not been assigned in the case of Fc(γ2a) as described in the text, both of Tyr resonances originating from the Tyr-Ser dipeptide (Tyr-407 and Tyr-423) were shifted less than 0.1 ppm in the presence of FB and therefore are included as such in the figure.

Fc(γ2b) bind to FB at almost identical regions on the interface between the C_H2 and C_H3 domains, which correspond to contact 1 in the crystal of the human Fc–FB complex, and (2) contact 2 forming on the C_H3 domain observed in the crystal of the human Fc–FB complex does not exist in solution.

One might expect that ionization of His residue(s) involved in contact 1 triggers the dissociation of the Fc–FB complex. In the case of Fc(γ2a) and Fc(γ2b), the dissociation of the complex occurs in the same pH range as the ionization of His-310 does (Fig. 1). Especially, His-310 is the only His residue of Fc(γ2b) involved in contact 1 (Table III). We suggest that His-310 is one of the strongest candidates responsible for the dissociation of the Fc(γ2b)–FB complex. On the other hand, Fc(γ1) dissociates from FB at higher pH than Fc(γ2a) does, although the pK_a values of the His residues in contact 1 except His-436 are almost identical between the two subclasses. Schneider et al. [22] have shown that hybrid mouse IgGs, which possess the N-terminal half of the C_H2 domain of γ1 and the C-terminal half of the C_H2 domain and the entire C_H3 domain of γ2a, do not bind protein A at pH 7.0. On the basis of this result, they suggested that differences in protein A binding between mouse IgG1 and IgG2a are due to amino acid substitutions at positions 252 (Thr → Met) and 254 (Thr → Ser). We suggest that, in the case of mouse IgG1, protonation of titratable groups in Fc(γ1) and/or FB taking place prior to the protonation of His-310 induces the dissociation of the Fc(γ1)–FB complex.

The ^{13}C NMR approach presented in this work will be useful for studying the interactions between IgG and a variety of other bacterial IgG-binding proteins including streptococcal protein G.

Acknowledgements. We thank Professor L.A. Herzenberg, Stanford University, and Dr. V.T. Oi, Becton Dickinson Immunocytometry systems, for generously providing us with the switch variant cell lines used in the present study. We are grateful to Dr. A. Saito and Dr. M. Sato, Kyowa Hakko Kogyo Co., Ltd., for generously providing us with *E.coli* strain W3110 containing the expression plasmid for FB. We thank Professor H. Yamada and Dr. T. Nagasawa for letting us use their facility and for their help in the preparation of β-tyrosinase. We thank Dr. H. Torigoe for his useful discussion. We also thank Mrs. A. Takahashi for her technical assistance. Financial supports by grants from the Ministry of Education, Science, and Culture of Japan (62870089, 63430022 and 04771899), and by Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency are gratefully ackowledged. K.K. is the recipient of a Terumo Life Science Foundation research award.

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