Human immunoglobulin E flexes between acutely bent and extended conformations

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Crystallographic and solution studies have shown that IgE molecules are acutely bent in their Fc region. Crystal structures reveal the Cε2 domain pair folded back onto the Cε3-Cε4 domains, but is the molecule exclusively bent or can the Cε2 domains adopt extended conformations and even ‘flip’ from one side of the molecule to the other? We report the crystal structure of IgE-Fc captured in a fully extended, symmetrical conformation and show by molecular dynamics, calorimetry, stopped-flow kinetic, surface plasmon resonance (SPR) and Förster resonance energy transfer (FRET) analyses that the antibody can indeed adopt such extended conformations in solution. This diversity of conformational states available to IgE-Fc offers a new perspective on IgE function in allergen recognition, as part of the B-cell receptor and as a therapeutic target in allergic disease.

Immunoglobulin E (IgE) antibodies play a central role in allergic disease1. They recognize allergens in two very different contexts, either in a membrane-bound form as part of the B-cell receptor (BCR) or bound to the receptor FcεRI on effector cells such as mast cells and basophils. FcεRI-bound IgE causes long-term sensitization of these cells, and cross-linking by allergens leads to cell degranulation, release of inflammatory mediators and an immediate allergic response. Disruption of the IgE–FcεRI interaction is a validated strategy for therapeutic intervention in allergic diseases including asthma: an anti-IgE monoclonal IgG antibody, omalizumab (Xolair, Novartis Pharmaceuticals), inhibits IgE binding to FcεRI and is effective in the treatment of severe persistent asthma and other allergic diseases2.

IgE consists of a dimer of two identical heavy and two identical light chains, but unlike IgG, in which the antigen-binding Fab region is separated from the receptor-binding Fc region by a flexible hinge, IgE contains an additional disulfide-linked pair of domains, (Cε2)2, forming a (Cε2-Cε3-Cε4)2 dimer1. Fluorescence depolarization studies to assess segmental flexibility have shown IgE to be less flexible than IgG3-4, and Förster resonance energy transfer (FRET) studies that determined distances both intramolecular and to the membrane led to a model of a compact, bent structure for IgE both when free in solution and when bound to FcεRI, consistent with FRET and fluorescence–depolarization studies that indicated reduced segmental flexibility6-15. At this point the existence of an extended conformation of IgE-Fc was all but dismissed.

Although the Cε2 domains are not directly involved in binding FcεRI, they do contribute to the kinetics of the interaction, decreasing both the association and dissociation rate constants14,17. Interest in their structural and functional roles intensified following the discovery that the Fab fragment of omalizumab binds to a partially unbent conformation of IgE-Fc, as detected in a FRET experiment16. This first indication that IgE-Fc may not always be bent raises the question of whether the molecule transiently explores more extended conformations, and perhaps even flips between bent structures with the Cε2 domains folded back on opposite sides of the Cε3-Cε4 domains. Trapping of transiently populated conformational states has previously been achieved by antibody binding18, and so to explore the potential conformational diversity of IgE-Fc, we generated an IgG antibody Fab fragment that binds to IgE-Fc (anti-ε-chain Fab; αεFab) and discovered that it captured an extended conformation.

RESULTS
Structure of IgE-Fc bound by two αεFab fragments
The αεFab–IgE-Fc crystal structure was solved at 2.9 Å resolution (Table 1). The IgE-Fc adopts a fully extended conformation, with two αFab molecules bound one on each side of the almost perfectly symmetrical IgE-Fc (αεFab1–IgE-Fc–αεFab2, with superscripted numbers denoting the two αFab molecules, Fig. 1b,c). Compared with the structure of IgE-Fc alone, the molecule has undergone a drastic ‘unbending’

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of 120° (Fig. 1a, c), completely losing the extensive intramolecular interface between the Ce2 and Ce3–Ce4 domains. This unbending seems to derive largely from movements in the Ce2–Ce3 linker region, in particular residues Pro333, Arg334 and Gly335 (Supplementary Video 1). Although the Ce2 domains show the greatest structural change, the Ce3 domains also undergo considerable movement. Conformational flexibility has been seen in a number of structures of the Fce3–4 subfragment of IgE-Fc, with the Ce3 domains described as ‘open’ or ‘closed’ (together with a ‘swinging’ of one Ce3 domain relative to the other, Supplementary Fig. 1a)33. In the bent structure of IgE-Fc alone, one Ce3 is open (chain B) and one is closed (chain A), whereas in the extended conformation of IgE-Fc revealed here, both Ce3 domains adopt an open conformation (Supplementary Fig. 1b). Ce3A (the Ce3 domain of chain A) thus moves more than Ce3B (the Ce3 domain of chain B) upon αFab binding, with a change in the Ce3–Ce4 interdomain angle of 15°. The (Ce4)2 pair are unchanged upon complex formation. Such is the symmetry of the IgE-Fc in the complex that the local two-fold axes of all three domain pairs are virtually coincident.

As a result of this symmetry, the two αFab interfaces are structurally equivalent (each ~1,400 Å²), mainly involving contact of the αFab heavy and light chains with Ce3, but with a small interaction with Ce2 (Supplementary Fig. 2a). Each αFab molecule principally contacts a single IgE-Fc chain (αFab1 to IgE-FcA and αFab2 to IgE-FcB), with the exception of a 315 Å2 interface with the Ce2–Ce3 linker region (including Ser331 and Asn332) of the other IgE-Fc chain (Supplementary Fig. 2b). The ‘hot spot’ of the αFab-binding surface on IgE-Fc appears to center on Ce3 residue Arg393, which protrudes into a pocket at the interface of the heavy and light chains of αFab (Supplementary Fig. 2c), forming a salt bridge (to Asp121 of the heavy chain) and hydrogen bonds (to Glu109 and Asn54 of the light chain; Supplementary Fig. 2d,e). The adjacent Ce3 residues also contribute extensively (Supplementary Fig. 2c).

Comparison of the αFab1–IgE-Fc–αFab2 complex with free IgE-Fc shows that the latter presents only one αFab binding site. When superimposed on the Ce3B domains, the αFab-binding hot spot is both unchanged and accessible in bent IgE-Fc, making it a likely point for αFab engagement. However, the Ce2 domains in the bent structure completely occlude αFab binding to the other side of the molecule (Ce3B) and would have to move substantially toward an extended conformation before αFab access would be possible (Fig. 1a,b).

**Molecular-dynamics simulation of IgE-Fc unbending**

The crystal structure of the αFab1–IgE-Fc–αFab2 complex reveals that an extended conformation of IgE-Fc is feasible. The key question, however, is whether this is just a consequence of αFab binding, or whether it (or other extended conformations) exists in solution as an intrinsic property of IgE. If the latter, then could the Ce2 domain pair make the transition from one side of the Ce3–Ce4 domains to the other via an extended structure—that is, ‘flip’ between two symmetrically related bent conformations (Supplementary Video 2)? We used metadynamics, an enhanced molecular-dynamics (MD) method20–23, to produce a detailed atomistic simulation of IgE-Fc unbending and its extended conformations. This simulation provides a free-energy surface (potential of mean force) calculated and presented in terms of the two principal components of the molecule’s unbending dynamics (Fig. 2a and Supplementary Fig. 3).

The lowest energy state is a well-defined minimum that corresponds to the bent conformation seen in the free IgE-Fc crystal structure13 (Fig. 2, conformation 1). A number of other energy minima may be seen at ~20 kJ/mol above that of the bent state, suggesting possible paths (one of which is indicated, Fig. 2a) leading away from the bent structure toward more extended conformations (Fig. 2a,b, conformations 2 and 3). The dynamic simulation identifies another minimum (Fig. 2a, conformation 4) symmetrically related to conformation 3 and separated by a small energy barrier that corresponds to a linear conformation (indicated in Fig. 2c). As IgE-Fc crosses this low barrier, the Ce2 domain pair passes from one side of the molecule to the other. Having traversed the midpoint (x = 0 in Fig. 2a), the Ce2 domains may fold onto the other side of the Ce3–Ce4 domains, completing a full transition from one

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**Table 1 Data collection and refinement statistics**

| Data collection | αFab1–IgE-Fc–αFab2 |
|----------------|---------------------|
| **Data collection** | | |
| Space group | P2_12_1 |
| Cell dimensions (Å) | a, b, c (Å) |
| Resolution (Å) | 2.91 (2.98–2.91) |
| Rmerge | 0.055 (0.796) |
| Completeness (%) | 99.7 (99.2) |
| Redundancy | 5.7 (6.0) |

| Refinement | | |
| Resolution (Å) | 67.01 – 2.91 |
| No. unique reflections | 41,987 (3,078) |
| Rmerge / Rfree | 0.236 / 0.284 |
| No. atoms | 11,712 |
| Protein | 11,541 |
| Ligand / ion | 122 |
| Water | 49 |
| B factors | | |
| Protein | 103.8 |
| Sugars | 110.9 |
| Water | 88.4 |
| r.m.s. deviations | | |
| Bond lengths (Å) | 0.010 |
| Bond angles (°) | 1.566 |

Values in parentheses are for highest-resolution shell.
residues that undergo the most substantial structural rearrangement systems. Local disorder or unfolding and re-folding within linkers, or regions of the domains themselves, can facilitate large-scale conformational changes in proteins, providing entropic compensation to reduce high enthalpic barriers.

The simulation additionally revealed a twisting of (Ce2)ε around Ser437 of the A chain are associated with the change in the interdomain angle. The simulation additionally revealed a twisting of (Ce2)ε relative to the Ce3-Ce4 domains during the unbending process. The mechanism by which domain rearrangements and unbending occur may not simply be rigid-body movements facilitated by linkers that undergo changes from one ordered structure to another (‘mechanical hinges’), but may involve order-disorder transitions as described in other systems. Local disorder or unfolding and re-folding within linkers, or regions of the domains themselves, can facilitate large-scale conformational changes in proteins, providing entropic compensation to reduce high enthalpic barriers.

Regardless of the mechanism, the highest barrier encountered in a complete flip, and thus the rate-determining step, is clearly that involved in leaving the bent conformation; once this is achieved, the barriers to exploring the extended conformations are relatively low. However, the energy difference between the wells corresponding to the bent and extended conformations is such that the fraction of molecules in the extended state will be very small, consistent with the experimental observation that the bent conformation predominates in solution.

aeFab binding to IgE-Fc by isothermal titration calorimetry

One way to establish whether IgE-Fc has an intrinsic capacity to unbend in solution is to determine the number of available aeFab binding sites. If IgE-Fc is rigidly and exclusively bent, only one site will ever be accessible, but if it is flexible, then two sites will be accessible. We therefore studied the interaction between aeFab and IgE-Fc by isothermal titration calorimetry (ITC) and compared this with the binding of aeFab to Fcε3-4 which, lacking the Ce2 domains, always has two accessible sites. The results for both IgE-Fc and Fcε3-4 are similar, showing that both molecules do indeed present two binding sites.

Assembly mechanism of aeFab1–IgE-Fc–aeFab2 in solution

The observed 2:1 stoichiometry demonstrates that in solution IgE-Fc can adopt the extended structures needed to expose both aeFab binding sites. The binding of aeFab1 mediates an allosteric change in IgE-Fc, which may occur through induced fit, conformational selection or a combination of both mechanisms. In order to investigate the assembly mechanism of the aeFab1–IgE-Fc–aeFab2 complex, we monitored the intrinsic tryptophan fluorescence upon binding in stopped-flow kinetic experiments. When IgE-Fc (or Fcε3-4) was in excess over aeFab, this restricted the stoichiometry of the aeFab–IgE-Fc complex to 1:1, a single binding event was observed. This has a fast association rate constant and a dissociation rate too slow to measure by this technique.

When repeated with aeFab in excess over IgE-Fc, two-step binding was observed for both IgE-Fc and Fcε3-4 (Supplementary Fig. 4ab), consistent with not only aeFab1 binding faster than aeFab2, but also the difference in affinities of the two sites determined by ITC.

Furthermore, a linear concentration dependence for the observed rate constants (Kobs) of the binding of both aeFabs to either IgE-Fc or Fcε3-4 suggests that aeFab1 does not cause a conformational change (such as unbending of IgE-Fc) that is rate-limiting for aeFab2 binding (Supplementary Fig. 4c–f). In the absence of any evidence for an induced conformational change, our results are most consistent with a conformational selection model, with extended structures selected from a dynamic population of IgE-Fc conformations.

To test whether aeFab1 binding alone traps IgE-Fc in an extended conformation or still allows it to flex between extended and
bent structures, we carried out intramolecular FRET, with IgE-Fc labeled with donor and acceptor fluorophores in the Ce2 and Ce4 domains, respectively. Upon titration into labeled IgE-Fc, a concentration of aFab sufficient to occupy the first binding site resulted in a partial decrease of FRET signal (Fig. 3e). This implies that under these conditions the IgE-Fc is not exclusively extended, as would be expected if aFab binding caused full linearization; nor does IgE-Fc remain bent. Taken together with the stopped-flow data (linear concentration dependence of the kinetics of aFab binding and similar behavior of IgE-Fc and Fc3-4), the FRET data show that IgE-Fc in the aFab1-IgE-Fc complex remains conformationally dynamic. It is only upon saturation of the second binding site that no further reduction in the FRET signal occurs (Fig. 3c), corresponding to a fully extended IgE-Fc.

These results are summarized in Figure 4, which depicts a possible mechanism for the formation of the aFab1-IgE-Fc-aFab2 complex (see also Supplementary Video 3). Initially, IgE-Fc is predominantly bent in solution (consistent with X-ray and neutron scattering13 and FRET16), but it may transiently adopt extended conformations such as those identified in the MD simulation (Fig. 4a). Next, aFab1 engages IgE-Fc in the bent or an extended conformation. With aFab1 bound, IgE-Fc can still flex between bent and extended conformations (Fig. 4b). Finally, when IgE-Fc is transiently extended, aFab2 engages and completes the aFab1-IgE-Fc-aFab2 complex (Fig. 4c).

**Effect of aFab on FceRI binding.** We have demonstrated the existence of a range of conformational states available to IgE-Fc, but how do these different states affect binding of IgE to FceRIα? Receptor-bound IgE-Fc is even more acutely bent than free IgE-Fc, and interacts with the receptor with high affinity (Kd ~ 0.1 nM) through two subsites, one on each Ce3 domain (Fig. 5a)14,31. In the extended conformation of IgE-Fc that results from aFab binding, both of the FceRIα-binding subsites are disrupted (Fig. 5b). In subsite 1 on IgE-FcA, Arg334 forms a critically important salt bridge with FceRIα, but this residue is part of the linker that undergoes structural rearrangement upon extension of (Ce2)2, and is no longer in a position to engage in FceRIα binding. The second subsite on IgE-FcB involves a proline (Pro426 of Ce3B) sandwiched between two tryptophan residues (Trp87 and Trp110 of FceRIα), but this proline moves 6 Å away from its receptor-bound conformation in the aFab complex. Even if receptor engagement with Pro426 of subsite 2 alone occurs, modeling of sFcRα in this position on the extended IgE-Fc results in clashes between both Ce3A and (Ce2)2 of IgE-Fc with the receptor (Fig. 5c).

Thus, aFab binding allosterically prevents FceRIα binding to IgE-Fc by trapping the molecule in an extended state that cannot engage with receptor. But does aFab1 alone compete with FceRIα binding to bent IgE-Fc? We compared the aFab-bound structure with that of IgE-Fc bound to sFcRα. Modeling of a single aFab

| Table 2 | ITC and stopped-flow analysis of the interaction between aFab and IgE-Fc or Fc3-4 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| aFab1–IgE-Fc    | Kd1 (µM)        | Kd2 (µM)        | Koff (M−1 s−1) | koff (s−1)     | Koff (M−1 s−1) | koff (s−1)     | ΔH1 ITC (kcal/mol) | ΔH2 ITC (kcal/mol) |
| 1aFab1–IgE-Fc   | 0.040 (±0.02)   | –               | 6.7 (±0.2) × 10^5 | n/m            | –               | –               | –3.5 (±0.1)            | –               |
| 2aFab1–IgE-Fc   | 0.076 (±0.07)   | 1.5 (±1.3)      | 3.5 (±0.2) × 10^5 | 3.2 (±0.1)     | 1.2 (±0.2) × 10^5 | n/m            | –3.5 (±0.1)            | −1.3 (±0.1)       |
| aFab1–Fc3-4     | 0.050 (±0.02)   | –               | 1.0 (±0.1) × 10^6 | n/m            | –               | –               | −3.9 (±0.1)            | −               |
| 2aFab1–Fc3-4    | 0.034 (±0.03)   | 0.98 (±0.45)    | 1.0 (±0.1) × 10^6 | 0.95 (±0.2)    | 3.7 (±0.3) × 10^5 | n/m            | −4.3 (±0.1)            | −2.3 (±0.1)       |

n/m, not measurable (too slow to measure).
onto the IgE–Fc–sFcRια structure shows that although the residues involved in the two interactions are distinct, the αFab and receptor molecules clash (Fig. 5d). Therefore, αFab1 and sFcRια compete for binding to the bent form of IgE–Fc. This was confirmed using SPR to determine whether αFab1 alone is capable of inhibiting sFcRια binding to IgE–Fc, or whether αFab2 is required to trap the molecule in an extended conformation before inhibition occurs.

αFab was first immobilized on an SPR biosensor surface and binding of IgE–Fc was tested over a range of concentrations (Supplementary Fig. 5a). The $K_\text{d}$ of αFab1 binding to IgE–Fc was found to be 95 nM (±6 nM), in agreement with ITC results (Table 2). Subsequently, binding of αFab2 to form αFab1–IgE–Fc–αFab2 was characterized. After immobilization of αFab on the SPR biosensor surface, IgE–Fc was flowed over the chip to form the αFab1–IgE–Fc complex, followed by αFab2 to complete the trimolecular complex (Supplementary Fig. 5b). On the basis of an equilibrium analysis, the $K_\text{d}$ of αFab2 binding was estimated to be 1.4 µM (±0.1 µM), again in good agreement with ITC results. To determine how binding of αFab1 to IgE–Fc affects the binding of sFcRια, the αFab1–IgE–Fc complex was first formed as described above, and before this complex could dissociate, sFcRια was passed over the surface in increasing concentrations. No binding of sFcRια was observed up to a concentration of 1 µM, confirming that binding of a single αFab is sufficient to inhibit receptor binding to IgE–Fc (Supplementary Fig. 5c). αFab thus inhibits receptor binding by a steric mechanism when IgE–Fc is bent and also allosterically when IgE–Fc is extended.

**Modeling membrane-bound IgE**

We previously modeled the conformations accessible to the Fab arms of IgE using the bent, receptor-bound structure of IgE–Fc16. This revealed little overlap between the spaces explored by the two Fab arms, one of which was directed away from, the other parallel to, the membrane, consistent with both allergen recognition and receptor cross-linking (Fig. 6a,b). However, it is difficult to see how a rigidly bent IgE molecule could function in allergen recognition in the BCR because the Fab arms would be directed toward the membrane (Fig. 6c). To test this, we oriented the ensemble of whole IgE models reported previously16 in a membrane-bound position, with a spacer to represent the extra membrane-proximal domains (EMPD) that separate the C termini of the Cε4 domains from the membrane (Fig. 6d).

Irrespective of spacer length (the EMPD structure is unknown), the Fab arms only explore space close to the membrane. To determine how an extended conformation of IgE–Fc would present the Fab arms, we implemented the same method of model generation and positioned 2,000 models of IgE relative to the membrane. Extended IgE–Fc
conformations orient the Fab arms away from membrane, optimally for allergen binding (Fig. 6e,f).

DISCUSSION

Ever since the first model of a bent structure for IgE6–9, the determination of the acutely and asymmetrically bent IgE-Fc by X-ray crystallography13 and demonstration by X-ray solution scattering and FRET that this structure predominates in solution9,11,12,16, the idea that the (Cε2)3 domain pair might flip from one side to the other has been only a tantalizing possibility. Fluorescence depolarization analyses of segmental flexibility in IgE and electron microscopy (EM) of immune complexes had shown that the molecule was less flexible than IgG3–6,15,32, and in the crystal structure of its complex with sFceRIα,14 IgE-Fc was found to be even more acutely bent, consistent with FRET studies in solution16 and when receptor-bound on cells6. Only the binding of omalizumab Fab to IgE-Fc in solution, monitored by FRET, indicated that a partial unbending of IgE-Fc was possible16.

The observation of a fully extended IgE-Fc in the αFab complex reported here was therefore a considerable surprise. We have now demonstrated by MD simulation and solution biophysical studies that IgE-Fc can indeed unbend and adopt a range of transiently extended conformations, suggesting a possible pathway for the Cε2 domains to flip from one side of the Ce3–Ce4 to the other.

The degree of flexibility described for IgE-Fc here is unlike any reported for other antibody classes. Flexibility between the V and C domains in Fab fragments (‘elbow bending’) and within the hinge separating Fab and Fc regions in IgG antibodies is well documented, but conformational variation within the Fc has to date been limited to minor changes in the orientation of the Cγ2 domains of IgG19 or the Ce3 domains of IgE19. Open and closed conformations for the latter have been reported in structures of IgE-Fc and Fcε3–4 both free and when bound to either sFceRIα14,16 or to sCD23, the soluble IgE-binding domain of the ‘low-affinity’ receptor on B cells34. The conformational diversity in IgE-Fc reported here is substantially greater than previously seen in Fc structures. In fact, the earlier fluorescence depolarization data on IgE should be reinterpreted in terms of bending at the Cε2-Cε3 linker regions in addition to bending between Fab and Fc.

IgM, an evolutionary precursor of IgE, also contains a domain pair, (Cμ2)3, rather than a flexible hinge, and is known to undergo substantial conformational changes. Free in solution it adopts planar, star-shaped pentameric or hexameric structures, as determined by small-angle X-ray and neutron scattering and EM studies35,36, but when bound by Fab arms of two or more subunits to an antigenic surface, it adopts ‘table-like’ structures with the disc of penta- or hexameric Fc regions at 90° to the Fab arms35. However, the location of the bend in the molecule is unknown. Modeling of IgM structures against solution-scattering data placed the bend between Cμ2 and Cμ3 (ref. 36), as suggested earlier in fluorescence depolarization studies37, whereas later modeling against cryo-EM data was consistent with bending between Cμ1 and Cμ2 (ref. 38). Recent crystallographic analyses of individual IgM-Fc domains leaves the question unresolved39. It is clear, however, that at least in the context of the polymeric structures, the monomeric IgM subunit can adopt either linear or bent conformations, and if the flexibility in IgE is evolutionarily related to that of IgM, bending of the latter would be predicted to occur between Cμ2 and Cμ3. Conformational change in IgM is critical for function, as dislocation upon antigen binding reveals sites for complement binding that are hidden in the planar structures. There is therefore a precedent for an extended conformation of an antibody with the ‘additional’, hinge-replacing domains that has a clear biological function.

That IgE evolved conformational diversity to allow both a compact bent and an ensemble of extended, flexible structures may be understood in terms of its allergen-binding functions in two distinct contexts. Bound to FceRI on effector cells (mast cells, basophils or antigen-presenting cells), bent IgE presents its Fab arms appropriately for allergen recognition and cross-linking16, which triggers the allergic response (Fig. 6a,b). In contrast, as part of the BCR, in which...
the role of membrane IgE is to capture allergen and ensure B-cell survival and proliferation, only an extended molecule presents the Fab arms optimally for detection of allergen (Fig. 6c–f). One recognized advantage of conformational flexibility is ‘fly casting’: that is, the ability to sample a greater range of conformational space and make ligand (allergen) capture more effective. Conventional depictions of the BCR for all antibody classes show extended Fab arms, and the extended IgE-Fc structure reported here demonstrates that such a conformation is feasible.

In the extensively studied IgM BCR, which contains a single IgM subunit, it has been assumed, in the absence of a crystal structure, that IgM adopts an extended conformation. It has also been proposed that conformational changes occur in the IgM-Fc upon antigen binding as part of the signaling function. Our results for IgE-Fc provide support for the notion of an extended IgM-Fc and suggest one way in which a conformational change might occur. Without knowing the structure of the EMPDs that connect the Ce4 domains to the transmembrane regions of the ε-chains, or how the accessory Igα and Igβ chains interact with membrane-IgE or membrane-IgM, it remains a matter for speculation how extended structures might be stabilized. A model for the association between Igα and Cα4 of IgM has been proposed on the basis of the crystal structure of Igκ, but it is hard to see how interaction with Cα4 alone could stabilize the extended conformation. Structural studies of the EMPD and the interactions between IgE-Fc and Igα and Igβ are clearly required.

We now consider the nature of the free IgE molecule. The MD analysis shows that the bent conformation exists in a relatively deep energy well (Fig. 2c, conformation 1) and that the rate-limiting step to reaching the extended conformations, and then flipping to the alternative bent conformation with the Ce2 domains packed against the other side of the Fcε3-4, is the escape from this well. Once this occurs, the energy barriers between various extended conformations, including the barrier as the IgE-Fc passes through the linear conformation, are much lower (Fig. 2b,c, conformations 2–4). Different pathways for unbending may be envisaged, but the most highly populated are expected to have free-energy profiles similar to that sketched in Figure 2c. Although kinetic information may not be directly extracted from the simulations presented here, as biasing forces were applied, an estimate for the rate of unbending may be made if we assume that the process of escape from the well that corresponds to the bent conformation can be described by passage over a single free-energy barrier. The resulting rate of 0.15 s⁻¹ (see Online Methods for equation and parameters used) must, however, be considered a very approximate calculation. The free-energy difference between the bent and the various extended states (≈8 k_BT, Fig. 2c) implies that only a very small fraction of the molecules occupy an extended conformation at any one time. This is in accord with studies of IgE-Fc in solution, which indicate that the bent conformation predominates.

Comparison of the structures of IgE-Fc or Fcε3-4 with sFcεRIα and sCD23 has shown that, although the binding sites are spatially distinct, occurring at either end of the Ce3 domains, they are allosterically linked such that IgE cannot bind both receptors simultaneously. This mutual exclusion is vital for controlling IgE function. For example, cross-linking of FcεRI-bound IgE by oligomeric CD23 would trigger the allergic response in the absence of allergen. Flexibility in IgE-Fc, in particular the relative disposition of the Ce3 domains, is key to determining their receptor-binding capacity, and therefore understanding this allostery and conformational flexibility is important for targeting IgE’s receptor interactions for therapeutic benefit.

We have shown here that aFab inhibits FcεRI binding both sterically and allosterically (Fig. 5). Because extended IgE-Fc conformations cannot bind FcεRI, it may be possible to exploit the intrinsic capacity of the molecule to unbend and enhance the dissociation of IgE from the receptor. Indeed, the recent demonstration that accelerated dissociation of IgE from FcεRI can occur upon binding of a DARPin may be explained in terms of the flexibility that we have identified in IgE-Fc. The reported structure of this DARPin complexed with a disulfide-constrained Fcε3-4 (ref. 45) (lacking Ce2 domains) leaves its mechanism of action upon IgE unclear. We do know, however, that the binding of the anti-IgE omalizumab Fab causes a partial unbending of IgE-Fc as monitored by FRET, which may contribute to its mechanism of action. In any event, the discovery that IgE can flex between bent and transiently occupied extended conformations provides a new framework for understanding IgE function in allergen recognition, and offers a more complete description of the structure of this important therapeutic target.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors for aFab–IgE-Fc–aFab have been deposited in the RCSB Protein Data Bank with accession code 4J4P.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

N.D. and B.P.C. contributed equally to this work. N.D. performed the crystallography and structure analysis, N.D. and J.M.M. conducted SPR experiments, and N.D. and B.J.S. wrote the manuscript. B.C. undertook the molecular dynamics and contributed to writing. A.H.K. was responsible for the ITC and stopped-flow analyses, M.W. generated the aFab molecule, K.C. performed antibody engineering, H.H. expressed the proteins, A.O. purified the proteins, J.D. and L.K.S. collected intramolecular FRET data using reagents made by M.W.-P.K. and A.J.H. J.M.M., A.J.H. and A.J.B. contributed to writing, data interpretation. B.J.S. designed and supervised the research.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Protein expression and purification. Anti-human IgE-Fc antibody was supplied by UCB and isolated V-region genes sub-cloned in the human IgG1-Fab format. arFab was expressed by transient transfection in CHO cells, cultured in CD-CHO medium with the addition of 10 mM glutamine at 37 °C with 8% CO2 and a rotation of 140 rpm. We resuspended 2 × 108 cells/ml in Earles Balanced Salt Solution before adding 400 µg of DNA. Cells were electropropated and resuspended in 100 mL of CD-CHO medium and incubated for 24 h. Incubation continued at 32 °C for 13 days and at 4 days post-transfection sodium butyrate (3 mM final concentration) was added to the culture. On day 14 post-transfection, cell culture supernatants were harvested by centrifugation (400g for 1 h) for purification.

arFab was purified by Protein G affinity chromatography (GE Healthcare) and bound arFab eluted in 100 mM glycine-HCl, pH 2.7, and fractions neutralized with 1/25th fraction volume of 2 M Tris-HCl, pH 8.0. arFab was further purified by size-exclusion chromatography (SEC) on a Superdex S200 column (GE Healthcare) in 25 mM Tris-HCl, 20 mM NaCl, 0.05% (w/v) NaN3, pH 7.5.

IgE-Fc(N265Q,N371Q) secreted from a stable NS-0 cell line was purified from tissue culture supernatant by cation exchange. Supernatant was buffer-exchanged at beamline I03, Diamond Light Source (Harwell, UK). Xia2 (ref. 47) was used assuming a single rate-limiting barrier according to equation (16) of Whitford et al. (ref. 26): 1/kt = 1/C exp(ΔG/RT) (1)

where k is the transition rate, C is the "attempt frequency" for barrier crossing and ΔG is the barrier height. A value for C was taken as 1014 s−1 since many protein folding and functional transitions have been found in the range of ~1–10 µs−1 (ref. 26) and the barrier height was taken as 18 k_BT (Fig 2c).

Crystallization. Sitting drop vapor diffusion crystallization experiments were set up with a protein complex concentration of 5 mg/mL in 20 mM NaCl, 25 mM Tris-HCl, pH 7.5, and 0.05% sodium azide. Crystals were grown at 18 °C using 12–22% PEG3350, 0.25 M sodium citrate, and 0.1 M Bis-Tris Propane, Tris-HCl, pH 7.5, and 0.05% sodium azide. Crystals were further purified by SEC on a Superdex S200 column (GE Healthcare) in PBS, pH 7.4.

Data collection and structure determination. Diffraction data were collected at beamline I03, Diamond Light Source (Harwell, UK). Xia2 (ref. 47) was used to index, integrate and merge data to 2.9 Å resolution65–54. The phases were solved using Phaser molecular replacement53. To generate the arFab search model, the RCSB PDB protein sequence search engine was used to find 3QHZ, from which nonconserved residues were removed using CHAINSAW56,57. IgE-Fc search models were generated by splitting the coordinates from the high-resolution IgE-Fc structure (PDB 2WQR4) into (Cε3-4), and (Cε3-4)2 fragments. Molecules were located in the asymmetric unit sequentially: arFab was followed by (Cε3-4), arFab, and finally (Cε3-4). The structure was initially rebuilt using the autobuild wizard of PHENIX58, and then refined using iterative cycles of PHENIX58, 59. Carbohydrate and refinement cycles, the structure was manually built into 2εFab and 2εFab, electron density maps using COOT59. Composite omit maps were generated using the autobuild wizard in PHENIX to prevent model bias58. Carbohydrate and water molecules were manually built into the structure. MolProbity60 and CARP61 were used to assess protein and carbohydrate geometry, respectively: 93.6% of residues are within the Ramachandran favored region, and 0.2% are outliers. PISA62, CONTACT and NCONT as part of the CCP4 program suite64 were used for analysis of protein-protein interfaces, and DynDom63 was used to calculate the domain motion involved in the conformational changes. Structure morphs for movies were calculated using the UCSF CHIMERA package64, and videos made using PyMOL (http://www.pymol.org/).

Enhanced molecular dynamics. The bent crystal structure (PDB 100V13) was used as the starting point for molecular dynamics simulation, after adding hydrogen atoms and removing terminal residues to symmetrize the two chains. Protonation states were predicted with Maestro (Schrödinger LLC). After initial exploratory simulations of unbending, manual checks were made for possible changes to protonation states in the unbent conformations. No changes in protonation states were found that could be justified, and thus the initial states were retained for the final simulation. The AMBER99SB-ildn and GLYCAM force fields were used for protein and carbohydrate, respectively. The structure was solvated in a truncated octahedron (dimensions 123 × 123 × 123 Å) such that no protein atom of the extended IgE-Fc conformation was within 8 Å of the edge after pressure equilibration. Monatomic ions were added to a salt concentration of 0.15 M. The final simulation system had 129,459 atoms.

Simulations were carried out with NAMD 2.8 (ref. 65) patched with Plumed-1.3 (ref. 46). Particle mesh Ewald68 was used for long-range electrostatics along with 9 Å cut-offs for Coulomb and Lennard-Jones potential functions. A preliminary 500 ns unbiased simulation was used to extract two collective variables (CVs) through principal component analysis (PCA). Only every other α-carbon was included in the PCA CVs. An exploratory metadynamics simulation then used these PCA CVs to explore unbending for 1,200 ns with Gaussians of height 8 kJ/mol and sigma of 0.5 Å added every 4 ps. This exploratory metadynamics run then provided new PCA CVs for a final simulation, hence the final CVs were extracted from a simulation that fully sampled unbending. The final multiple-walker metadynamics simulation converged after 3,755 ns using Gaussians of height 4 kJ/mol added every 4 ps. Convergence of this calculation was gauged by the reduction of the Gaussian heights being added (50–100 times smaller than at the beginning) and a convergence in the relative depths of the lowest free energy wells. Additionally, the converged simulation walkers were able to move through CV space relatively freely, suggesting the suitability of the CVs.

To confirm the results of the metadynamics simulation, we carried out a short (250 ns) unbiased simulation starting from the extended crystal conformation of IgE-Fc (Supplementary Fig. 3d).

An estimation of the rate of transition from a bent to an unbent conformation was made assuming a single rate-limiting barrier according to equation (16) of Whitford et al. (ref. 26): 1/kt = 1/C exp(ΔG/RT) (1)

where k is the transition rate, C is the "attempt frequency" for barrier crossing and ΔG is the barrier height. A value for C was taken as 1014 s−1 since many protein folding and functional transitions have been found in the range of ~1–10 µs−1 (ref. 26) and the barrier height was taken as 18 k_BT (Fig 2c).

Isothermal titration calorimetry. Experiments were carried out using a Microcal iTC200 calorimeter (GE Healthcare) at 20 °C in PBS buffer pH 7.4. Depending on the final ratio required, 25–30 µM of protein were used in the cell with 10–20 times higher concentrations in the syringe. The number and volume of injections were varied as appropriate. Heats of dilution were subtracted from the data before analysis. Analyses were carried out using MicroCal Origin, using a 1:1 binding model when IgE-Fc or Fcε3-4 was titrated into arFab, or a sequential 2:1 (non-identical) binding model when arFab was titrated into IgE-Fc or Fcε3-4. The non-identical 2:1 binding model was selected based on prior knowledge of the crystal structure, and observation that the curve clearly deviated from an identical 2:1 binding mode.

Stopped-flow fluorescence. Experiments were carried out using a Chirascan Plus (Applied Photophysics) with a stopped-flow attachment at 20 °C in PBS buffer, pH 7.4, and with pseudo-first order protein concentrations varied as required. Fluorescence was excited at 280 nm (1 nm slit width) and emitted fluorescence above 305 nm detected with a long-band pass. We averaged 6–10 runs for each experiment. Data were collected and analyzed using supplied software according to the manufacturer’s instructions. Experimental transients were fitted either to single-exponential (equation (2)): [IgE-Fc] or [Fcε3-4] > [arFab] and only arFab
binding observable) or double-exponential (equation (3); [arFab] > [IgE-Fc] or [Fcε3-4] and binding of both arFab1 and arFab2 observable) equations:

\[ F = \Delta F_1 \exp(-k_{obs1}t) + F_e \]  
(2)

\[ F = \Delta F_2 \exp(-k_{obs2}t) + F_e \]  
(3)

where \( F \) is the observed fluorescence, \( \Delta F \) is the fluorescence amplitude change for the nth transient, \( k_{obs} \) is the pseudo-first order rate constant for the nth step and \( F_e \) is the end-point fluorescence. The bimolecular association rate constants for arFab1 (\( k_{1} \)) and arFab2 (\( k_{2} \)) binding were determined by fitting the linear concentration dependences of \( k_{obs1} \) and \( k_{obs2} \) to equation (4):

\[ k_{obs} = k_{1n}[\text{ligand}] + k_{2n} \]  
(4)

where \( k_{obs} \) is the pseudo-first order rate constant for the nth transient at the ranges of ligand concentrations used, \( k_{1n} \) is the association rate constant for the nth arFab binding event and \( k_{2n} \) is the dissociation rate constant for the nth Fab binding.

FRET. Intramolecular FRET was carried out using IgE-FcE289C-BirA (IgE-Fc with the BirA recognition motif added to the C terminus and biotinylated according to the manufacturer’s instructions (Avidity)), labeled with thiol-reactive terbium chelate (Invitrogen) and monovalent streptavidin67 labeled with amine-reactive Alexa488 (Invitrogen), each according to the manufacturer’s instructions. Terbium-labeled IgE-Fc and Alexa488-labeled streptavidin were mixed in equimolar ratios (final assay concentration 25 nM) with arFab titrated to 30 µM in PBS and incubated for 120 min at room temperature. FRET was measured on an Analyst LT-LHT with excitation 330 nm (80 nm slit width), emission 485 nm in running buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM CaCl2, and 0.005% (v/v) Surfactant P-20). For sandwich assays, IgE-Fc at 100 nM in running buffer was injected over the sensor chip for 180 s at a flow rate of 25 µl min\(^{-1}\). Measurements were performed at 25 °C. Double referencing data subtraction methods were performed68.

Molecular modeling of whole, extended IgE. FPMOD69 was used to model whole, extended IgE molecules. The Fab domains from an IgE-like antibody (PDB 2R56) were fused onto the extended IgE-Fc structure, and allowed to move rigidly about a flexible linker until 2,000 models had been generated.

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