Accelerated disassembly of IgE–receptor complexes by a disruptive macromolecular inhibitor

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IgE antibodies bind the high-affinity IgE Fc receptor (FceRI), found primarily on mast cells and basophils, and trigger inflammatory cascades of the allergic response1–3. Inhibitors of IgE–FceRI binding have been identified and an anti-IgE therapeutic antibody (omalizumab) is used to treat severe allergic asthma4,5. However, preformed IgE–FceRI complexes that prime cells before allergen exposure dissociate extremely slowly6 and cannot be disrupted by strictly competitive inhibitors. IgE–Fc conformational flexibility indicated that inhibition could be mediated by allosteric or other non-classical mechanisms7–9. Here we demonstrate that an engineered protein inhibitor, DARPin E2_79 (refs 9–11), acts through a non-classical inhibition mechanism, not only blocking IgE–FceRI interactions, but actively stimulating the dissociation of preformed ligand–receptor complexes. The structure of the E2_79–IgE-Fc3,4 complex predicts the presence of two non-equivalent E2_79 sites in the asymmetric IgE–FceRI complex, with site 1 distant from the receptor and site 2 exhibiting partial steric overlap. Although the structure is indicative of an allosteric inhibition mechanism, mutational studies and quantitative kinetic modelling indicate that E2_79 acts through a facilitated dissociation mechanism at site 2 alone. These results demonstrate that high-affinity IgE–FceRI complexes can be actively dissociated to block the allergic response and suggest that protein–protein complexes may be more generally amenable to active disruption by macromolecular inhibitors.

The IgE antibody Fc, comprising three domains (Cε2–Cε3–Cε4), binds the α-chain of FceRI (FceRIα) with subnanomolar affinity (<1 nM)4,12. The IgE–Fc Cε3 domains contact receptor directly and can adopt multiple conformational states, ranging from closed to open forms13–15, which could have an impact on FceRI binding and potential receptor complex dynamics. In an effort to characterize different IgE ligands and mechanisms of FceRI inhibition, we developed a fluorescence binding assay that distinguishes IgE ligands using a site-specific reporter fluorophore. A double mutant (C328A/K367C) of the IgE–Fc Cε3–Cε4 protein (IgE-Fc3,4) was labelled with Alexa Fluor 488 at residue 367 (referred to as AF488–Fc), which is adjacent to the FceRIα binding site (Supplementary Fig. 1). AF488–Fc exhibited systematic fluorescence quenching with increasing concentrations of FceRIα (Fig. 1a), yielding a dissociation constant (Kd) of ~22 nM (Supplementary Table 1), consistent with the lower affinity of the C328A mutation16. FceRIα-directed inhibitors, such as unlabelled IgE-Fc3,4 and anti-FceRIα antibody (monoclonal antibody 15.1)17,18, reversed receptor-induced fluorescence quenching (Fig. 1b, c and Supplementary Table 1).

IgE-directed inhibitors, including the anti-IgE antibody omalizumab (Xolair)19, a 34-nucleotide DNA aptamer (D17.4)20,21, and DARPin E2_79 (refs 9–11), yielded three inhibition profiles. Omalizumab induced fluorescence quenching comparable to FceRIα (Fig. 1d and Supplementary Table 1), consistent with its binding an epitope overlapping the FceRIα site16,17. E2_79 restored the receptor-quenched fluorescence signal (Fig. 1e and Supplementary Table 1), similar to FceRIα binding inhibitors (Fig. 1b, c). D17.4 did not quench or compete with FceRIα, but in an indirect competitive binding experiment with AF488–Fc, FceRIα and unlabelled wild-type IgE-Fc3,4, D17.4 induced systematic fluorescence quenching (Fig. 1f and Supplementary Table 1).

Figure 1 | A fluorescence-quenching assay reveals different classes of IgE-directed inhibitors. a, AF488–Fc fluorescence is quenched by FceRIα. RFU, relative fluorescence units. b, Unlabelled IgE-Fc3,4 competes for FceRIα binding (filled circles, solid line) but has no effect on AF488–Fc fluorescence (open circles, dotted line). c, The anti-FceRIα antibody mAb15.1 competes for FceRIα binding (filled circles, solid line) but has no effect on AF488–Fc fluorescence (open circles, dotted line). d, Omalizumab quenches AF488–Fc fluorescence similar to FceRIα. e, E2_79 competes for FceRIα binding (filled circles, solid line) but does not affect AF488–Fc fluorescence (open circles, dotted line). f, D17.4 competes in assays containing AF488–Fc, FceRIα and unlabelled wild-type IgE-Fc3,4, by binding IgE-Fc3,4 competitor (filled circles, solid line). Error bars represent standard deviations of replicate measurements.

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consistent with D17.4 binding to wild-type IgE-Fc \(_4\), but not AF488–Fc. These data indicated that D17.4 and omalizumab act as direct competitive inhibitors, but E2_79 was a candidate allosteric inhibitor.

We determined the 4.3 Å crystal structure of E2_79 bound to IgE-Fc \(_4\) (Supplementary Table 2) using a cysteine mutant (C335) that locks the Fc into a closed conformational state\(^{26}\). E2_79 binds the IgE C\(_3\) domain and does not directly engage residues involved in Fc:Rl binding (Fig. 2a, b). E2_79 interactions extend throughout the C\(_3\) domain, including the C\(_3\)–C\(_4\) domain linker and encroaching on Fc:Rl binding loops (Fig. 2a, c).

To examine the structural basis for E2_79 inhibition, we superimposed the E2_79 structure onto the IgE–Fc–Fc:Rl complex using the IgE C\(_3\) domains. The IgE–Fc–Fc:Rl complex is asymmetric, defining two distinct E2_79 sites (Fig. 2b). In the complex, site 1 is entirely exposed, with E2_79 and Fc:Rl separated by ~20 Å and no steric overlap (Fig. 2b), indicating the potential for simultaneous E2_79 and Fc:Rl binding. For site 2, three E2_79 and five Fc:Rl residues make contacts <3.5 Å (Supplementary Table 3), causing partial steric overlap.

We generated three E2_79 double mutants (E20A/R23A, Y45A/R23A, and E20A/R23A/Y45A) to probe the inhibition mechanism (Fig. 2c). E20 and R23 are located adjacent to the C\(_3\)–C\(_4\) domain linker and could affect the C\(_3\) domain conformational state, allosterically inhibiting Fc:Rl. Y45 and W46 are in the hydrophobic interface with the IgE Fc, and are probably important for binding affinity. E126 and D127 account for most of the predicted steric conflicts with Fc:RI at site 2 (Supplementary Table 3) and could potentially interact with the Fc:RI FG binding loop containing R427, contributing to the inhibition. The E20A/R23A and E126A/D127A mutants exhibited similar binding affinity to IgE Fc compared to wild-type E2_79 (Fig. 3a, b), whereas the Y45A/W46A mutant showed significantly reduced binding affinity (Fig. 3c). The inhibition activities of the E20A/R23A and E126A/D127A mutants remained similar compared to that of wild-type E2_79 (Fig. 3d, e), indicative of a non-allosteric model. The Y45A/W46A mutant exhibited no inhibition (Fig. 3f), as expected from its lack of binding (Fig. 3c). The mutants validated the crystal structure, and indicated that E2_79 inhibition is not due to C\(_3\)–C\(_4\) linker or Fc:RI binding loop interactions.

To investigate binding of E2_79 to the IgE–Fc:Rl complex, we conducted Biacore experiments that revealed a marked acceleration of complex disassembly. Fc:Rl was coupled to the chip, then loaded with either full-length IgE (Sus11) or IgE–Fc \(_4\) proteins (Supplementary Fig. 2). The monoclonal anti-IgE antibody Le27 recognizes a non-inhibitory epitope in the IgE C\(_4\) domain, and binding to the preformed IgE–Fc:Rl complexes was readily observed (Supplementary Fig. 2a, e). In contrast, E2_79, but not controls, stimulated rapid dissociation of both Sus11 and IgE–Fc \(_4\) complexes (Supplementary Fig. 2b–d, f–h).
E2_79 and FcεRIα binding to IgE are linked by a six state coupled binding reaction scheme (Fig. 4a, b), in which two E2_79 molecules and one FcεRIα protein can potentially assemble into a quaternary complex with IgE. We considered three potential mechanisms to explain the accelerated complex dissociation: (1) a classic competitive inhibition mechanism operating solely through steric conflicts at site 2; (2) an allosteric mechanism operating through both site 1 and site 2; (3) a facilitated dissociation mechanism in which E2_79 binding to IgE–FcεRIα complexes only at site 2 stimulates complex dissociation. Model 3 represents a competitor-induced dissociation mechanism in which a subset of ligand attachment points that become exposed during partial complex dissociation can be engaged by an exogenously added competitor, thereby accelerating the intrinsic dissociation rate. This has been named ‘facilitated dissociation’.

Figure 4 | Kinetic modelling of SPR data points to a facilitated dissociation mechanism for E2_79. a, Reaction scheme for the allosteric model. Blue arrows indicate that IgE–FcεRIα complex dissociation is accelerated by E2_79 binding to site 1 and site 2. b, Reaction scheme for the facilitated dissociation model. The blue arrow indicates that IgE–FcεRIα complex dissociation is only accelerated by E2_79 binding to site 2. c, d, Best global curve fitting result for the allosteric model, shown with seven concentrations of E2_79 (c), or the 100 nM and 200 nM E2_79 concentration curves (d). e, f, Best global curve fitting result for the facilitated dissociation model, shown with seven concentrations of E2_79 (e), or the 100 nM and 200 nM E2_79 concentration curves (f). Rate and equilibrium dissociation constants highlighted in yellow were fit as described in Methods.

The classic competitive inhibition mechanism is not consistent with the SPR data, but predicts high-affinity binding of E2_79 to site 1 and no acceleration of IgE release from the chip surface (Supplementary Fig. 3a, b). Simulations for this model are similar to the experimental observations with the Le27 antibody (Supplementary Fig. 2a, e). In this model, E2_79 competition is only mediated by site 2, where steric hindrance prevents E2_79 binding to IgE–FcεRIα complexes. Relaxation to a new equilibrium occurs with the unaccelerated, slow dissociation rate of the complex.

For the allosteric model, we posited that both site 1 and site 2 would contribute to FcεRIα inhibition and the acceleration of complex dissociation. In this model, E2_79 binding to sites 1 and 2 in the IgE–FcεRIα complex should both exhibit reduced affinity associated with the allosteric coupling. Similarly, both site 1 and site 2 binding by E2_79 would induce reduced affinity and increased dissociation rates
for FccRIx (Fig. 4a). Owing to this allosteric coupling, IgE–FccRIx complexes should not have any high-affinity binding sites for E2_79.

For the facilitated dissociation model, we posited that E2_79 binding to site 1 is not affected by FccRIx binding, consistent with the predicted >20 Å distance between these proteins (Figs 2b and 4b). There is also correspondingly no change in FccRIx binding affinity associated with E2_79 binding to site 1. The rate constants for both of these binding steps were initially set to the experimentally determined rate constants for E2_79 and FccRIx binding in the absence of the other ligand. In this model, only the binding affinity of E2_79 at site 2 is reduced by FccRIx, through this non-classical competition mechanism. Similarly, there must be a corresponding reduction in FccRIx binding affinity in the quaternary E2_79–IgE–FccRIx–E2_79 complex. Importantly, in this model, site 1 retains native high affinity for E2_79 in the IgE–FccRIx complex, whereas only site 2 exhibits reduced E2_79 affinity and the ability to accelerate FccRIx dissociation. This distinguishes it from the allosteric model, which lacks any high-affinity E2_79 sites in the complex.

The initial 120 s of SPR data for IgE–Fc_{3-a} (Supplementary Fig. 2h), covering E2_79 concentrations from 100 nM to 10 μM, were simultaneously fit using the allosteric and facilitated dissociation COPASI models. Curve fitting (Fig. 4c–d) demonstrates that only the facilitated dissociation mechanism captures the biphasic behaviour of the SPR traces accurately. The SPR data exhibit a ~50 resonance units (RU), E2_79-concentration-dependent association phase within the first 10–20 s that cannot be modelled by the full allosteric mechanism (Fig. 4c, d). Whereas both models fit the later concentration-dependent dissociation rates for the IgE–FccRIx complexes, the initial 10–20 s demonstrate an association that corresponds to the presence of the high-affinity binding of E2_79 to the exposed site 1 within the IgE–FccRIx complex. Given the robustness of the fitting, we allowed site 1 kinetic constants in the facilitated dissociation model to vary from experimental values for both E2_79 and FccRIx (Supplementary Table 4). This provided slightly improved curve fitting, and the fitted E2_79 site 1 kinetic constants remained close to those determined for the independently binding ligand, further validating this model over an allosteric one.

Non-classical or allosteric inhibitors have the general potential to not only block receptor-ligand binding, but also to engage and actively disassociate preformed receptor complexes. For the IgE–FccRIx interaction, the activated release of receptor-bound antibody on the surface of effector cells might prove beneficial in treating acute allergic reactions. Here we demonstrated that the E2_79 DARPin is a disruptive inhibitor that binds to IgE–FccRIx complexes, markedly accelerating their dissociation orders of magnitude over the unaccelerated intrinsic rate.

It has been proposed that facilitated dissociation occurs through competition for subsite attachment points within a ligand–binding site, which becomes partially exposed before full ligand dissociation. In principle, competitor-induced acceleration of ligand dissociation can be explained by multiple mechanisms, such as allosteric or facilitated dissociation mechanisms considered here, but few observations have been fully examined by both structural and kinetic methods. Small-molecule inhibitors of TNF have been shown to accelerate the dissociation of TNF trimers, potentially intercalating into the trimeric interface. It has been suggested, and it is probably commonly assumed, that larger macromolecular inhibitors depend on complete dissociation of protein complexes and would be unlikely to engage partially dissociated intermediates, potentially providing a ‘kinetic advantage’ to small-molecule protein interaction inhibitors. However, the E2_79-induced dissociation of IgE–FccRIx complexes indicates that macromolecular therapeutics may have untapped potential as disruptive inhibitors. Antibody-induced complex dissociation has been observed, but specific mechanisms and requirements for such accelerated dissociation have not been fully investigated. Facilitated dissociation may also have an unappreciated role in naturally evolved macromolecular complex disassembly processes.

**METHODS SUMMARY**

IgE and FccRIx were produced in insect cells, whereas E2_79 was produced in *Escherichia coli* as described. Other proteins or DNA ligands (omalizumab, Mab15-1, D17.4, Sus11) were purchased or obtained as described in Methods. IgE–Fc_{3-a} and E2_79 mutants were generated by site-directed mutagenesis and confirmed by DNA sequencing. Fluorescence binding experiments and ELISA assays were performed using a Synergy 4 multi-mode plate reader (BioTek), with labelled AF488–Fc produced as described in Methods. Diffraction data for the IgE–Fc_{3-a}–C335–E2_79 complex was collected at the Advanced Light Source beamline 8.3.1 and the structure solved by molecular replacement. SPR assays were conducted with a BIACore X100 machine and evaluated with BIAevaluation and COPASI software.

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

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1. Kraft, S. & Kinet, J. P. New developments in FcRI regulation, function and inhibition. *Nature Rev. Immunol.* 7, 365–378 (2007).
2. Gould, H. J. & Sutton, B. J. IgE in allergy and asthma today. *Nature Rev. Immunol.* 8, 205–217 (2008).
3. D’Amato, G., Bucchioni, E., Oldani, V. & Canonica, W. Treating moderate-to-severe allergic asthma with a recombinant humanized anti-IgE monoclonal antibody (Omalizumab). *Treat. Respir. Med.* 5, 393–398 (2006).
4. Chen, T. W. The pharmacological basis of anti-IgE therapy. *Nature Biotechnol.* 18, 157–162 (2000).
5. Holdom, M. D. et al. Conformational changes in IgE contribute to its uniquely slow dissociation rate from receptor FcRI. *Nature Struct. Mol. Biol.* 18, 571–576 (2011).
6. Dhaliwal, B. et al. Crystal structure of IgE bound to its β-cell receptor CD23 reveals a mechanism of reciprocal allosteric inhibition with high affinity receptor FcRI. *Proc. Natl Acad. Sci. USA* 109, 12686–12691 (2012).
7. Wurzburg, B. A. & Jardetzky, T. S. Conformational flexibility in immunoglobulin E-Fc_{3-a} revealed in multiple crystal forms. *J. Mol. Biol.* 393, 176–190 (2009).
8. Wurzburg, B. A., Garman, S. C. & Jardetzky, T. S. Structure of the human IgE-Fc_{3-a} Cα4 reveals conformational flexibility in the antibody effector domains. *Immunity* 13, 375–385 (2000).
9. Eggel, A., Baumann, M. J., Amstutz, P., Stadler, B. M. & Vogel, M. DARPin as bispecific receptor antagonist for IgE-induced mast cell responses. *Omalizumab.* *Nature* 406, 259–266 (2000).
10. Besu, M. et al. Purification and characterization of human recombinant IgE–Fc_{3-a} fragments that bind to the human high affinity IgE receptor. *J. Biol. Chem.* 268, 13118–13127 (1993).
11. Wang, B. et al. Epidermal Langerhans cells from normal human skin bind monomeric IgE via FcRI. *J. Exp. Med.* 175, 1353–1365 (1992).
12. Mendonsa, S. D. & Bowers, M. T. in vitro selection of high-affinity DNA ligands for human IgE using capillary electrophoresis. *Anal. Chem.* 76, 5387–5392 (2004).
13. Wiegand, T. W. et al. High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc receptor I. J. Immunol. 157, 221–230 (1996).
14. Zheng, L. et al. Fine epitope mapping of humanized anti-IgE monoclonal antibody omalizumab. *Biochem. Biophys. Res. Commun.* 375, 619–622 (2008).
15. Wright, J. D. & Lim, C. Prediction of an anti-IgE binding site on IgE. *Proc. Natl Acad. Sci. USA* 109, 120–128 (2007).
16. Garman, S. C., Wurzburg, B. A., Tarchevskaya, S. S., Kinet, J. P. & Jardetzky, T. S. Structure of the Fc fragment of human IgE bound to its high-affinity receptor FcRI. *Nature* 406, 259–266 (2000).
17. Wang, C. et al. Positive and negative site-site interactions, and their modulation by pH, insulin analogs, and monoclonal antibodies, are preserved in the purified insulin receptor. *Proc. Natl Acad. Sci. USA* 85, 8400–8404 (1988).
27. Lowenthal, J. W. et al. High and low affinity IL 2 receptors: analysis by IL 2 dissociation rate and reactivity with monoclonal anti-receptor antibody PC61. *J. Immunol.* **135**, 3988–3994 (1985).

28. Boulain, J. C. & Menez, A. Neurotoxin-specific immunoglobulins accelerate dissociation of the neurotoxin-acetylcholine receptor complex. *Science* **217**, 732–733 (1982).

**Supplementary Information** is available in the online version of the paper.

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**Author Information** The structure factors and model for the E2_79–C335 IgE complex have been deposited in the Protein Data Bank under accession code 4GRG. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to T.S.J. (tjardetz@stanford.edu).
from Invitrogen/Molecular Probes and dissolved in DMSO (dimethyl sulphoxide) (QuickChange kit, Stratagene) and confirmed using a thiol and sulphide quantification kit (Molecular
ature for 1 h. After TCEP treatment, the presence of free thiol groups in the molar equivalents of TCEP (Tris(2-Carboxyethyl)phosphine) at room temper-
Dye labelling of AF488–Fc. 50 nM Fc3-4 (0–200 nM), mAb15.1 (0–100 nM) and E2_79 (0–200 nM). Fc
competition assays, each well in a black 96-well plate was coated with 1 µg ml-1 Fc3-4, 50 nM unlabelled wild-type IgE-Fc3-4, 50 nM FcRz, and increasing concentrations of D17.4 (0–200 nM) in the fluorescence buffer in a final volume of 100 µl. D17.4 ligand was annealed at 70 °C and cooled before use. The AF488–Fc and unlabelled wild-type IgE-Fc3-4 were mixed together, and then 50 nM FcRz and D17.4 were added last together.
Biacore binding assays. SPR assays were conducted with a BiACore X100 machine and evaluated with the BiAevaluation Software, as described in the
method of every binder with 50 mM NaOH. The anti-IgE molecules were injected
8. To remove the C-terminal tag residues, purified IgE-Fc3-4. The binding of E2_79 containing the His-tag to plate-bound
Ri (a 1.32 cm-1 (mg ml-1) at 280 nm). To remove the C-terminal tag residues, purified
expression and purification of proteins. Expression and purification of the soluble FcRI α-chain ectodomain was carried out as previously described5,6. Omalizumab (Xolair) was purchased from Novartis. MAb15.1 (anti-FcRz) was a gift from the Kinet laboratory. Selection and characterisation of the DARPin E2_79 gene was reported elsewhere18. Expression and purification of the E2_79 mutants were carried out as previously described6. The IgE-Fc3-4 cysteine mutants were expressed in insect cells. The wild-type IgE-Fc3-4, C367 and C335 IgE-Fc3-4 proteins were cloned into pENTR1A (Invitrogen) using a KpnI–XhoI fragment and transferred into the BaculoDirect C-term Linear DNA by using LR Clonase II Enzyme Mix (Invitrogen). Recombinant baculo-
viruses expressing wild-type and C367 IgE-Fc3-4 proteins were generated using BaculoDirect C-term Transfection Kit (Invitrogen). Recombinant virus was selected and amplified following standard protocols. The wild-type and C367 IgE-Fc3-4 proteins include 3 non-native residues (ADP) generated by the construct at the N terminus and 5 non-native residues including a V5 epitope and a histidine affinity tag (His tag) at the C terminus. For protein production, Hi5 supernatants were introduced into the original E2_79 gene ranging from 0 nM to 200 nM or 400 nM in duplicate to wells coated with the purified IgE-Fc3-4. The binding of E2_79 containing the His-tag to plate-bound IgE-Fc3-4 was monitored using anti-His tag antibody (Novagen, 70796-3) as a primary antibody and anti-mouse IgG HRP conjugated antibody (R&D system, HA007) as the secondary antibody. The anti-His tag antibody in a 1:1,000 dilution was incubated after washing for 1 h at room temperature, and then, the
mouse IgG HRP conjugated antibody was incubated after washing for 1 h at room temperature. Plates were washed and developed using TMB single solution (Invitrogen, 00-2023). Microplates were read using a Synergy 4 multi-mode plate reader (BioTek) at 650 nm.
COPASI modelling. The COPASI software package for quantitative modelling of biochemical networks22 was used to build the full reaction schemes presented in Fig 4 and Supplementary Fig. 3. The model consists of eight species and seven reactions within a single compartment. Global quantities were defined, corresponding to on/off
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rates for the reaction steps associated with each mechanism, a consolidated ‘Biacore output’ (RU_{calc}) corresponding to reaction intermediates bound to the chip surface, a fractional mass weighting constant (F_{ME}) to account for E2_79 chip-bound species and overall scaling (k_{ov}) and baseline constants (C_0) for fitting the experimental SPR data. The Biacore output (RU_{calc}) represents a combination of the three receptor-bound species affecting the SPR measurements and was defined mathematically as:

\[ RU_{calc} = k_{ov} \cdot (IgE-FCRRI)_t + (1 + F_{ME}) \cdot (E2_{79}-IgE-FCRRI)_t + (1 + 2 \cdot F_{ME}) \cdot (E2_{79}-IgE-FCRRI-E2_{79})_t + C_0 \]

where (IgE-FCRRI)_t, (E2_{79}-IgE-FCRRI)_t, and (E2_{79}-IgE-FCRRI-E2_{79})_t, represent the time-dependent evolution of these reaction species as numerically simulated by COPASI, using the specific model restrictions. Biacore data were extracted using the BiaEvaluation software and imported directly into COPASI for parameter estimation. Each kinetic time course includes 120 data points (seconds), corresponding to a total of 840 data points for the simultaneous fitting of the model parameters. Data fitting was carried out with all available convergence methods, with best results obtained with simulated annealing, particle swarm, Hook & Jeeves, and Levenberg–Marquardt algorithms. The stable convergence of these multiple methods to similar end points was taken as an indicator of the robustness of the fitting and the convergence to a common, global minimum. Experimentally determined rate constants for E2_{79} and FcεRIα were determined in independent Biacore experiments and allowed to vary by 2–3-fold during the data fitting. E2_{79} concentrations used experimentally were also allowed to vary up to 25% and F_{ME} was allowed to vary from 0 to 1. For the allosteric model, kinetic rate constants for site 1 and site 2 E2_{79}–IgE–FcεRIα complexes were set to be equivalent. These four parameters were allowed to vary freely, along with k_{ov} and C_0, yielding a total of six freely varying parameters. For the initial fitting of the facilitated dissociation model, kinetic rate constants for site 1 were restricted to experimentally determined values for E2_{79} binding in the absence of FcεRIα, and only the four kinetic constants associated with site 2 E2_{79}–IgE–FcεRIα–E2_{79} complexes were allowed to vary freely, also yielding a total of six freely varying parameters. For the full fitting of the facilitated dissociation model, kinetic rate constants for both site 1 and site 2 complexes were allowed to vary widely, yielding a total of 10 highly varying parameters.

29. Garman, S. C., Kinet, J. P. & Jardetzky, T. S. Crystal structure of the human high-affinity IgE receptor. Cell 95, 951–961 (1998).
30. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. in Methods Enzymol. Vol. 276 (eds Carter, J. C. W. & Sweet, R. M.) 307–326 (Academic, 1997).
31. McCoy, A. J. et al. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674 (2007).
32. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D 60, 2126–2132 (2004).
33. Chen, V. B. et al. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr. D 66, 12–21 (2010).
34. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R. & Thornton, J. M. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J. Biomol. NMR 8, 477–486 (1996).