

Disulfide Linkage Controls the Affinity and Stoichiometry of IgE Fcε3–4 Binding to FcεRI*

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James Hunt, Rebecca L. Beavil, Rosaleen A. Calvert, Hannah J. Gould, Brian J. Sutton,
and Andrew J. Beavil‡

From the Randall Division of Cell and Molecular Biophysics and the Division of Asthma, Allergy and Lung Biology,
King's College London, Guy's Campus, London SE1 1UL, United Kingdom

IgE antibodies cause long-term sensitization of tissue mast cells and blood basophils toward allergen-induced cross-linking and triggering of allergic inflammation. This persistence of IgE binding is due to its uniquely high affinity for the receptor FcεRI and in particular its slow rate of dissociation once bound. The binding interface consists of two subsites, one contributed by each Cε3 domain of IgE Fc in a 1:1 complex. We have investigated the contributions of Cε3 disulfide linkage and glycosylation to the kinetics and affinity of binding of an Fc subfragment (Fcε3–4) to a soluble receptor fragment (sFcεRIα). In contrast to IgG Fc where deglycosylation abrogates receptor binding activity, the removal of the N-linked carbohydrate at Asn-394 in Fcε3–4 only reduces binding affinity by a factor of 4, principally because of a faster off-rate. Removal of the inter-heavy chain disulfide bond unexpectedly resulted in a fragment with a much faster off-rate and the potential to form a complex with a 2:1 stoichiometry (sFcεRIα:Fcε3–4). This permitted the determination of the affinity of a single, natively folded Cε3 domain for the first time. The low affinity $K_a \approx 10^5\text{--}10^6\text{ M}^{-1}$, similar to that determined previously for an isolated and partially folded Cε3 domain, demonstrates that substantial reduction in affinity can be achieved by preventing the engagement of one of the two Cε3 domains. Recent structural data indicate that conformational change in IgE is required to allow both Cε3 domains to bind, and thus an allosteric inhibitor that prevents access to the second Cε3 has the potential to reduce the ability of IgE to sensitize allergic effector cells.

Immunoglobulin E (IgE)¹ is the antibody class that plays a central role in the allergic response (1). Mast cells and ba-

sophils express a high-affinity receptor for IgE, FcεRI, and it is the cross-linking of receptor-bound IgE on these cells by multivalent allergens that triggers the immediate release of pre-formed inflammatory mediators. The affinity of IgE for FcεRI is uniquely high among Ig-receptor complexes, two to five orders of magnitude higher than that of IgG for its receptors FcγRI-III (2). This is due principally to a very slow off-rate. The half-life of IgE bound to FcεRI is hours (3) compared with only minutes for IgG bound to its receptors (4). Inhibition of IgE binding to FcεRI or at least modulation of its binding kinetics is a potential therapeutic strategy.

IgE Fc binds to the receptor with a 1:1 stoichiometry (5) using both Fc heavy chains (6). The crystal structure of the complex between the IgE fragment, Fcε3–4 (a homodimer of ε-chains consisting of the Cε3 and Cε4 domains), and a soluble fragment of the IgE-binding α-chain of the receptor, sFcεRIα (6), revealed the precise details of the involvement of the two Cε3 domains in the complex. The extensive binding interface consists of two subsites, one contributed by each Cε3 domain, and thus the maintenance of the dimeric site might be expected to be important for high-affinity binding. The Cε3 domain also contains an attachment site (Asn-394) for N-linked carbohydrate that is conserved in other antibody classes (e.g. Asn-297 in the Cγ2 domain of IgG Fc). Although the crystal structure did not reveal any contact with carbohydrate (6), an indirect effect of glycosylation upon receptor binding via stabilization of the polypeptide conformation cannot be ruled out. Indeed, an isolated Cε3 domain expressed in *Escherichia coli* and lacking carbohydrate was found to be only partially folded (7) and NMR analysis of the same fragment indicates that it may have a molten-globule-like structure (8).

The aim of this study was to determine the contributions of these two factors, dimerization and glycosylation of the Cε3 domains, to the kinetics and affinity of FcεRI binding. We have analyzed these effects using four variants of the Fcε3–4 fragment: (i) disulfide-linked and glycosylated (Fcε3–4); (ii) lacking only the disulfide link (*red*Fcε3–4); (iii) lacking only glycosylation (*degly*Fcε3–4); and (iv) lacking both structural features (Fcε3–4ΔC). Fcε3–4 lacks the Cε2 domains of the complete IgE Fc, but we have shown previously that not only does this fragment display a 1:1 stoichiometry of binding (9), it also retains full binding affinity (10), although the kinetics of binding are affected (3). It has been reported previously that deglycosylation of IgE has little effect upon receptor binding (11)

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‡ To whom correspondence should be addressed: Randall Division of Cell and Molecular Biophysics, New Hunt's House, King's College London, Guy's Campus, London SE1 1UL, United Kingdom. Tel.: 44-20-78488064; Fax: 44-20-78486410; E-mail: andrew.beavil@kcl.ac.uk.

¹ The abbreviations used are: IgE, immunoglobulin E; SPR, surface plasmon resonance; CD, circular dichroism; MS, mass spectrometry; Cε2, Cε3, and Cε4, second, third, and fourth constant region domains of the IgE ε-heavy chain, respectively; Fcε3–4, mammalian-expressed homodimer of the third and fourth constant region domains of the IgE ε-heavy chain disulfide linked at the N terminus by Cys³²⁸ and glycosylated at Asn³⁹⁴; *degly*Fcε3–4, as Fcε3–4 but enzymatically deglycosylated; *red*Fcε3–4, as Fcε3–4 but with the disulfide linkage at the N terminus removed by partial reduction with dithiothreitol; Fcε3–4ΔC,

E. coli expressed homodimer of the third and fourth constant region domains of the IgE ε-heavy chain with a Cys-Ser mutation at position 328; IgE Fc, dimer of the second, third, and fourth constant region domains of the IgE ε-heavy chain; Cγ2 and Cγ3, second and third constant region domains of the IgG γ-heavy chain; sFcεRIα, the soluble fragment of the high-affinity IgE receptor FcεRI α-chain.

and that *E. coli* expressed fragments retain high-affinity receptor binding activity (12). This is in contrast to the behavior of IgG where the loss of carbohydrate from the Fc destroys receptor binding altogether (13). Here we report the first full kinetic characterization of a fully folded, carbohydrate-free IgE Fc subfragment. We show that glycosylation makes a minor contribution to its affinity for Fc ϵ RI. Furthermore, irrespective of glycosylation, we show that a loss of the inter-heavy chain disulfide bond has a more profound effect, generating, remarkably, a fragment that can bind two molecules of receptor. This unexpected stoichiometry of complex formation enabled us to determine for the first time the affinity of a single folded C ϵ 3 domain for receptor. The result, an affinity 10^3 – 10^4 times lower than native and comparable to that of the isolated and partially unfolded C ϵ 3 domain (7, 8), has implications for the development of inhibitors of the IgE–Fc ϵ RI interaction.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The production and purification of recombinant human Fc ϵ 3–4 (residues 328–547), sFc ϵ RI α (residues 1–176), and IgG4–Fc (sFc ϵ RI α)₂ fusion protein from NS-0 cells has been described previously (14, 15). A further recombinant Fc ϵ 3–4 fragment with the C328S mutation was made in *E. coli*. This construct, Fc ϵ 3–4 Δ C, was produced by PCR using an IgE Fc cDNA clone isolated from U266 cells (16) and was presented in pSC213 (17). The PCR fragment was subcloned into the NdeI and BamHI sites of the pET5a (EMD Biosciences Inc., Madison, WI) *E. coli* expression vector. The integrity of the clone was verified by DNA sequencing. Recombinant Fc ϵ 3–4 Δ C was expressed in the *E. coli* host strain BL21 (DE3). Inclusion body protein was extracted from cell pellets using a procedure adapted from Bohmann and Tjian (18) and refolded according to the method of Taylor *et al.* (19). We have previously described in detail the production and purification of an IgE Fc domain using these methods (7).

Deglycosylation of Fc ϵ 3–4—Native deglycosylation of mammalian Fc ϵ 3–4 was carried out using peptide *N*-glycosidase F according to the manufacturer's instructions (New England Biolabs, Beverly, MA). Material was then re-purified by size-exclusion chromatography on a Superdex S75 gel filtration column (Amersham Biosciences). Deglycosylation was assessed by SDS-PAGE and a combination of Coomassie blue and Schiff's staining. Complete deglycosylation of Fc ϵ 3–4 was also confirmed by MS.

CD Spectroscopy—The folded status of each protein was examined by CD as described previously (14). CD measurements were performed on a Jobin-Yvon CD6 spectrophotometer (Longjumeau, France) calibrated for wavelength and ellipticity using *D*-10-camphorsulfonic acid. Samples were analyzed in cylindrical quartz cells of 0.05-cm path length in the concentration range of 100–500 μ g/ml in 20 mM sodium phosphate, 50 mM sodium fluoride, pH 7.4, at 20 °C in a thermostated cell holder. Spectra were recorded in 0.2-nm steps with an integration time of 4 s and corrected by subtraction of the solvent spectrum obtained under identical conditions. The units of $\Delta\epsilon$ are $\text{M}^{-1} \text{cm}^{-1}$ per backbone amide unit.

Kinetics of Fc ϵ 3–4 Fragments Binding to Fc ϵ RI—The binding of all of the fragments to IgG4–Fc(sFc ϵ RI α)₂ fusion protein was measured by SPR. All of the experiments were performed at 24 °C on either a Biacore 1000, 2000, or 3000 instrument (Biacore Int. SA, Switzerland). Methods and kinetic analysis have been described previously (3, 15, 20). In these experiments, coupling density was typically restricted to 500 response units, flow rate of 20 μ l/min, and exposure time to analyte of 360 s.

Analytical Ultracentrifugation—The interaction between sFc ϵ RI α and the various Fc ϵ 3–4 fragments was studied by sedimentation equilibrium in an Optima XL-A analytical ultracentrifuge (Beckman Coulter Inc., Fullerton, CA) as described previously (14). Samples were dialyzed into phosphate-buffered saline with 0.05% azide and mixed in molar ratios of 1:1, 2:1, and 1:2 sFc ϵ RI α :Fc ϵ 3–4 with overall loading corresponding to an absorbance $A_{280} \sim 0.6$. The mixtures were spun at 4 °C at rotor speeds of 8000, 11,000, and 14,000 r.p.m. until equilibrium was reached. Data were collected as an average of 25 A_{280} measurements at a radial spacing of 0.001 cm. The equilibrium data for the mixtures were then simultaneously fitted to a range of models using the experimentally determined buoyant molecular masses of Fc ϵ 3–4 ($14,000 \pm 300$), Fc ϵ 3–4 Δ C ($14,115 \pm 480$), and sFc ϵ RI α ($10,200 \pm 400$) from which the association constants were derived. To study the reduced Fc ϵ 3–4 species, the minimum level of dithiothreitol required to

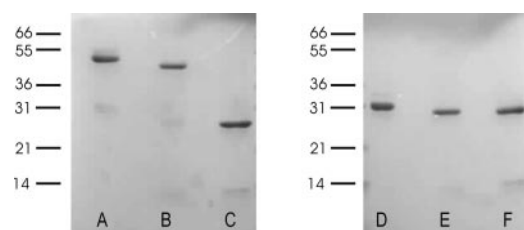


FIG. 1. **SDS-PAGE analysis of Fc ϵ 3–4 fragments.** Non-reducing (left panel) and reducing (right panel) 15% polyacrylamide gels. A–F, Fc ϵ 3–4 (NS-0) (A and D), deglyFc ϵ 3–4 (NS-0) (B and E), and Fc ϵ 3–4 Δ C (*E. coli*) (C and F).

reduce the interchain disulfide bond was determined by observing the change in apparent molecular weight by SDS-PAGE for a range of dithiothreitol concentrations. The concentration of dithiothreitol used (0.4 mM) to break the inter-heavy chain bridge was far lower than that required to break intradomain bridges (between 2 and 10 mM) (21). Nitrogen-purged ultracentrifugation cells containing mixes of Fc ϵ 3–4 and sFc ϵ RI α were then prepared as described with the addition of dithiothreitol to the phosphate-buffered saline-azide immediately prior to loading. Maintenance of the reduced form of Fc ϵ 3–4 was confirmed by non-reducing SDS-PAGE following the centrifuge run.

RESULTS

Expression and Purification of Fc ϵ 3–4 and Fc ϵ 3–4 Δ C—Fc ϵ 3–4 with N-terminal residue Cys-328 was expressed in mammalian NS-0 cells (14), and Fc ϵ 3–4 Δ C containing the C328S mutation was expressed in *E. coli*. Therefore, Fc ϵ 3–4 is glycosylated and is a covalent dimer (Fig. 1, lanes A and D), whereas Fc ϵ 3–4 Δ C is unglycosylated and unable to form the inter-heavy chain disulfide bridge. The refolded Fc ϵ 3–4 Δ C was purified by size-exclusion chromatography and confirmed as monomeric by SDS-PAGE (Fig. 1, lanes C and F), but it behaved as a monodisperse, non-covalent dimer by analytical ultracentrifugation (data not shown) and analytical gel filtration chromatography (Fig. 2). The integrity of the *E. coli* product was checked by MS, which yielded a molecular mass of 24,559 Da within 1 Da of the theoretical value (24,558). The slower migration on SDS-PAGE for Fc ϵ 3–4 Δ C non-reduced (Fig. 1, lane C) and fully reduced (lane F) was indicative of the formation of the intradomain disulfide bonds during the refolding process.

Deglycosylation of Fc ϵ 3–4—Deglycosylation of the NS-0-expressed Fc ϵ 3–4 fragment (deglyFc ϵ 3–4) was assessed by Schiff's staining and the change in apparent molecular weight on SDS-PAGE (Fig. 1, lanes B and E). Complete deglycosylation was confirmed by further peptide *N*-glycosidase F digestion under denaturing conditions and the release of no further sugar residues as detected by MS.

Gel Filtration Chromatography—The three fragments, Fc ϵ 3–4, deglyFc ϵ 3–4, and Fc ϵ 3–4 Δ C, were run sequentially on a gel filtration column as shown in Fig. 2. The two disulfide-linked dimers behaved similarly, but the retention time of Fc ϵ 3–4 Δ C was ~ 0.5 min longer, indicating that, although it too behaved as a (non-covalent) dimer, there was a discernable reduction in its effective volume. This suggests that it forms a somewhat more compact structure. Because deglyFc ϵ 3–4 ran identically to Fc ϵ 3–4, it appears to be the loss of the disulfide bond rather than the carbohydrate that is the cause of this conformational difference.

CD Spectroscopy—The CD spectra of Fc ϵ 3–4, deglyFc ϵ 3–4, and Fc ϵ 3–4 Δ C (Fig. 3) were all typical of a folded protein consisting principally of β -structure and in agreement with data previously published for Fc ϵ 3–4 fragments (10, 14, 20). This indicates that neither the refolding of the protein expressed in *E. coli* nor deglycosylation of the NS-0 material affected the folding of the fragment.

Surface Plasmon Resonance Analysis—A comparison of rep-

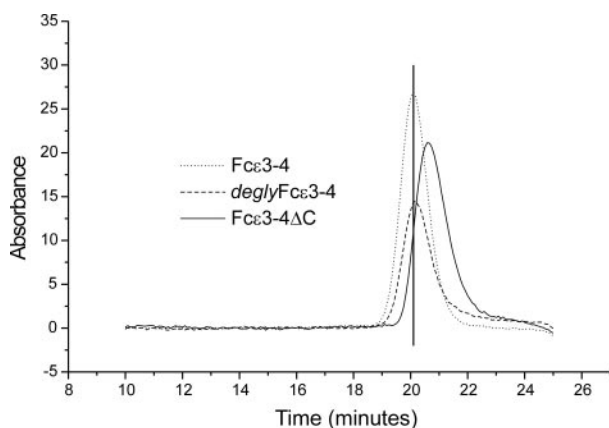


FIG. 2. Gel filtration elution profiles of the Fcε3-4 fragments. Superdex S200 HR column run at 0.75 ml/min in 500 mM Tris, pH 7.2, plus 250 mM NaCl.

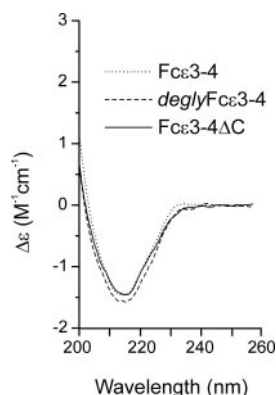


FIG. 3. Circular dichroism spectra of the Fcε3-4 fragments. Data were acquired at 20 °C in a 0.5-mm cell at a protein concentration of 0.5 mg/ml.

representative sensorgrams for the three Fcε3-4 species (Fig. 4) shows that there are major differences between their binding properties for receptor (immobilized on the biosensor surface as a fusion protein IgG4-Fc(sFcεRIα)₂), especially in their off-rates. *deglyFcε3-4* (Fig. 4B) clearly dissociates faster than Fcε3-4 (Fig. 4A), and for Fcε3-4ΔC, the off-rate is faster still (Fig. 4C). As we found in our previous studies of Fcε3-4 fragments (15, 20), only a biphasic model could satisfactorily fit the observed binding curves for Fcε3-4 and *deglyFcε3-4*. Representative curve fits and residuals are shown in Figs. 4, A and B, and the resulting kinetic parameters are summarized in Table I. Although the on-rates (k_{a1} and k_{a2}) for Fcε3-4 and *deglyFcε3-4* are very similar, the chief component of the off-rate, k_{d2} , is faster for the latter protein, leading to a 4-fold lower affinity constant ($K_{a2} = 4.92 \times 10^8 \text{ M}^{-1}$ for Fcε3-4 *c.f.* $K_{a2} = 1.14 \times 10^8 \text{ M}^{-1}$ for *deglyFcε3-4*) for the principal binding component. This difference is accentuated by the fact that the contribution of the first (lower affinity K_{a1}) component of the fit (given by the R_1/R_0 ratio, Table I) is higher for *deglyFcε3-4* (38%) than for Fcε3-4 (15%).

Kinetic analysis of the Fcε3-4ΔC fragment was possible with a 1:1 model of association. Representative curve fits and residuals are shown in Fig. 4C, and the resulting kinetic parameters are summarized in Table I. The rate of dissociation of this fragment is over two orders of magnitude faster than that of the major component of the Fcε3-4 biphasic analysis ($k_{d2} = 6.26 \times 10^{-4} \text{ s}^{-1}$ for Fcε3-4 *c.f.* $k_d = 6.63 \times 10^{-2} \text{ s}^{-1}$ for Fcε3-4ΔC). The rate of association is also faster however, leading to only an order of magnitude difference in overall affinity ($K_{a2} = 4.92 \times 10^8 \text{ M}^{-1}$ for Fcε3-4 *c.f.* $K_a = 4.17 \times 10^7 \text{ M}^{-1}$ for Fcε3-4ΔC).

Sedimentation Equilibrium Analysis—We previously studied the binding of sFcεRIα to NS-0-derived Fcε3-4 in the analytical ultracentrifuge and found that it formed a single species with 1:1 stoichiometry and an affinity too high to measure by this technique (9). As observed in SPR experiments, Fcε3-4ΔC lacking both carbohydrate and the interchain disulfide bond behaved very differently under the same conditions (Fig. 5, A and B). The data were collected at three different molar ratios (1:1, 2:1, and 1:2 sFcεRIα:Fcε3-4ΔC), and each curve was fitted to three different binding models: a simple 1:1 interaction; a mixture of 1:1 and 2:1 (sFcεRIα:Fcε3-4ΔC); and a solely 2:1 interaction. It is clear from the curve fits and residual plots (Fig. 5A) that a 1:1 sFcεRIα:Fcε3-4ΔC model of association does not fit the data. However, the curves did fit a 1:1 + 2:1 model, residuals for which are shown in Fig. 5B. The equilibrium constants for the formation of the 1:1 and then the 2:1 complex, $A + B \rightleftharpoons AB + B \rightleftharpoons AB_2$ (where A is Fcε3-4ΔC and B is sFcεRIα) are $K_{AB} = 1.8 (\pm 0.4) \times 10^5 \text{ M}^{-1}$ and $K_{AB2} = 7.2 (\pm 2.2) \times 10^5 \text{ M}^{-1}$.

To determine whether this difference in behavior was a result of the absence of the carbohydrate or the interchain disulfide bond at Cys-328, the *deglyFcε3-4* and a partially reduced Fcε3-4 preparation (*redFcε3-4*) were examined in the analytical ultracentrifuge in the same way. The residuals for these two species are shown in Fig. 5, C and D. *deglyFcε3-4* simply formed a high-affinity 1:1 complex and behaved in the same way as Fcε3-4 (Fig. 5C). The data for *redFcε3-4*, however, could not be fitted by a 1:1 model (data not shown) but were instead fitted to a 1:1 + 2:1 model similar to Fcε3-4ΔC (Fig. 5D). Clearly, it is the loss of the disulfide bond that leads to the formation of a 2:1 complex with the soluble receptor. The corresponding equilibrium constants for the association of sFcεRIα with *redFcε3-4* are $K_{AB} = 1.3 (\pm 0.05) \times 10^6 \text{ M}^{-1}$ and $K_{AB2} = 1.0 (\pm 0.16) \times 10^4 \text{ M}^{-1}$. After centrifugation, the partial reduction process was judged from SDS-PAGE analysis (data not shown) to leave a residual amount (<5%) of the material in the unreduced state. The presence of this species, which would bind with higher affinity and 1:1 stoichiometry may explain the higher value for the K_{AB} component. The affinity may have been enhanced further by the presence of glycosylation in *redFcε3-4*, consistent with the Biacore data presented under the previous section.

DISCUSSION

The recent crystal structures of IgE Fc (22), the Fcε3-4 fragment (23), and the latter complexed with sFcεRIα (6) have not only provided a description of the binding interface but also suggested that engagement of the receptor by IgE involves a substantial conformational change in IgE Fc. The interaction surface is extensive and consists of two subsites, one on each Cε3 domain. However, in neither the free IgE Fc nor the Fcε3-4 fragment are both subsites accessible. In IgE Fc, the Cε2 domains are acutely and asymmetrically bent back against the Cε3 domains so that one subsite is accessible while the other is hidden (22). In Fcε3-4, neither site is accessible and the Cε3 domains adopt a closed quaternary conformation (23). Thus, a conformational change involving the Cε3 domains (and probably also Cε2 (3)) must occur upon receptor binding to allow contact at both sites and full affinity to be achieved. We and others (22) have suggested that the inhibition of the conformational change and thus engagement of both sites may offer an alternative strategy to blocking the binding interaction directly. However, it was not known what the individual contributions of the two subsites were to the overall affinity and, thus, how effective such a strategy of restricting the interaction to only one subsite (such as the only accessible subsite in IgE Fc) might be. The present study answers this question.

FIG. 4. **SPR analysis of Fc ϵ 3-4 fragments.** A, Fc ϵ 3-4 (100–6.25 nM). B, *degly*Fc ϵ 3-4 (100–6.25 nM). C, Fc ϵ 3-4 Δ C (200–12.5 nM), all binding to immobilized IgG4-Fc(sFc ϵ RI α)₂. Data were recorded using either a Biacore 3000, 2000, or 1000, respectively (flow rate 20 μ l min⁻¹ in Hepes-buffered saline). All of the traces are base line-corrected for nonspecific binding by the subtraction of data obtained from an IgG4 control surface treated in an identical manner. Curves corresponding to the fitted interaction model (Fc ϵ 3-4- and *degly*Fc ϵ 3-4-biphasic; Fc ϵ 3-4 Δ C-monophasic) are plotted as *white dots* on the same axes as the experimental data with the residuals shown beneath. All of the values derived from the fitting procedures are given in Table I. RU, response units.

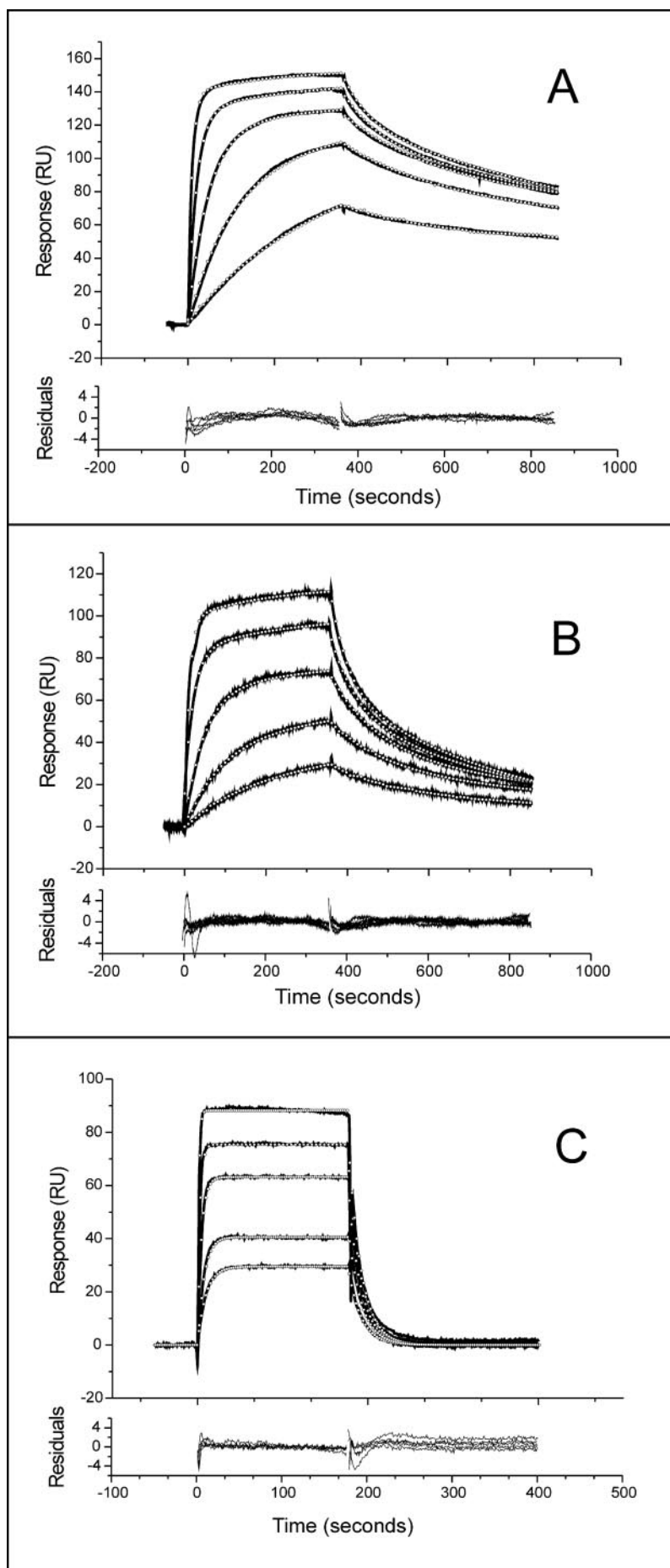


TABLE I

Kinetic parameters and affinity constants derived from the SPR analyses of different Fcε3-4 fragments binding to immobilized IgG4-(sFcεRIα)₂

Fcε3-4 and deglyFcε3-4 were analyzed using a biphasic interaction model from which association and dissociation constants were derived for each component (shown ± S.D. for at least ten determinations in the concentration range of 6.25–100 nM). R_1/R_0 describes the fractional contribution of the first component to the overall fit. The Fcε3-4ΔC parameters (shown ± S.E.) were derived from a global analysis using a 1:1 model of association simultaneously fitted to five different analyte concentrations (12.5–200 nM).

Fcε3-4		deglyFcε3-4	Fcε3-4ΔC	
k_{a1} (M ⁻¹ s ⁻¹)	$(4.77 \pm 1.88) \times 10^5$	$(4.88 \pm 1.97) \times 10^5$	k_a (M ⁻¹ s ⁻¹)	$(2.76 \pm 0.04) \times 10^6$
k_{d1} (s ⁻¹)	$(1.21 \pm 0.19) \times 10^{-2}$	$(1.50 \pm 0.29) \times 10^{-2}$	k_d (s ⁻¹)	$(6.63 \pm 0.05) \times 10^{-2}$
k_{a2} (M ⁻¹ s ⁻¹)	$(3.08 \pm 0.65) \times 10^5$	$(1.97 \pm 0.15) \times 10^5$	K_a (M ⁻¹)	4.17×10^7
k_{d2} (s ⁻¹)	$(6.26 \pm 1.29) \times 10^{-4}$	$(1.73 \pm 0.23) \times 10^{-3}$		
K_{a1} (M ⁻¹)	3.94×10^7	3.25×10^7		
K_{a2} (M ⁻¹)	4.92×10^8	1.14×10^8		
R_1/R_0	0.15 ± 0.03	0.38 ± 0.03		

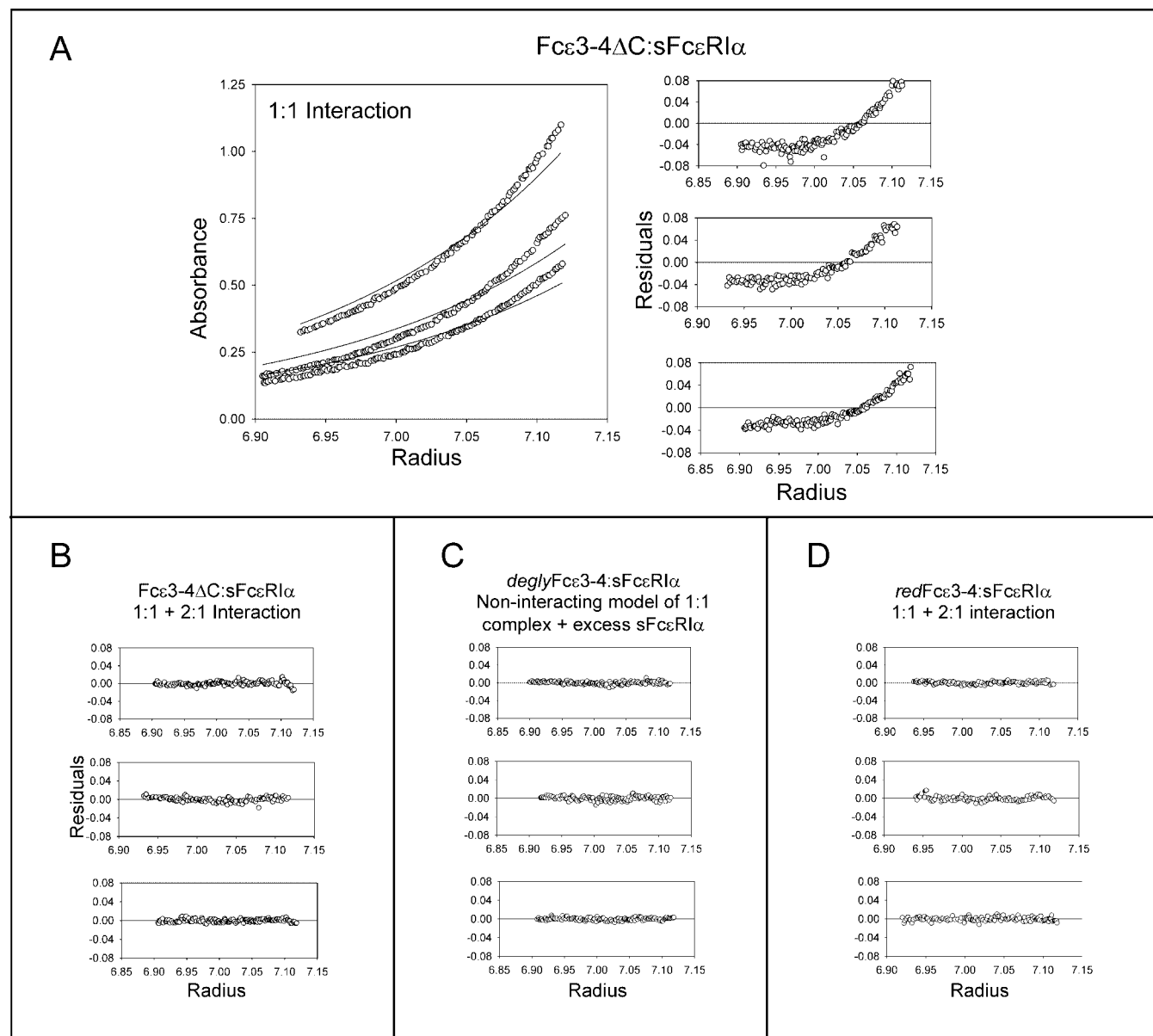


FIG. 5. Analytical ultracentrifugation of Fcε3-4 fragments in complex with sFcεRIα. All of the data shown were collected at 11,000 rpm. A, the three curves and corresponding residuals for fitting to a 1:1 interaction model are for different loading ratios of Fcε3-4ΔC and sFcεRIα in the mixtures (1:1, 2:1, and 1:2). The residuals are non-randomly distributed, indicating a poor fit. B, residuals only for a 1:1 + 2:1 model fitted to the same data as A with random residuals indicating a good fit. C, residuals only for three mixtures of deglyFcε3-4ΔC and sFcεRIα fitted to a model in which deglyFcε3-4ΔC and sFcεRIα form a high-affinity 1:1 complex that does not then interact with an excess of sFcεRIα. D, residuals only for a 1:1 + 2:1 model fitted to three mixtures of redFcε3-4 and sFcεRIα indicating a good fit.

A further question concerns the importance of glycosylation for IgE binding to FcεRI. The crystal structure of the complex between IgG Fc and Fcγ RIII (24, 25) shows a structurally

homologous interaction, yet it is known that glycosylation of the IgG Fc is essential for receptor binding activity (13), whereas it is not for IgE (12). Therefore, we set out to explore

in more detail the contribution of glycosylation to the binding kinetics and uniquely high affinity of IgE for its receptor using Fcε3–4, the fragment structurally homologous to IgG Fc.

To identify the contributions to folding and receptor binding of Cε3 glycosylation and covalent dimerization by the inter-chain disulfide, we generated and tested variants of the Fcε3–4 fragment of IgE lacking one or both of these structural features. The native-like protein, Fcε3–4, expressed in mammalian NS-0 cells contained both features. *degly*Fcε3–4 prepared from Fcε3–4 lacked glycosylation but retained the disulfide bridge. Fcε3–4ΔC produced in *E. coli* lacked both glycosylation and the disulfide bridge. A fourth preparation derived from Fcε3–4 by partial reduction and termed *red*Fcε3–4 (glycosylated and with at least 95% molecules lacking the disulfide bridge) was used only to confirm the conclusions derived from the other three fully isolated and characterized molecules.

The Role of Glycosylation—The effect of removing the carbohydrate at Asn-394 in Fcε3–4 on the kinetics of receptor binding can be seen from the results of the SPR analysis (Fig. 4 and Table I) with *degly*Fcε3–4 clearly displaying a faster dissociation rate than Fcε3–4. Although this is a striking result, leading to an overall 4-fold reduction in the affinity of the principal binding component, it is very different to the behavior of IgG Fc for which removal of the structurally homologous carbohydrate at Asn-297 leads to total loss of receptor binding (13, 26). The crystal structures of both the IgG Fcγ3 RIII (24, 25) and the Fcε3–4 sFcεRIα complexes (6) reveal that there are only tenuous, if any, direct interactions between Fc carbohydrate and receptor, but clearly indirect effects are possible. Indeed, the effect upon the IgG Fc structure of progressive removal of the carbohydrate has been investigated crystallographically (27) and a gradual “closing” of the Cγ2 domains is found as the carbohydrate chains are shortened. Because the Cγ2 domains must adopt a more “open” conformation to form the complex and changes in the mobility of loop regions that make contact with receptor can also be detected as carbohydrate is removed (27), a (indirect) structural link between glycosylation and receptor binding in IgG Fc has been established.

In IgE Fc, the role of carbohydrate is different. Although the regions of contact with their respective Cε3 and Cγ2 domains are similar, the fact that deglycosylation of Fcε3–4 has a much less profound effect upon receptor binding than in IgG Fc may depend upon other structural differences, such as the more extensive interface between Cε3 and Cε4 compared with Cγ2 and Cγ3, or the different degree of conformational change required for receptor binding (reviewed in Ref. 28). Our observation that, in analytical gel filtration experiments, the Fcε3–4 and *degly*Fcε3–4 behaved identically also contrasts with published data for native and deglycosylated IgG Fc that show a difference in retention time that is interpreted as a more compact conformation for the deglycosylated IgG Fc (26). Thus, although the Cγ2 domains of IgG may move closer together upon removal of carbohydrate (consistent with the crystallographic observations (27)), this appears not to occur for the Cε3 domains of IgE. This observation is supported by the examination of the Fcε3–4 crystal structure (23) in which it can be seen that the glycosylated Cε3 domains are already in a “closed” conformation. In contrast, we observed that the removal of the interchain disulfide bridge led to a marked change in mobility in gel filtration experiments (Fig. 2), indicating that disulfide linkage of the Cε3 domains, rather than glycosylation, is likely to be the major contributor to maintenance of the correct conformation for receptor engagement.

The Role of Disulfide Linkage between the Two Cε3 Domains—The comparative SPR binding studies showed that Fcε3–4ΔC, lacking both the inter-heavy chain bridge at Cys-

328 and Cε3 glycosylation at Asn-394, displayed an even faster dissociation rate than either Fcε3–4 or *degly*Fcε3–4 (Fig. 4). More surprisingly, it fitted a monophasic model. The fitting of this model returned a K_a value of $4.17 \times 10^7 \text{ M}^{-1}$ (Table I). When studied in the analytical ultracentrifuge, the reason for the change in mode of interaction became apparent as Fcε3–4ΔC behaved very differently from Fcε3–4 and *degly*Fcε3–4. We had reported earlier that Fcε3–4 formed a high-affinity 1:1 complex with sFcεRIα (9) but that the Fcε3–4ΔC fragment can form a 2:1 complex, *i.e.* two molecules of sFcεRIα binding to a single Fcε3–4ΔC molecule (Fig. 5, A and B). The data were fitted to an equilibrium between the unbound components, a 1:1 and a 2:1 stoichiometric complex, with two approximately equal binding constants for the addition of the first ($K_{AB} = 1.8 (\pm 0.4) \times 10^5 \text{ M}^{-1}$) and the second ($K_{AB2} = 7.2 (\pm 2.2) \times 10^5 \text{ M}^{-1}$) sFcεRIα molecules. These values represent the affinity of a single folded Cε3 domain for the receptor, *i.e.* the affinity contributed by each of the two subsites.

Confirmation that this stoichiometry and dramatically reduced affinity are the result of removing the inter-heavy chain disulfide bond came from the parallel analysis under identical conditions of the mildly reduced preparation of glycosylated Fcε3–4, *red*Fcε3–4 (Fig. 5D). This species behaved in the same way as Fcε3–4ΔC fitting only to a 1:1 + 2:1 stoichiometry, yielding values of $K_{AB} = 1.3 (\pm 0.05) \times 10^6 \text{ M}^{-1}$ and $K_{AB2} = 1.0 (\pm 0.16) \times 10^4 \text{ M}^{-1}$. The analytical ultracentrifugation analysis of the fragment that maintained the disulfide bond at position 328 but lacked carbohydrate (*degly*Fcε3–4) demonstrated a high-affinity complex that behaved as a single species (Fig. 5C). This finding was consistent with the drop in affinity of only a factor of four determined by Biacore analysis and further confirmed that the disulfide bridge is the key determinant of stoichiometry.

We envisage that this change in stoichiometry upon removal of the disulfide bond occurs because the two subsites, one in each Cε3 domain, that together constitute the high-affinity binding site are now presented in such a way that each can bind independently to an FcεRIα molecule. This implies that the Cε3 domains must move sufficiently far relative to each other so that there is no steric hindrance between the two FcεRIα molecules. The crystal structures of free (23) and complexed Fcε3–4 (6) and Fcε2–4 (22) demonstrate that there is substantial flexibility in the disposition of the Cε3 domains. The gel filtration profiles (Fig. 2) indicate that this relative movement of the Cε3 domains in Fcε3–4ΔC results in a structure that is considerably more compact, even than that seen in the Fcε3–4 crystal structure (23), yet one in which the subsites of both Cε3 domains are exposed.

The affinities measured for Fcε3–4ΔC by analytical ultracentrifugation ($K_a \approx 10^5\text{--}10^6 \text{ M}^{-1}$) and the value obtained by Biacore ($K_a = 4.17 \times 10^7 \text{ M}^{-1}$) differ, presumably because of differences between the assay formats. In the Biacore, the receptor is immobilized on the biosensor surface, whereas in the analytical ultra-centrifuge, both species are free to associate in solution. An earlier study in which a cell binding assay was used to measure the ability of an Fcε3–4 fragment lacking Cys-328 altogether (residues 329–547) to compete with the binding of IgE to native surface FcεRI ($\alpha\beta\gamma_2$) gave an IC_{50} value of $32.9 \pm 25.8 \text{ nM}$ (11). When Fcε3–4ΔC was tested in a competition enzyme-linked immunosorbent assay with immobilized receptor, we recorded an IC_{50} value of $89.6 \pm 1.5 \text{ nM}$ (29). The assay format clearly affects the affinity values that are returned, but the Biacore and solution phase analytical ultracentrifugation measurements enabled us to detect differences in stoichiometry and affinity due to glycosylation and disulfide linkage in the Fcε3–4 fragments.

Implications for Inhibitor Design—These affinities recorded for a single folded Cε3 domain in Fcε3-4ΔC (1.8×10^5 and $7.2 \times 10^5 \text{ M}^{-1}$) are similar to those measured for an isolated recombinant Cε3 domain ($\sim 5 \times 10^6 \text{ M}^{-1}$ in a Biacore assay), which we reported in an earlier study (7). This isolated Cε3 domain is not fully folded as judged by CD spectroscopy (7) and NMR (8); therefore, it may be inferred that the binding event involves substantial interaction with linear or unstructured regions of the Cε3 domain. The crystal structure of the Fcε3-4-sFcεRIα complex (6) shows that many of the contacts are indeed made by residues in the extended N-terminal region and loops of Cε3, and this may also explain why short peptides have been reported to have inhibitory activity (12, 30). However, the present study demonstrates that, even though complete folding may not be necessary, optimized dimerization of the Cε3 is essential for full binding affinity and also that the contribution of glycosylation is not critical for the formation of a high-affinity complex. Thus, peptides or non-peptide analogues if presented as a dimer may attain sufficiently high inhibitory activity.

Furthermore, these affinity values of 10^5 - 10^6 M^{-1} represent the contribution of just one of the two subsites that constitute the total binding interface seen in the crystal structure of the Fcε3-4-sFcεRIα complex (6). The recently determined crystal structure of the complete IgE Fc including the Cε2 domains (22) revealed that only one of the two subsites was accessible in the free IgE Fc and that a conformational change is required for access to the second site. Therefore, if an inhibitor can be found that restricts the conformational change, its effect would be to reduce the affinity of the interaction by three to four orders of magnitude with a correspondingly faster dissociation rate. This is very likely to be sufficient to result in a therapeutically beneficial reduction in mast cell sensitization.

REFERENCES

- Gould, H. J., Sutton, B. J., Beavil, A. J., Beavil, R. L., McCloskey, N., Coker, H. A., Fear, D., and Smurthwaite, L. (2003) *Annu. Rev. Immunol.* **21**, 579–628
- Ravetch, J. V., and Kinet, J.-P. (1991) *Annu. Rev. Immunol.* **9**, 457–492
- McDonnell, J. M., Calvert, R., Beavil, R. L., Beavil, A. J., Henry, A. J., Sutton, B. J., Gould, H. J., and Cowburn, D. (2001) *Nat. Struct. Biol.* **8**, 437–441
- Maenaka, K., van der Merwe, P. A., Stuart, D. I., Jones, E. Y., and Sondermann, P. (2001) *J. Biol. Chem.* **276**, 44898–44904
- Keown, M. B., Ghirlando, R., Young, R. J., Beavil, A. J., Owens, R. J., Perkins, S. J., Sutton, B. J., and Gould, H. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 1841–1845
- Garman, S. C., Wurzburg, B. A., Tarchevskaya, S. S., Kinet, J.-P., and Jardetzky, T. S. (2000) *Nature* **406**, 259–266
- Henry, A. J., McDonnell, J. M., Ghirlando, R., Sutton, B. J., and Gould, H. J. (2000) *Biochemistry* **39**, 7406–7413
- Price, N. E., Price, N. C., Kelly, S. M., and McDonnell, J. M. (2005) *J. Biol. Chem.* **280**, 2324–2330
- Keown, M. B., Ghirlando, R., Mackay, G. A., Sutton, B. J., and Gould, H. J. (1997) *Eur. Biophys. J.* **25**, 471–476
- Keown, M. B., Henry, A. J., Ghirlando, R., Sutton, B. J., and Gould, H. J. (1998) *Biochemistry* **37**, 8863–8869
- Basu, M., Hakimi, J., Dharm, E., Kondas, J. A., Tsien, W. H., Pilson, R. S., Lin, P., Gilfillan, A., Haring, P., Braswell, E. H., and Kochan, J. P. (1993) *J. Biol. Chem.* **268**, 13118–13127
- Helm, B., Marsh, P., Vercelli, D., Padlan, E., Gould, H., and Geha, R. (1988) *Nature* **331**, 180–183
- Mimura, Y., Sondermann, P., Ghirlando, R., Lund, J., Young, S. P., Goodall, M., and Jefferis, R. (2001) *J. Biol. Chem.* **276**, 45539–45547
- Shi, J., Ghirlando, R., Beavil, R. L., Beavil, A. J., Keown, M. B., Young, R. J., Owens, R. J., Sutton, B. J., and Gould, H. J. (1997) *Biochemistry* **36**, 2112–2122
- Cook, J. P., Henry, A. J., McDonnell, J. M., Owens, R. J., Sutton, B. J., and Gould, H. J. (1997) *Biochemistry* **36**, 15579–15588
- Kenten, J. H., Molgaard, H. V., Houghton, M., Derbyshire, R. B., Viney, J., Bell, L. O., and Gould, H. J. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 6661–6665
- Kenten, J., Helm, B., Ishizaka, T., Cattini, P., and Gould, H. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 2955–2959
- Bohmann, D., and Tjian, R. (1989) *Cell* **59**, 709–717
- Taylor, M. A., Pratt, K. A., Revell, D. F., Baker, K. C., Sumner, I. G., and Goodenough, P. W. (1992) *Protein Eng.* **5**, 455–459
- Henry, A. J., Cook, J. P., McDonnell, J. M., Mackay, G. A., Shi, J., Sutton, B. J., and Gould, H. J. (1997) *Biochemistry* **36**, 15568–15578
- Takatsu, K., Ishizaka, T., and Ishizaka, K. (1975) *J. Immunol.* **114**, 1838–1845
- Wan, T., Beavil, R. L., Fabiane, S. M., Beavil, A. J., Sohi, M. K., Keown, M., Young, R. J., Henry, A. J., Owens, R. J., Gould, H. J., and Sutton, B. J. (2002) *Nat. Immunol.* **3**, 681–686
- Wurzburg, B. A., Garman, S. C., and Jardetzky, T. S. (2000) *Immunity* **13**, 375–385
- Radaev, S., Motyka, S., Fridman, W.-H., Sautes-Fridman, C., and Sun, P. D. (2001) *J. Biol. Chem.* **276**, 16469–16477
- Sondermann, P., Huber, R., Oosthuizen, V., and Jacob, U. (2000) *Nature* **406**, 267–273
- Radaev, S., and Sun, P. D. (2001) *J. Biol. Chem.* **276**, 16478–16483
- Krapp, S., Mimura, Y., Jefferis, R., Huber, R., and Sondermann, P. (2003) *J. Mol. Biol.* **325**, 979–989
- Wurzburg, B. A., and Jardetzky, T. S. (2001) *Mol. Immunol.* **38**, 1063–1072
- Henry, A. J. (1996) *Human IgE and the Interaction with Its High Affinity Receptor*. Ph.D. thesis, University of London, London
- Stamos, J., Eigenbrot, C., Nakamura, G. R., Reynolds, M. E., Yin, J., Lowman, H. B., Fairbrother, W. J., and Starovasnik, M. A. (2004) *Structure* **7**, 1289–1301