

An Intermediate pH Unfolding Transition Abrogates the Ability of IgE to Interact with Its High Affinity Receptor FcεRIα*

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Stephen J. Demarest¹, Jennifer Hopp, Julie Chung, Karen Hathaway, Elisabeth Mertsching, Xianjun Cao, Judy George, Konrad Miatkowski, Michael J. LaBarre, Michael Shields, and Marilyn R. Kehry

From Biogen Idec, San Diego, California 92122

The interaction between IgE-Fc (Fcε) and its high affinity receptor FcεRI on the surface of mast cells and basophils is a key event in allergen-induced allergic inflammation. Recently, several therapeutic strategies have been developed based on this interaction, and some include Fcε-containing moieties. Unlike well characterized IgG therapeutics, the stability and folding properties of IgE are not well understood. Here, we present comparative biophysical analyses of the pH stability and thermostability of Fcε and IgG1-Fc (Fcγ). Fcε was found to be significantly less stable than Fcγ under all pH and NaCl conditions tested. Additionally, the Cε3Cε4 domains of Fcε were shown to become intrinsically unfolded at pH values below 5.0. The interaction between Fcε and an Fcγ-FcεRIα fusion protein was studied between pH 4.5 and 7.4 using circular dichroism and a combination of differential scanning calorimetry and isothermal titration calorimetry. Under neutral pH conditions, the apparent affinity of Fcε for the dimeric fusion protein was extremely high compared with published values for the monomeric receptor ($K_D < 10^{-12}$ M). Titration to pH 6.0 did not significantly change the binding affinity, and titration to pH 5.5 only modestly attenuated affinity. At pH values below 5.0, the receptor binding domains of Fcε unfolded, and interaction of Fcε with the Fcγ-FcεRIα fusion protein was abrogated. The unusual pH sensitivity of Fcε may play a role in antigen-dependent regulation of receptor-bound, non-circulating IgE.

Immunoglobulin E (IgE) is important for host defense against parasites and for protective inflammation. Yet, IgE-mediated signaling through its receptors is also a focal point of inflammatory allergic disease (1). The constant domain of IgE (Fcε) is responsible for binding to its two receptors, FcεRI and CD23 (also known as FcεRII). FcεRI is expressed on the surface of mast cells, basophils, and some antigen presenting cells in humans (2, 3), whereas FcεRII is expressed primarily on antigen presenting cells, including B-cells (4). Binding of IgE to FcεRI, in particular, leads to increased levels of FcεRI on the cell surface, up-regulation of proteins involved in cell survival pathways, and cellular sensitization for an allergic event (2, 5–7).

Cross-linking of cell surface IgE-FcεRI complexes with multivalent antigens or anti-IgE antibodies leads to receptor signaling and cellular degranulation, the release of preformed secretory granules containing histamine, serotonin, various lipids, proteases, and other acute inflammatory agents, and the expression and release of inflammatory cytokines (1). Receptor cross-linking also leads to endocytosis of aggregated IgE-FcεRI complexes and exposure to endosomes and lysosomes. Endocytosis is a potentially important pathway for IgE regulation and FcεRI turnover during an antigen-mediated allergic response (8–10).

FcεRI expressed on mast cells and basophils is a heterotetramer consisting of one α, one β, and two γ chains (1). The α chain, an immunoglobulin family member, is the subunit responsible for high affinity binding of IgE ($K_D \sim 10^{-10}$ M) (11). It has been shown that mice lacking B cells and passively sensitized with anti-trinitrophenyl IgE respond to antigen treatment or anti-IgE for more than 1 month following IgE administration even with the absence of detectable serum IgE (12). This implies that high affinity between IgE and FcεRI translates into an incredibly long *in vivo* half-life of the complex on the surface of mast cells and basophils.

The Fcε/FcεRIα interaction occurs in a structurally homologous manner to what has been observed for IgG₁ constant domain (Fcγ) binding to FcγRI-III (13–15). Domain 2 of the FcεRIα subunit interacts with the homodimeric interface of the Cε3 domains of Fcε. Cε4 of Fcε does not participate directly in the binding but is crucial for maintaining high affinity (16–18).² The Cε2 domain, which is unique to IgE and virtually replaces the hinge found in IgG, is much less involved in binding but plays an important role in controlling the proper IgE/FcεRIα stoichiometry and binding kinetics (17, 19).

The production and purification of IgGs, particularly human IgG1, for diagnostic or therapeutic applications is now fairly routine. IgE-based therapeutics, however, have only recently begun to build some momentum (20–22). Unlike IgG therapeutics, standard methods for producing, handling, and formulating IgE or Fcε, particularly at an industrial scale, have not been established. In particular, it is not known whether Fcε is similar to Fcγ in terms of its thermostability and pH sensitivity. Exposure to destabilizing conditions could lead to denaturation or aggregation. Even low levels of Fcε dimer or higher order aggregates could have profound effects on cellular or *in vivo*

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¹ To whom correspondence should be addressed. Dept. of Protein Chemistry, Biogen Idec, 5200 Research Place, San Diego, CA 92122. Tel.: 858-401-5261; Fax: 858-401-5031; E-mail: stephen.demarest@biogenidec.com.

² M. Shields and S. J. Demarest, unpublished data.

functional assays by inadvertently cross-linking FcεRI in the absence of antigen (5, 7). Our initial studies revealed an unusual sensitivity of Fcε to various buffer and pH conditions. This led us to characterize in detail the biophysical properties of Fcε important for maintaining IgE-FcεRI interactions and for IgE half-life.

Here we describe detailed biophysical analyses of Fcε focused on deriving domain-specific unfolding information for Fcε and how buffer conditions, particularly pH, affect FcεRIα binding. Using circular dichroism (CD)³ and differential scanning calorimetry (DSC), we demonstrate that Fcε is much less thermostable than Fcγ. The native structure of Fcε is also highly sensitive to intermediate pH levels (5.0 and below) and high ionic strength. Binding of Fcε to an Fcγ-FcεRIα fusion protein is extremely high affinity at all pH values where Fcε remains folded. Unfolding of the Cε3 and Cε4 domains below pH 5.0 abolishes Fcε binding to the Fcγ-FcεRIα fusion. This pH-dependent unfolding property of Fcε may be important for providing a mechanism of IgE release from its high affinity receptor inside the cell and may help define a unique pathway for the regulation of receptor-bound IgE *in vivo*.

EXPERIMENTAL PROCEDURES

Subcloning of Fcε, Fcγ, Fcγ-Cε2, Fcγ-Cε2Cε3, and Fcγ-FcεRIα-Fcε was subcloned from mRNA extracted from human blood B cells (CD20+ and CD20+ IgM-). Complementary DNA primers, GTCACATATGCCACCATCAGCTTG and TTTACCGGGATTACAGACACCGC, which amplify from the C terminus of the Cε1 domain to the C terminus of the Cε4 domain, were used to create an Fcε vector insert via PCR. The resulting insert was cloned into a TA vector (TOPO TA cloning kit, Invitrogen). The modified TA vector was used as template for PCR using primers GCGGCCGCCTCACCATGGGCTGGAGCCTGATCCTGCTGTTCTTGGTGGCCGTGGCCACCCGCGTGCTGAGCTTACCCCGCCACCCGTGAA and CGTCGTTTAATTAATCATTTACCGGGATTACAG. The resulting PCR product was also introduced into the TA vector. After sequencing the 2nd generation TA vector, a fragment was digested from the vector using the enzymes NotI and PacI and ligated into the expression vector PV-90 (Biogen Idec). The theoretical subcloned sequence following signal processing was (in Kabat numbering) ²⁴⁸FTPP... PGK⁶¹⁰ (23). The plasmid was introduced into CHO cells by electroporation. Clones were selected with geneticin (Invitrogen). An N-terminal signal sequence, MGWSLILLFLVAVATRVLS, was built-in to the expression vector for secretion into the media and was included for the majority of the protein constructs described

here (24). Cells were cultured for 14 days at 28 °C, 5% CO₂ humidified air, in BCM16 serum-free media. A secreted, dimeric protein of the expected molecular mass, ~71.3 kDa (as per SDS-PAGE and static light scattering analysis), was found in the media following culture. The Fcε protein identity was also confirmed by Edman degradation and intact mass analysis.

An in-house vector containing the constant region of human IgG₁ was used as template for PCR amplification of human Fcγ using primers GCGGCCGCCTCACCATGGGCTGGAGCCTGATCCTGCTGTTCTTGGTGGCCGTGGCCACCCGCGTGCTGAGCGAGCCCAATCTTGTGACAA and TTAATTAATCATTTACCCGGAGACAGG. The PCR product was cloned into the TA vector and sequenced in-house. The remaining plasmid manipulations, transfections, and cell culture were performed as described above for Fcε. The mature sequence of the Fcγ protein was predicted to be ²²⁶EPK... PGK⁴⁷⁸. The secreted, dimeric protein was found to have an average mass ~56.4 kDa by SDS-PAGE and static light scattering analyses. Mass analysis with the purified protein reduced with dithiothreitol and treated with PNGase F yielded a dominant ion at 25,964 kDa corresponding to mature Fcγ with the C-terminal lysine cleaved at >90%. Cys²³⁰ (residue 5 in the mature protein) forms a disulfide bond with light chain in full-length IgG₁. No nonnative inter- or intra-molecular disulfides were detected following long term (*i.e.* several months) incubation at 4 °C.

The chimeric protein, Fcγ-FcεRIα, containing human Fcγ connected at its C terminus (Nterm-²²⁶EPK... SPG⁴⁷⁷-Cterm) by a 9-amino acid linker, SRENLYFQG, to the N terminus of human FcεRIα (starting with -VPQ...), was created for enhanced receptor expression. The human FcεRIα DNA insert encoding the extracellular domain without its signal peptide (Val²⁶ to Tyr²⁰², residue numbering includes signal peptide) was obtained by PCR from IMAGE clone 4294467 (ATCC) using the primers GCGGCGTCTAGAGAGAACCTGTACTTCCAGGGCGTCCCTCAGAAACCTAAGGTC and GCGGCGGTCGACGAATTCTTAGTACTTCTCACGCGGAGCTT. The PCR product was subsequently digested with XbaI and SalI and subcloned into a modified INPEP4-Fc vector containing a signal peptide of the mouse κ-light chain fused to the human immunoglobulin heavy chain IgG₁ constant region (Fc) (Kabat number 226-477). The Fcγ portion contained a T318A (EU299) mutation that abolishes glycosylation at Asn³¹⁴ (EU297). The C-terminal Lys on Fcγ was not included because this residue was cleaved at >90% within the cell culture for Fcγ (see above). The plasmid was transfected into dihydrofolate reductase-CHO DG44 cells using electroporation (25) and cloned in medium containing CHO-S-SFMII (Invitrogen) supplemented with HT (Invitrogen) in the presence of 400 μg/ml G418 (Invitrogen) using the limiting dilution method. Cell culture was performed using the same protocols as used for the Fcε producing CHO cells.

The chimeric proteins, Fcγ-Cε2 and Fcγ-Cε2Cε3, were prepared by linking the Cε2 or Cε2Cε3 domains of IgE to the C terminus of Fcγ via a 15-amino acid linker GSGGS(GGGGS)₂. The C-terminal Lys on Fcγ was also not included here. The sequences of the Fcγ-Cε2 and Fcγ-Cε2Cε3 protein domains began at the same N terminus as the above Fcε construct and

³ The abbreviations used are: CD, circular dichroism; Fcγ, IgG constant domain fragment; IgG, immunoglobulin G; Cε2, Cε3, and Cε4, second, third, and fourth constant region domains of the IgE heavy chain, respectively; DSC, differential scanning calorimetry; Cγ2 and Cγ3, second and third heavy chain constant domains of IgG, respectively; ANS, 1-anilino-8-naphthalene sulfonate; ITC, isothermal titration calorimetry; CHO, Chinese hamster ovary; SEC, size exclusion chromatography; LC/MS, liquid chromatography/mass spectrometry; HPLC, high pressure liquid chromatography; SPR, surface plasmon resonance; PBS, phosphate-buffered saline; ΔH_A⁰(T), molar enthalpy of association; ΔC_p⁰, molar heat capacity of association; T_m, midpoint of thermal unfolding transition; ΔS_A⁰(T), molar entropy of association.

contained the following C termini: ... SNP³⁶⁴ for Fcγ-Cε2 and ... GPR⁴⁹⁹ for Fcγ-Cε2Cε3. CHO cell lines producing high titers of Fcγ-Cε2 and Fcγ-Cε2Cε3 were subcloned from the bulk transformants using the method of Brezinsky *et al.* (26) and cultured using the same protocol as described for the Fcε CHO clone.

Protein Purification—Purifications were performed using an AKTA Explorer (GE-Healthcare). CHO cell media containing Fcε was titrated to pH 9.0 using 1 M Tris base, diluted 10 times with distilled deionized H₂O, and loaded on a Q Sepharose column equilibrated in 20 mM Tris, pH 9.0, at 10 ml/min. The column was washed with running buffer, and Fcε was eluted using a linear gradient to 200 mM NaCl. Fcε eluted between 110 and 170 mM. To concentrate the protein, the eluant was diluted 10 times, passed over a 60-ml Q Sepharose column, washed, and eluted in a single step using 20 mM Tris, 1 M NaCl, pH 9.0. The concentrated eluant was titrated to pH 8.0 using 2 M NaCH₃COO, pH 5.0, and further concentrated using an Amicon stir unit with a 10,000 *M_r* cut-off YM10 membrane, and passed over a 350-ml Superdex 200 column equilibrated with PBS to remove aggregates, multimers, and remaining contaminants. Purified Fcε was concentrated using an Amicon Ultra-15 centrifugal filter device, 10,000 *M_r* (Millipore). Protein concentration was measured by AU_{280 nm} using an extinction coefficient of 1.4 ml mg⁻¹ cm⁻¹.

CHO supernatants containing recombinant Fcγ, Fcγ-FcεRIα, or Fcγ-Cε2 were loaded onto a Protein A-Sepharose FF (Amersham Biosciences) column equilibrated with 50 mM Tris, 500 mM NaCl buffer at pH 7.5. The column was washed and protein was eluted using a step gradient to 100% 100 mM glycine, pH 3.0. Protein-containing fractions were titrated to about pH 7.0 using 1 M Tris base, pooled, and dialyzed against PBS. The Fcγ-FcεRIα fusion protein was further purified by size exclusion chromatography (SEC) as described above for Fcε. Purified Fcγ, Fcγ-FcεRIα, and Fcγ-Cε2 were concentrated in the same manner as Fcε. Fcγ and Fcγ-FcεRIα concentrations were determined by AU_{280 nm} using extinction coefficients of 1.4 and 1.9 ml mg⁻¹ cm⁻¹, respectively. Fcγ-Cε2 concentration was determined using the modified Lowry method and bovine IgG as the standard.

Analytical Size Exclusion Chromatography with In-line Static Light Scattering—Various pH samples of Fcε and Fcγ were prepared by dilution of 1.8 and 4.0 mg/ml stocks, respectively, into 25 mM phosphate, 25 mM citrate, 150 mM NaCl buffers at pH 2, 3, 4, 4.5, 5.0, 5.3, 5.7, 6.0, 6.5, 7.0, 8.0, and 9.0. Final protein concentrations were 320 μg/ml. Additional samples were prepared by diluting the Fcε and Fcγ stocks into 25 mM phosphate, 25 mM citrate, 750 mM NaCl at pH 4, 5, and 6. The stock solutions were in PBS (10 mM phosphate, pH 7.4, 140 mM salt). All samples were incubated for 3 or more hours at room temperature before analysis. Eighty μl of each sample was injected onto a TSKgel G3000SW XL, 5 mm, 250-Å Analytical SEC column (Tosoh Biosciences) equilibrated in 10 mM phosphate, 150 mM NaCl, 0.02% sodium azide at pH 7.2 using an Agilent 1100 HPLC system. Whereas injection of the various pH samples onto the SEC column using a similar running buffer may result in some protein refolding or re-equilibration, the running buffer was amenable for the column and allowed for uniform

comparison of elution times via the use of a single running buffer. Light scattering data for material eluting from the SEC column were collected using a miniDAWN static light scattering detector coupled to an in-line refractive index meter (Wyatt Technologies). Light scattering data were analyzed using the ASTRA V software provided by the manufacturer.

ANS Binding—The fluorescence of 1-anilino-8-naphthalene sulfonate (ANS, Sigma) was measured using a Victor3 multilabel fluorescence plate reader (PerkinElmer Life Sciences) with a 360 ± 20 nm excitation cutoff filter and a 460 ± 20 nm emission cutoff filter. All fluorescence measurements were made using 96-well fluorescence plates (NUNC MaxisorbF) with 150 μl/well and a 0.1-s sample averaging period per well. ANS (20 μM) was incubated with 20, 40, 80, 160, and 320 μg/ml bovine α-lactalbumin (Sigma) at pH 2.0 and 7.0 to establish appropriate protein concentrations to obtain adequate signal to noise. At 80 μg/ml α-lactalbumin at pH 2.0, the ANS fluorescence signal was ~20-fold higher than the noise; therefore, 80 μg/ml protein concentrations were utilized for the Fcε and Fcγ measurements. To study ANS binding to Fcε or Fcγ, each protein was diluted into a 25 mM phosphate, 25 mM citrate, 150 mM NaCl buffer series at pH 2, 3, 4, 4.5, 5.0, 5.3, 5.7, 6.0, 6.5, 7.0, 8.0, and 9.0 and incubated with the fluorescent dye. Additional Fcε and Fcγ samples were prepared in 25 mM phosphate, 25 mM citrate, 750 mM NaCl at pH 4.0, 5.0, and 6.0.

CD Spectroscopy—CD measurements were performed using a Jasco J-810 spectropolarimeter equipped with a thermoelectric Peltier device for temperature control and an external water bath as a heat sink. Fcε, Fcγ, and Fcγ-FcεRIα spectra (195–260 nm) were taken using the continuous scan mode at 100 nm/min. Bandwidth was set to 1 nm and data pitch to 0.2 nm. The response time of the instrument was set to 1 s. Five spectra were averaged for increased signal to noise. All spectra were taken at 25 °C. Fcε, Fcγ, and Fcγ-FcεRIα samples were dialyzed against 10 mM sodium citrate, 10 mM NaCl at pH 4.5, 5.0, 5.5, and 6.0. Additional Fcε, Fcγ, and Fcγ-FcεRIα samples at pH 7.4 were prepared by dialysis against PBS. To map the apparent unfolding transition of Fcε in detail, additional Fcε samples were prepared by dialysis against the same citrate buffer at pH 4.8 and 5.2. Background buffer scans at pH 5.0, 6.0, and 7.4 were virtually identical; therefore, all background subtractions were performed using the pH 5.0 background spectrum. All CD spectra were obtained using 3.75 μM protein with the dimer molecular mass (71.8 kDa) used for Fcε and the monomer molecular mass used for Fcγ (24.7 kDa) and the divalent Fcγ-FcεRIα fusion protein (49.8 kDa). Thermal melts of Fcε were performed in the cuvette by heating the samples at 5 °C intervals at a rate of 1 °C/min. Between each 5 °C heating interval, three replicate scans were performed using the parameters described above.

Limited Proteolysis and LC/MS—Fcε was digested by trypsin and Glu-C at pH 4.5, where significant protein structural changes away from the natively folded material occurred. Five samples were prepared by diluting 1 mg of Fcε into 0.4 ml of 70 mM sodium acetate solutions at pH 4.5. The pH of the acetate solution was unperturbed subsequent to the addition of Fcε in PBS. Various concentrations of trypsin (30, 300, and 3000 units, Sigma T-1426) and endoproteinase Glu-C (10 and 100 units,

Sigma P-2922) were added to individual pH 4.5 Fcε samples. Digestions were incubated at 37 °C. Time points (100 μl) were taken for LC/MS analysis after 0, 2, 10, 60, and 300 min and after overnight incubation. Proteolysis was halted by the addition of 50 mM EDTA and 10 mM phenylmethylsulfonyl fluoride. Dithiothreitol was then added to >10 mM for disulfide reduction, and each time point was subsequently frozen at −80 °C. Reagent blanks containing trypsin or Glu-C but excluding Fcε were also prepared for identifying non-Fcε peaks in the LC chromatograms. Based on the level of proteolysis observed by SDS-PAGE analysis, the 30-unit trypsin and 100-unit Glu-C samples were chosen for LC/MS peptide mass mapping. All time points were analyzed by HPLC MS as described previously (27). Total ion chromatograms were analyzed using the Agilent Deconvolution and Peptide Tools software programs.

DSC—DSC scans were performed using an automated capillary DSC (capDSC, MicroCal, LLC). Protein and reference solutions were sampled automatically from 96-well plates using the robotic attachment. Prior to each protein scan, two buffer scans were performed to define the baseline for subtraction. All 96-well plates containing protein were stored within the instrument at 6 °C.

For the Fcε and Fcγ pH titrations (Figs. 3, B and C, and 4, A and B), three separate pH panels were created with 15, 150, or 750 mM NaCl. The buffers included 70 mM glycine at pH 2.8, 3.3, and 3.5; 70 mM sodium acetate at pH 4.0, 4.5, 4.8, 5.0, and 5.2; 35 mM sodium citrate at pH 5.5, 6.0, and 6.5; and 35 mM Tris at pH 7.0, 7.5, and 8.0. Stock solutions of Fcε and Fcγ at 1.8 and 4.0 mg/ml, respectively, in PBS were diluted to 0.5 mg/ml in each buffer solution. Identical PBS dilutions into all members of the buffer panel were made for baseline measurements, and it was confirmed that the pH of each sample was not altered by the residual PBS. Scans were performed from 10 to 90 °C at 1 °C/min using the medium feedback mode for enhanced peak resolution. Additionally, the Fcε and Fcγ-Cε2 proteins were co-dialyzed against 10 mM sodium phosphate, 15 mM NaCl, pH 2.5, diluted to 1 mg/ml with dialysate, and scanned under identical conditions as described for the buffer panel.

For measurement of the thermodynamic interaction parameters between Fcε and Fcγ-FcεRIα, each protein was dialyzed against a panel of buffer solutions at pH 4.5, 5.0, 5.5, 6.0, and 7.4 as described above for the CD experiments. Using the dimeric molecular weight for apo- and holo-Fcε and the monomeric molecular weight for apo- and holo-Fcγ-FcεRIα, 2.5 μM protein solutions were prepared using matched dialysates for dilution and incubated for at least 2 h at 4 °C prior to DSC analysis. Dialysates were used within the reference cell of the calorimeter to define the baseline of each protein scan. Additional baseline corrections were performed by scanning with dialysate in both the reference and sample cells. For the binding experiments, scans were performed from 20 to 95 °C using a scan rate of 4.0 °C/min and the low feedback mode.

Scans were analyzed using the Origin software supplied by the manufacturer. Subsequent to the subtraction of reference baseline scans, non-zero protein scan baselines were corrected using a third order polynomial. The unfolding parameters for the multidomain unfolding profiles of Fcε, Fcγ, Fcγ-FcεRIα,

and Fcε/Fcγ-FcεRIα were deconvoluted using the multipeak fitting routine within the software.

Isothermal Titration Calorimetry (ITC)—Fcε and Fcγ-FcεRIα were dialyzed against 10 mM citrate, 10 mM NaCl at pH 5.5 and 6.0 as described above for the CD experiments. Additional samples were also prepared by dialyzing Fcε and Fcγ-FcεRIα against 1× PBS. Fcε stock solutions were concentrated to ~100 μM using 6 ml of Vivaspin MW5000 centrifugal concentration units (VivaSciences). ITC experiments were performed on a VP-ITC microcalorimeter (MicroCal, LLC). Aliquots of 70 μM Fcε (15 μl) were injected into the reaction cell containing 5 μM solutions of Fcγ-FcεRIα to obtain a final Fcε/Fcγ-FcεRIα ratio of ~2:1. Dialysates were used in the reference cell. A 4-min equilibration period was used between all Fcε injections with an initial delay of 60 s. All samples were degassed for ~10 min prior to each experiment. The ITC internal sample jacket was set 10 °C below the constant temperature within the reaction and reference cells, except for the 10 °C titrations where the bath was set to 1 °C. Numerical integration of the data were performed using the ITC data analysis software supplied by MicroCal (Origin). $\Delta H_A^0(T)$ values were calculated based on the difference between the average heat liberated/absorbed during the binding phase of the injections and the average heat of dilution found once the receptor, Fc-FcεRIα, was saturated with Fcε. The titration midpoints that defined the stoichiometry, *n*, were determined without data fitting. None of the ITC curves were utilized further for determination of binding constants due to the lack of titration points in the transition region. The absence of multiple titration points at the concentration where Fcε saturated the receptor was an indication that the affinity was too high to be measured by ITC alone.

Evaluation of the apparent binding thermodynamics for Fcε/Fcγ-FcεRIα using DSC and ITC- $K_U(T_M)$ values for the isolated Cε3Cε4 domains of Fcε and the apodomains of FcεRIα were extrapolated from the measured calorimetric enthalpies and T_M values of the protein domains (*i.e.* with Fcε alone or Fcγ-FcεRIα alone in the calorimeter) using theoretical heat capacities, 7.2 and 3.1 kcal mol^{−1} K^{−1}, respectively, based on the molecular weight of their cooperatively folded domains (28). ΔC_p^0 and $\Delta H_A^0(25\text{ °C})$ values for the Fcε/Fcγ-FcεRIα interaction were determined by ITC and estimated at pH 5.0 by fitting the experimentally derived values at pH 5.5, 6.0, and 7.4 to a second order polynomial. All other thermodynamic parameters were measured directly by fitting the DSC peaks within the Origin Software. The parameters were input into expressions derived by Brandts and Lin (29) for two proteins that demonstrate independent unfolding transitions in isolation but whose unfolding becomes thermodynamically coupled upon formation of a complex.

RESULTS

pH-dependent Unfolding of Fcε—Concentrated Fcε was diluted into a set of buffers ranging from pH 2 to 9 and chromatographed on an SEC column (Fig. 1A). The molecular weight(s) of the eluted Fcε peak(s) was determined by static light scattering and refractive index analyses. Following incubations between pH 5.3 and 9.0, Fcε eluted as a single peak with

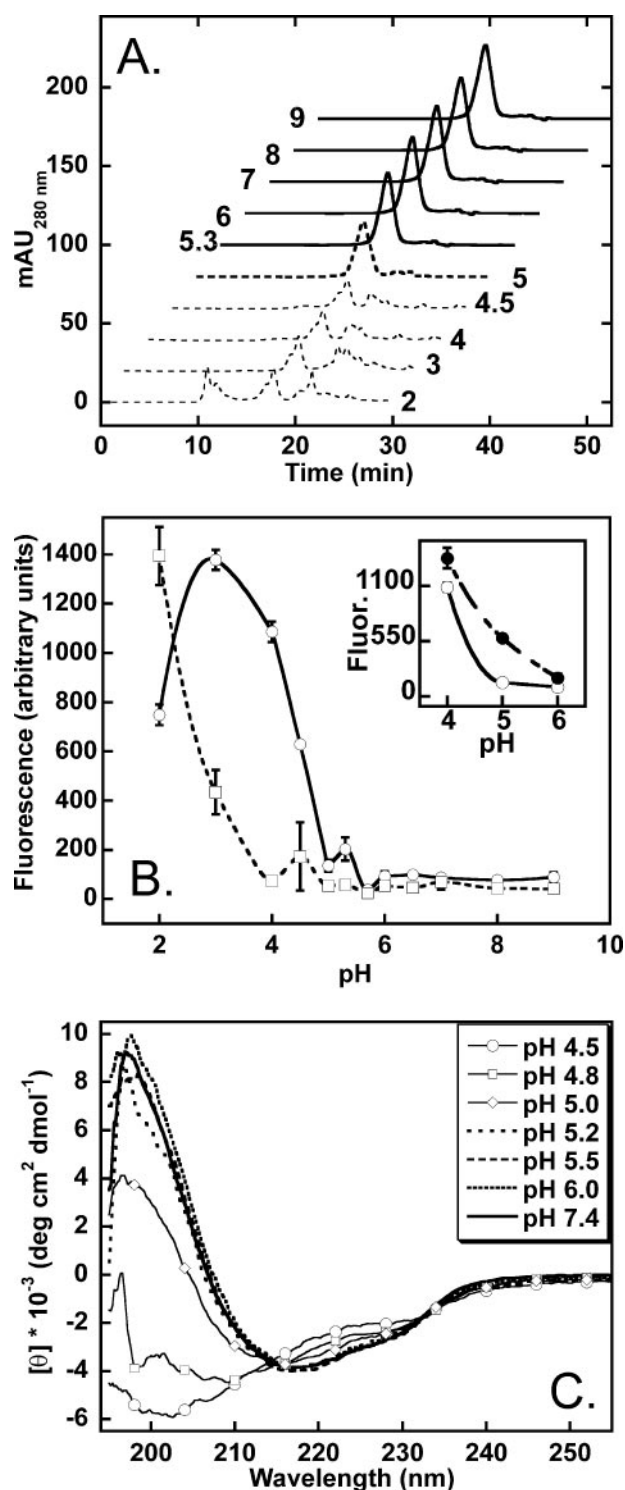


FIGURE 1. A, SEC chromatograms of Fcε samples incubated at various pH values (indicated next to each line) before injection over the SEC column. Chromatograms of Fcε samples with multiple peaks in the elution profile are shown with dotted lines. B, fluorescence intensity of ANS in the presence of Fcγ (□) or Fcε (○) and at various pH values. Inset, fluorescence intensity of ANS/Fcε mixtures at various pH values in 150 mM NaCl (○) and 750 mM NaCl (●). C, CD spectra of Fcε at various pH values.

the expected molecular mass of 71 kDa. At pH 5.0 and below, multiple peaks were observed within the chromatogram (Fig. 1A). Between pH 3.0 and 5.0, the additional peaks were limited to small multimers (*i.e.* dimers and tetramers) and apparent

monomeric material, which eluted later than the folded Fcε protein did. At pH 2.0, higher order aggregates were observed.

Using the same buffer set, the fluorescent hydrophobic dye ANS was added to solutions containing either Fcγ or Fcε. ANS is a hydrophobic dye used to probe for non-native or partially folded protein species. Its fluorescence is significantly quenched in aqueous solution; however, upon incorporation into the “fluid” hydrophobic interior of unfolding or molten globule-like proteins, the dye exhibits a sharp increase in fluorescence. Under neutral pH conditions, neither Fcγ nor Fcε associated with ANS as judged by fluorescence (Fig. 1B). In samples containing Fcγ, titration to pH 3.0 and below led to significant increases in ANS fluorescence corresponding to the loss of tertiary structure due to unfolding (Fig. 1B). For Fcε samples, sharp increases in ANS fluorescence were observed at pH values below 5.0 (Fig. 1B), 2 pH units above the pH where ANS bound Fcγ. In 750 mM NaCl and pH values below 6, the dye bound to Fcε (Fig. 1B, inset), suggesting that high ionic strengths may facilitate the unfolding event.

To more directly evaluate secondary structure, CD spectra of Fcε were taken under buffer conditions ranging from pH 4.5 to 7.4. Between pH 5.2 and 7.4, the spectra of Fcε were found to be identical and contained a single minimum between 216 and 217 nm indicative of significant β -sheet and typical of Ig domains (Fig. 1C). At pH 5, the Fcε spectrum shifted in a random coil direction (the minimum shifted toward 200 nm), and by pH 4.5, the spectrum was predominantly random coil. Based on the remaining negative signal at wavelengths between 210 and 220 nm and the overall spectral minimum, which did not shift entirely to 197 nm as would be expected for a random coil polypeptide, residual secondary structure was present at pH 4.5 (Fig. 1C).

The Receptor-binding Domains of Fcε, Cε3Cε4, Unfold Below pH 5—To determine whether Fcε unfolding observed at pH values below 5.0 was limited to particular domains or occurred over the entire Fcε region, limited proteolysis with either Glu-C or trypsin was performed at pH 4.5. Digestion time courses were analyzed by reverse phase HPLC MS. The first peaks appeared within 10 min of Glu-C or trypsin digestion and corresponded predominantly to peptides from the Cε4 domain and to a lesser extent the Cε3 domain. Several peptides from the Cε3 domain appeared at a moderately slower rate. After 5 h, peptides covering >80% of the sequences of the Cε3 and Cε4 domains were observed (Fig. 2A). After 24 h of proteolysis, several small peaks corresponding to initial Cε2 proteolytic events appeared (not shown).

To confirm that the Cε2 domain was stable at low pH values relative to the Cε3 and Cε4 domains, a recombinant Cε2 domain without Cε3 and Cε4 was created by fusing the domain to the C terminus of Fcγ. Both Fcε and the Fcγ-Cε2 fusion protein were subjected to DSC analysis. DSC is capable of deconvoluting individual protein unfolding events within multidomain proteins and was used to investigate the stability of Cε2 within the context of the multidomain proteins Fcε and Fcγ-Cε2. Both Fcε and Fcγ-Cε2 were analyzed by DSC at multiple pH values between 2.5 and 8.0 (data not shown). Under all pH conditions, a single transition with a midpoint of unfolding (T_M) between 60 and 70 °C corresponding to the denaturation

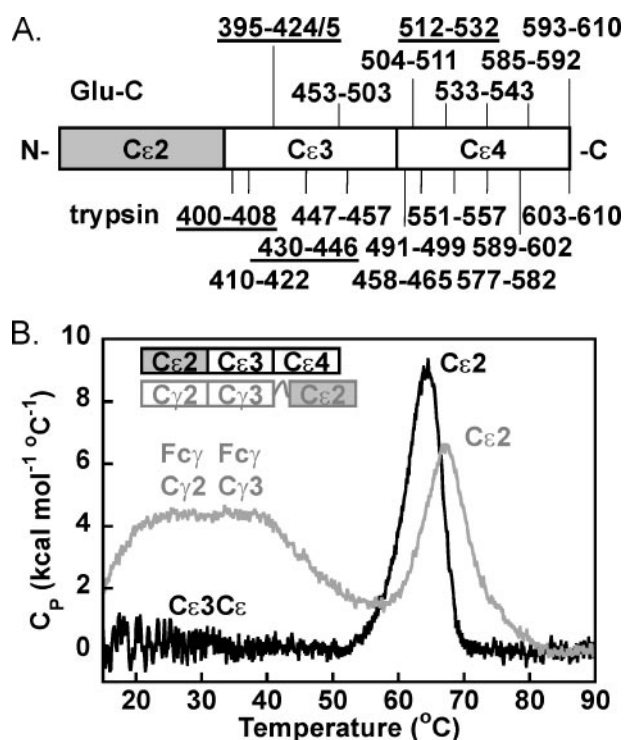


FIGURE 2. A, schematic (rectangle) diagram of Fcε with peptides detected by LC/MS subsequent to digestion at pH 4.5, 37 °C labeled above (Glu-C) and below (trypsin). Peptides/domains with masses <800 or >10,000 Da were not included in the diagram as the small peptides could correspond to multiple sequences, and the large peptides were too large for precise intact mass determination. Peptide sequences that appeared slowly (after 1 day) upon Glu-C or trypsin digestion at pH 4.5 are underlined. All non-underlined peptide sequences were detectable within 1 h of digestion. B, DSC traces of Fcε (black line) and Fcγ-Cε2 (gray line) performed using samples dialyzed against the same pH 2.5 phosphate buffer. Schematic diagrams of the Fcε and Fcγ-Cε2 proteins are shown above the DSC curves.

of Cε2 within both Fcε and Fcγ-Cε2 was observed. Fcε and the Fcγ-Cε2 construct were additionally dialyzed against a sodium phosphate buffer at pH 2.5, and the matched solutions were analyzed by DSC (Fig. 2B). Both proteins displayed a Cε2 unfolding transition near 65 °C indicating that the Cε2 domain was stably folded even at very low pH values.

pH-dependent Stability of Fcε—Based on the pH-dependent unfolding described above, we investigated whether Fcε may have an attenuated stability between pH 7.0 and 5.0 (*i.e.* above the pH where unfolding is observed). Thermal denaturation was chosen as the method for measuring Fcε stability under the different conditions. Thermal denaturation of Fcε at various pH values was first monitored by far-UV CD (Fig. 3A). At pH 7.0, there was one transition for the unfolding of all three domains (Cε2–4). A similar transition was observed at pH 6.0, although the apparent T_M decreased by 1 °C. Thermal unfolding of Fcε at pH 5.2 resulted in a much broader transition(s) that began 6 °C lower than at neutral pH. At pH 4.8, two clear transitions were evident, with the lower temperature transition at ~42 °C corresponding to the unfolding of the Cε3Cε4 domains and the higher temperature transition at ~70 °C corresponding to the unfolding of Cε2. The Cε2 transition was identified based on the Fcγ-Cε2 DSC analyses and the Fcε limited proteolysis studies at pH 4.5.

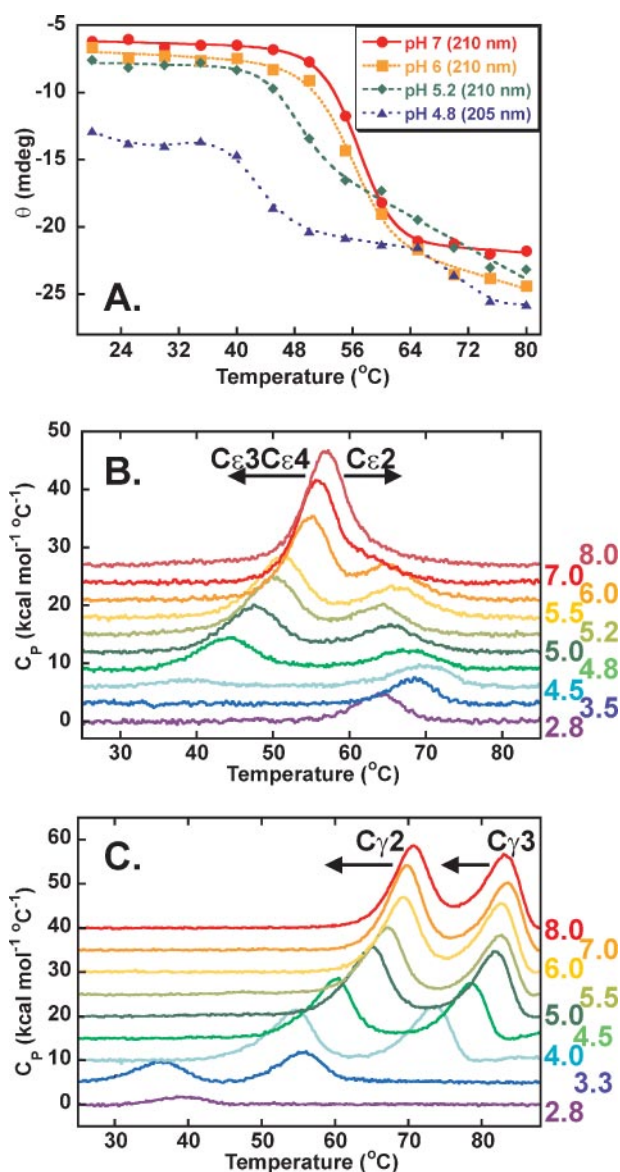


FIGURE 3. A, pH-dependent thermostability of Fcε examined by CD spectroscopy. The curves represent the ellipticity at chosen wavelengths from spectra taken between 20 and 80 °C using 5 °C intervals. B and C, pH-dependent DSC traces of Fcε (B) and Fcγ (C) in the presence of 15 mM NaCl.

The pH-dependent stabilities of both Fcε and Fcγ were compared using DSC. The unfolding transitions of both Fcε and Fcγ were found to be irreversible and scan rate dependent (data not shown), suggesting that irreversible aggregation affects the apparent T_M values of both proteins (30, 31). Therefore, we compared the *relative* stabilities of the Fcε and Fcγ domains by measuring T_M values under identical instrument conditions. The Cε2 domain of Fcε was pH stable and exhibited an unfolding transition very similar to Cε2 measured within the Fcγ-Cε2 fusion protein at pH values between 2.5 and 8.0 (compare Figs. 2B and 3B). At pH 8.0, the Cε2 transition occurred at a similar T_M as the Cε3Cε4 domains suggesting possible unfolding cooperativity; however, its T_M was similar when measured in the context of Fcε or Fcγ-Cε2. Because the Cε3Cε4 domains did not raise the thermostability of Cε2, the apparent overlap of the unfolding transitions of Cε2 and Cε3Cε4 was unlikely due to

cooperativity between these domains at pH 8.0. The Cε3Cε4 domains of Fcε were pH labile (Fig. 3B). The Cε3Cε4 domains unfolded cooperatively with a maximum T_M of 57 °C at pH 8. As the pH was lowered, the single unfolding transition of the Cε3Cε4 domains shifted to lower temperatures, whereas the unfolding transition of the Cε2 domain remained relatively static. As the pH was lowered to pH 4.8, the Cε3Cε4 unfolding transition decreased to 44 °C, and at pH 4.5 the unfolding transition disappeared entirely, presumably because the domains became intrinsically unfolded at all temperatures.

The Cγ2 and Cγ3 domains of Fcγ were generally much more stable than the homologous Cε3Cε4 domains of Fcε (Fig. 3C). Unlike the cooperative unfolding observed for the Cε3Cε4 domains of Fcε, the Cγ2 and Cγ3 domains unfolded in two separate transitions. The Cγ2 domain was significantly less thermostable than the Cγ3 domain. The Cγ2 transition was identified by the effect that deglycosylation had on its thermostability.⁴ The Cγ3 domain was identified by its high thermostability, a characteristic of the isolated domain as investigated previously (32). The stability of both domains decreased with decreasing pH. Unlike the Cε3Cε4 domains, however, the sharp transition toward an intrinsically unfolded state at all temperatures only occurred below pH 3.

In the presence of high salt, the Cγ2 and Cγ3 domains of Fcγ and the Cε3 and Cε4 domains of Fcε were slightly destabilized. This was seen as a small shift in their T_M values at 150 and 750 mM NaCl relative to 15 mM NaCl in the intermediate pH range between 5.0 and 7.0 (Fig. 4A). These small stability differences are unlikely to have a major effect on the *in vitro* half-life of Fcγ within this pH range because the T_M of both the Cγ2 and Cγ3 domains remained above 60 °C. Interestingly, in high salt, the Cε2 domain of Fcε appeared to be slightly more stable. Cε2 was especially stabilized at neutral pH and 750 mM NaCl with a T_M more than 7 °C higher than the T_M measured using 15 mM NaCl (Fig. 4B). In contrast, NaCl significantly destabilized the Cε3Cε4 domains between pH 5 and 6 (Fig. 4B). Cε3Cε4 began to unfold (*i.e.* a measurable fraction of intrinsically unfolded material at all temperatures) at pH 5.0 in low salt. In high salt, the unfolding transition was shifted 0.5 pH units (to pH 5.5).

pH Dependence of the Fcε/FcεRIα Interactions—Many protein-protein interactions are highly pH-dependent. Such pH dependencies often have significant biological implications. For example, the pH dependence of the interaction between IgG1-Fcγ and FcRn is important for serum IgG recirculation and half-life (33). Because marked decreases in Fcε stability were observed as the pH was decreased, we investigated whether pH-dependent stability changes attenuated the ability of IgE to interact with its high affinity receptor, FcεRIα. To facilitate the expression of soluble FcεRIα, the receptor subunit was fused to Fcγ (26). The resulting protein was a dimeric chimera of Fcγ and FcεRIα. A mutation within Fcγ (T299A) that abrogated glycosylation was utilized to prevent potential nonspecific binding of Fcγ to FcεRIα.

We first investigated whether FcεRIα alone exhibited pH-dependent unfolding similar to the Cε3Cε4 domains of IgE.

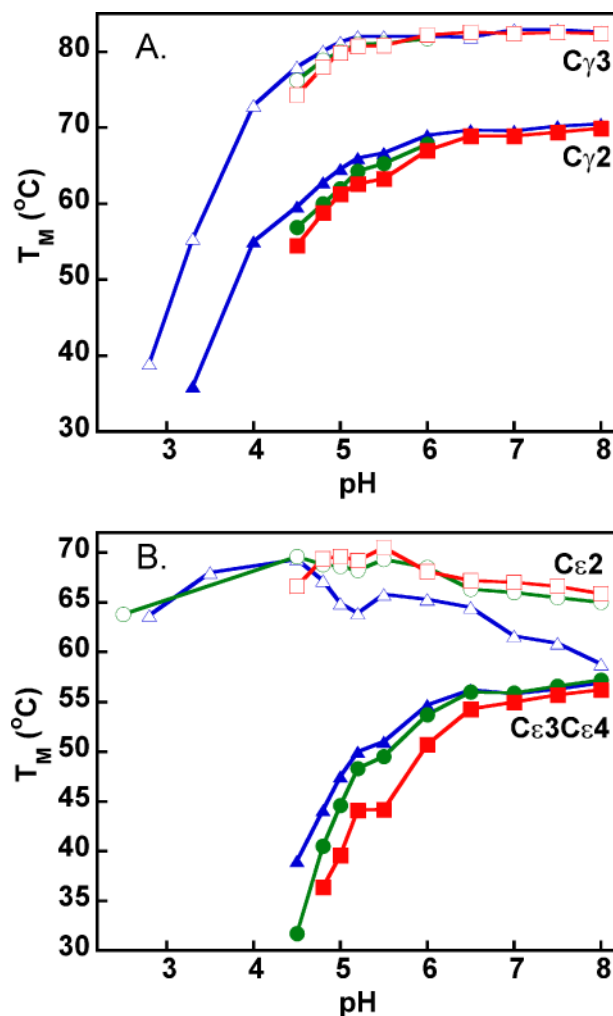


FIGURE 4. pH dependence of the Fcε and Fcγ domain thermostabilities as represented by their T_M values. A, Fcγ domain T_M values: Cγ2 at 15 (▲), 150 (●), and 750 (■) mM NaCl; Cγ3 at 15 (△), 150 (○), and 750 (□) mM NaCl. B, Fcε domain T_M values: Cε3Cε4 at 15 (▲), 150 (●), and 750 (■) mM NaCl; Cε2 at 15 (△), 150 (○), and 750 (□) mM NaCl.

CD spectra of Fcγ-FcεRIα in buffers ranging from pH 4.5 to 7.4 indicated no major structural changes under these conditions. Fig. 5A shows the differences between the spectra of Fcγ-FcεRIα and Fcγ and is representative of the CD spectra of FcεRIα. The resulting difference spectra closely resembled the FcεRIα CD spectrum published at pH 7.2 (34). Thus, FcεRIα did not appear to undergo a pH-dependent unfolding at intermediate pH levels.

Combining Fcε and Fcγ-FcεRIα in solution led to changes in the CD spectra of the complexed proteins (Fig. 5, B–IE). Between pH 5 and 7.4, the spectra of the complex were compared with added spectra of the isolated Fcε and Fcγ-FcεRIα proteins. Across these pH values, the absolute value of the spectral intensity of the complex decreased slightly, and the minimum shifted from 216 to 217 nm indicating potential β-sheet stabilization and possibly the loss of some dynamic flexibility. The spectral changes upon complex formation were highly similar to the spectral changes observed by Sechi and co-workers (34) for the Fcε-FcεRIα complex at pH 7.2. While reproducible, the changes in the CD spectra upon Fcε-FcεRIα complex formation were fairly minimal and would certainly not reflect

⁴ S. J. Demarest, unpublished results.

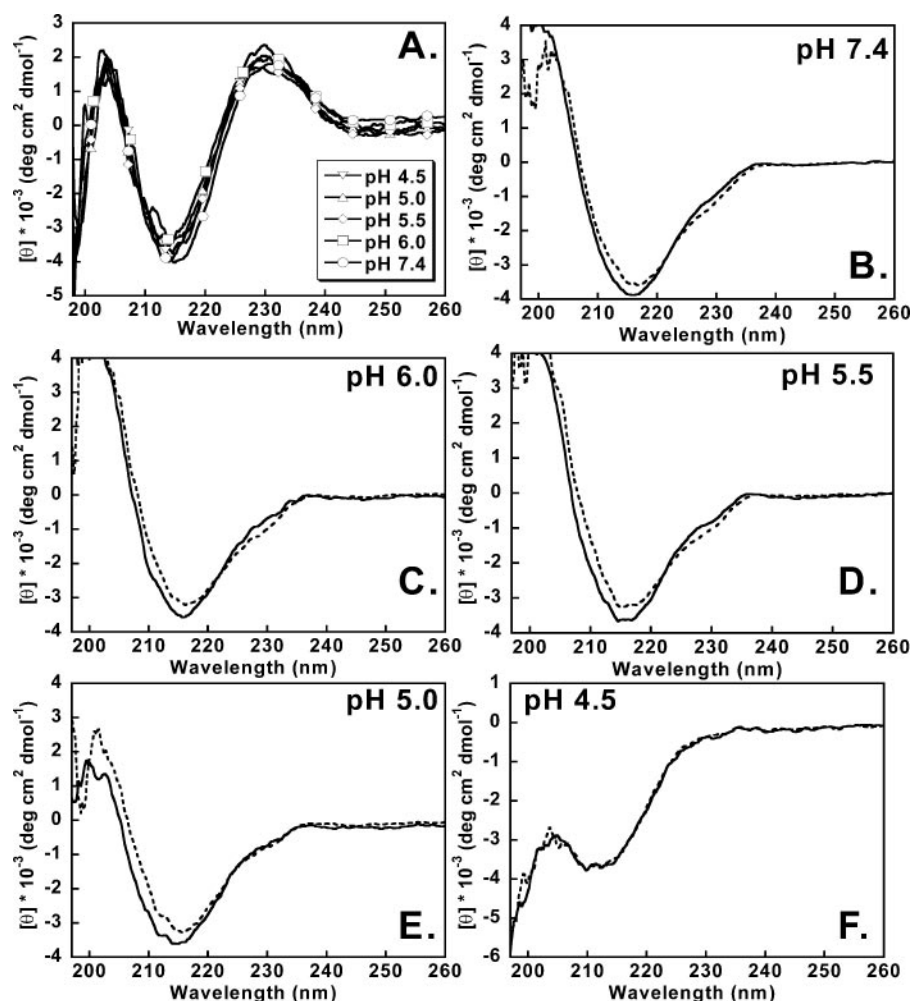


FIGURE 5. pH-dependent structural changes within Fcε/Fcγ-FcεRIα upon complex formation. A, difference CD spectra obtained by subtracting Fcγ spectra from Fcγ-FcεRIα spectra at the indicated pH values. The difference spectra reflect the expected CD spectra of FcεRIα alone. B–F, CD spectra of equimolar mixtures of Fcε and Fcγ-FcεRIα (dotted lines) and the mathematical addition of the spectra of apo-Fcε and apo-Fcγ-FcεRIα taken in isolation (solid lines) at the indicated pH values.

large structural changes such as the folding/unfolding of an entire Ig domain. Due to the unfolded nature of Fcε at pH 4.5, the summed spectra of the apoproteins changed significantly from that observed at higher pH values (Fig. 5F). The spectrum of the physical mixture of Fcε and Fcγ-FcεRIα was identical to the summed spectra of the apoproteins indicating that mixing the proteins together at pH 4.5 produced no changes in structure.

pH Dependence of the Fcε/FcεRIα Binding Thermodynamics—Quantification of Fcε-FcεRIα binding affinities was attempted by surface plasmon resonance (SPR). Although we were able to reproduce results in the literature (11), the sensorchip surfaces were poorly behaved at and below pH 6.0 and precluded obtaining interpretable results. Additionally, the kinetics obtained at pH 7.4 did not fit to a single exponential and were highly mass transfer limited, suggesting that the measured K_D (2.6×10^{-10} M) represented an upper limit of the K_D value (*i.e.* the limit of detection for SPR kinetic measurements) between the two proteins.

We therefore investigated the relative binding strengths of Fcε to Fcγ-FcεRIα at various pH values using DSC. The DSC

curves of the individual Fcε and Fcγ-FcεRIα proteins at pH 7.4 are shown in Fig. 6. An Fcγ(T299A) construct was also analyzed to differentiate the unfolding transitions of Fcγ from the single transition observed for FcεRIα in the Fcγ-FcεRIα fusion protein,⁵ as the two transitions could not be separated by deconvolution (not shown). In particular, the two domains of FcεRIα were found to unfold at the same temperature as the Fcγ(T299A) Cγ2 domain. The DSC curve of complexed Fcε/Fcγ-FcεRIα was significantly different from the simple addition of the isolated Fcε and Fcγ-FcεRIα curves (dotted lines in Fig. 6) indicative of a strong interaction between the two proteins. The transitions corresponding to the Cε3Cε4 domains of Fcε and domains 1 and 2 of FcεRIα coalesced into a single transition that occurred at a higher temperature ($\sim 8^\circ\text{C}$ higher) than the unfolding transitions of Cε3Cε4 and FcεRIα in isolation. Additionally, there was a significant increase in the calorimetric enthalpy of the transition (Fig. 6).

A mathematical treatment of DSC changes resulting from protein complex formation has been developed to obtain approximate binding affinities for high affinity interactions (29). The equations used by Brandts and Lin (29) were applied to Fcε and Fcγ-FcεRIα interactions

with the following assumptions: 1) unfolding in all cases was two-state and reversible; and 2) the temperature-dependent equilibrium of the interaction could be described by, $K_{eq}(T) = [U_{FcεRIα}][U_{Cε3Cε4}]/[F_{FcεRIα-Cε3Cε4}]$, where $[U_{FcεRIα}]$ and $[U_{Cε3Cε4}]$ are the concentrations of unfolded FcεRIα and Cε3Cε4, respectively, and $[F_{FcεRIα-Cε3Cε4}]$ is the concentration of the complex that was folded at all temperatures. Although the unfolding was not two-state for Fcε or FcεRIα due to aggregation at temperatures where unfolded protein exists, the scans were performed at maximal rates ($4^\circ\text{C}/\text{min}$) to reduce aggregation and approximate two-state behavior.

Most of the necessary parameters for determining the affinity were obtained from the DSC experiment; however, the heat capacity of the interaction (ΔC_p^0) and the enthalpy of the interaction at 25°C ($\Delta H_A^0(25^\circ\text{C})$), the temperature where we report the affinity, needed to be determined by ITC. The ΔC_p^0 ($-760 \text{ cal mol}^{-1} \text{ K}^{-1}$) and $\Delta H_A^0(25^\circ\text{C})$ ($-20.4 \text{ kcal mol}^{-1}$) values for binding of Fcε to Fcγ-FcεRIα at pH 7.4 were measured by ITC

⁵ S. J. Demarest and F. Taylor, manuscript in preparation.

and found to be similar to previously determined values (35). The remaining thermodynamic parameters derived by DSC for determination of the apparent binding affinity of Fcε to Fcγ-FcεRIα are shown in Table 1. An extremely high affinity was calculated using the DSC method ($K_D < 10^{-12}$ M) that was significantly stronger than the value measured by SPR at pH 7.4 ($K_D = 2.6 \times 10^{-10}$ M, Table 2). However, for reasons described above, the SPR experiments likely reflect the lower limit (*i.e.* weakest possible K_D) of the interaction.

The interaction between Fcε and Fcγ-FcεRIα remained extremely strong at pH 6.0 and 5.5 (Table 2). Measurements of ΔC_p^0 ($-670 \text{ cal mol}^{-1} \text{ K}^{-1}$) and ΔH_A^0 (25 °C, $-8.9 \text{ kcal mol}^{-1}$) at pH 6.0 and ΔC_p^0 ($-520 \text{ cal mol}^{-1} \text{ K}^{-1}$) and ΔH_A^0 (25 °C, $-6.9 \text{ kcal mol}^{-1}$) at pH 5.5 by ITC were utilized in the affinity calculations in Table 2 (Fig. 7, A–C). No changes in binding stoichiometry were observed. At pH 7.4, 6.0, and 5.5, the binding isotherms at all temperatures were too sharp (*i.e.* no points in the transition region) for accurate determination of the bind-

ing affinity by ITC (Table 2) and indicated a $K_D < 10^{-9}$ M, consistent with the values obtained using DSC.

DSC experiments with Fcε, Fcγ-FcεRIα, and Fcε-Fcγ-FcεRIα complexes were performed at pH 6.0 and 5.5 to obtain parameters for determining the affinity at these pH values (Tables 1 and 2, and Fig. 8, A and B). Dropping the pH from 7.4 to 6.0 did not attenuate the affinity within the error of the experiment (Table 2; compare Figs. 6 and 8A). At pH 5.5, however, the affinity appeared to be attenuated significantly ($K_D < 10^{-10}$ M; Table 2 and Fig. 8B). Complex formation between Fcε and Fcγ-FcεRIα at both pH 6.0 and 5.5 resulted in large increases in the apparent T_M values of the Cε3Cε4 domains of Fcε and domains 1 and 2 of FcεRIα similar to what was observed at pH 7.4 (compare Figs. 6 and 8, A and B), indicating the persistence of a strong interaction.

Titration to pH 5.0 and 4.5 profoundly weakened the interaction between Fcε and Fcγ-FcεRIα. DSC experiments clearly indicated that the interaction between the two proteins was highly attenuated at pH 5.0 and completely abrogated at pH 4.5 (Fig. 8, C and D). At pH 5.0, a structural change within Fcε (Figs. 1C and 5E) appeared coincidentally with a reduced stability of the Cε3Cε4 domains (Fig. 3B). Formation of the complex between Fcε and Fcγ-FcεRIα at pH 5.0 resulted in a marginal increase in the T_M of the Cε3Cε4 domains and the apodomains of FcεRIα over what was observed for the two proteins alone (Fig. 8C). The integrated enthalpy under the DSC curve of the Fcε-Fcγ-FcεRIα complex at pH 5.0 was also greatly decreased compared with that observed at higher pH values. At pH 4.5, the mathematically combined DSC traces of apo-Fcε and apo-Fcγ-FcεRIα were nearly identical to the DSC trace measured for the mixture of the two proteins indicating that the interaction was completely abolished at pH 4.5 (Fig. 8D).

We speculated that Fcε bound to FcεRI (as if on the surface of a cell) may be afforded some protection to pH-dependent denaturation via its strong interaction with the receptor. To test this idea, Fcε and Fcγ-FcεRIα were mixed at pH 7.4 prior to dialysis at pH 4.5. The *potential* complex was removed from dialysis tubing and analyzed by DSC. Fcε was found to be completely

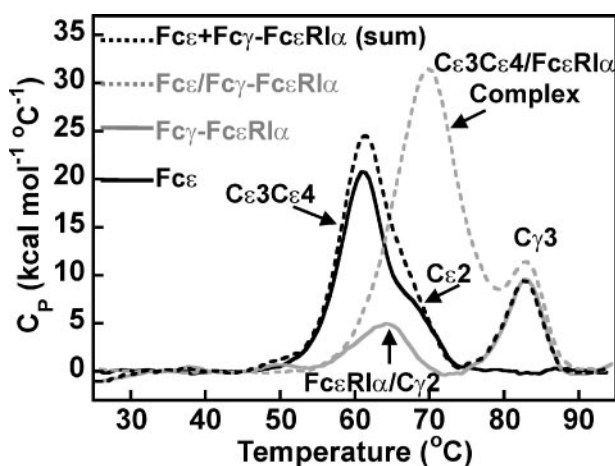


FIGURE 6. DSC traces of apo-Fcε (solid black line), apo-Fcγ-FcεRIα (solid gray line), the summation of the apo-Fcε and apo-Fcγ-FcεRIα traces (black dotted line), and the DSC trace of an equimolar complex of the two proteins (gray dotted line). The identity of each transition (*i.e.* peak) within the DSC curves is indicated. All scans were performed at pH 7.4 using identical instrument parameters.

TABLE 1

Thermodynamic values determined for apo-Fcε, apo-Fcγ-FcεRI, and the Fcε-Fcγ-FcεRI complex by DSC

Construct (pH)	Cε3Cε4 Fcε T_M	Cε2 Fcε T_M	FcεRI T_M	Cγ2 Fcγ T_M	Cγ3 Fcγ T_M
Fcε (7.4)	60.8 (225) ^a	67.2 (78)			
FcεRIα (7.4)			63.7 (60-30/30) ^b	Overlap FcεRI ^b	82.7 (62)
FcεRIα/Fcε (7.4)	70.3 (413) ^c	67.2 (78)		63.7 (31)	83.4 (72)
Fcε (6.0)	57.22 (156)	70.1 (78)			
FcεRIα (6.0)			62.3 (67-33/34) ^b	Overlap FcεRI ^b	82.1 (40)
FcεRIα/Fcε (6.0)	69.6 (279) ^c	70.1 (78)		62.3 (34)	82.6 (37)
Fcε (5.5)	53.5 (95)	71.8 (79)			
FcεRIα (5.5)			60.4 (80-40/40) ^b	Overlap FcεRI ^b	82.1 (48)
FcεRIα/Fcε (5.5)	66.9 (200) ^c	71.8 (79)		60.4 (40)	82.9 (45)
Fcε (5.0)	51.2 (34)	72.8 (80)			
FcεRIα (5.0)			59.5 (78-38/40) ^b	Overlap FcεRI ^b	81.4 (67)
FcεRIα/Fcε (5.0)	63.7 (200) ^c	73.0 (80)		59.5 (40)	83.0 (67)
Fcε (4.5)		74.2 (78)			
FcεRIα (4.5)			54.3 (44)	61.0 (27)	80.0 (48)
FcεRIα/Fcε (4.5)		74.5 (96)	54.0 (52)	62.3 (53)	81.1 (38)

^a Calorimetrically determined unfolding enthalpy in kcal/mol.

^b FcεRIα T_M determined by curve fitting the two peaks under the single DSC peak where Fcγ-Cγ2 and FcεRIα unfold to a single transition. The integrated calorimetric enthalpy of the Fcγ-Cγ2 peak fit was held constant based on the independent measurement of the Fcγ-Cγ2 calorimetric enthalpy measured for the non-fusion Fcγ construct. Values in parentheses are (total integrated enthalpy-FcεRIα calorimetric enthalpy/Fcγ-Cγ2 calorimetric enthalpy).

^c Cε3Cε4 domain unfolding transition merges with the unfolding transition of the FcεRIα domains upon binding. Both domains demonstrate increases in their overall melting temperatures (T_M values) upon binding.

TABLE 2

pH-dependent binding affinity of Fcε for Fcγ-FcεRIα

pH (method)	K_D , 25 °C
7.4 (SPR)	$<10^{-10}$
7.4 (ITC, 10, 20, 30, 37 °C) ^a	$<10^{-9}$
7.4 (DSC)	$<10^{-12}$
6.0 (ITC, 20, 30, 37 °C) ^{a,b}	$<10^{-9}$
6.0 (DSC)	$<10^{-12}$
5.5 (ITC, 20, 30, 37 °C) ^{a,b}	$<10^{-9}$
5.5 (DSC)	$<10^{-10}$
5.0 (DSC)	10^{-8-9}
4.5 (DSC)	$>10^{-5}$

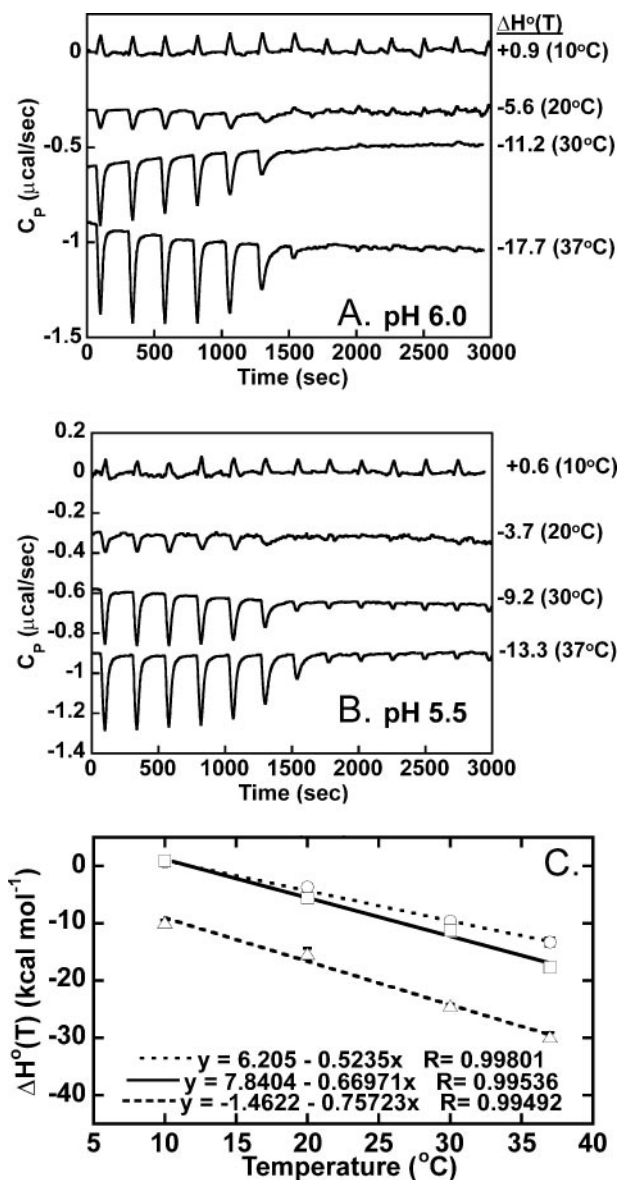
^a ITC curves all contained a maximum of 1 or 2 points in the transition region.^b 10 °C data impossible to analyze due to the lack of enthalpy of during binding.

FIGURE 7. Examples of ITC measurement of $\Delta H_A^0(T)$ and ΔC_p^0 of the interaction between Fcε and Fcγ-FcεRIα. A and B, titration of Fcε into solutions containing Fcγ-FcεRIα at pH 6.0 (A) and pH 5.5 (B). C, determination of ΔC_p^0 of the interaction by linear regression of $\Delta H_A^0(T)$ measured between 10 and 37 °C at pH 7.4 (Δ), 6.0 (□), and 5.5 (○).

dissociated from the Fcγ-FcεRIα fusion protein suggesting that the unfolding behavior of Fcε at pH 4.5 disrupted the pre-formed complex. Thus, FcεRIα would not be predicted to pro-

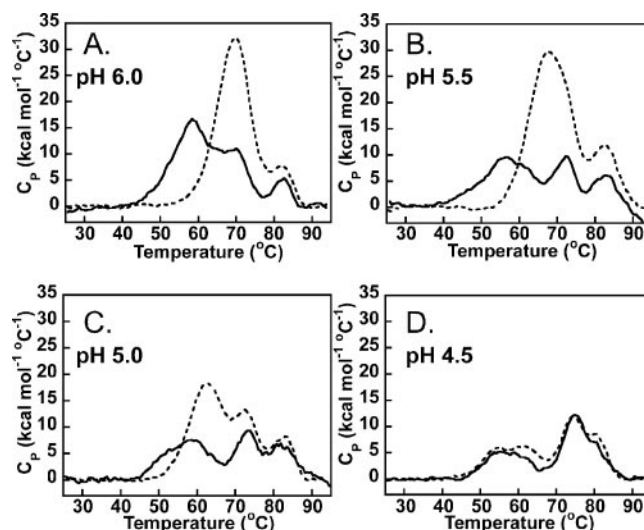


FIGURE 8. Sum of the DSC traces of the apo-Fcε and apo-Fcγ-FcεRIα proteins (solid line) and the DSC trace of an equimolar mixture of the two proteins (dotted line). A, pH 6.0; B, pH 5.5; C, pH 5.0; and D, pH 4.5.

tect IgE from denaturation if the complex were exposed to low pH values.

DISCUSSION

In this study, we showed that the IgE-Fc demonstrated an unusual pH-dependent instability unlike that found for the IgG₁-Fc. The pH-dependent unfolding of Fcε was localized to the Cε3Cε4 domains and abolished the ability of IgE to interact with its high affinity receptor, FcεRI, below pH 5.0. The thermostability of Cε3Cε4 decreased significantly as the pH was reduced from pH 7.4 to 5.0. Attenuated Fcε thermostability did not significantly attenuate binding to FcεRI until intrinsically unfolded Fcε became present at pH 5.0 and below.

Unfolding Cooperativity and Stability Differences between Fcε and Fcγ—The Cγ2 and Cγ3 domains of Fcγ were found to unfold independently of one another, whereas the Cε3 and Cε4 domains of Fcε unfolded cooperatively under all conditions tested. Analyses of the structures of Fcε (14) and Fcγ (36) reveal a similar amount of surface area buried at the Cε3/Cε4 interface compared with the Cγ2/Cγ3 interface (500 Å² for the Cε3/Cε4 interface versus 460 Å² for the Cγ2/Cγ3 interface). There is also a strong homology between the Cγ2/Cγ3 and Cε3/Cε4 interfaces, with near identical Fc residue positions burying surface area between the domains (in terms of the Kabat numbering system). However, the amino acid identity at the two interfaces is moderate and similar to the overall sequence identity between Fcγ and Fcε (34% identity, 64% homology). It is likely that differences between the cooperativity of unfolding for the IgG and IgE Fc regions are a consequence of specific amino acid interactions at the interfaces of the Fc domains because the contact areas and structural compositions are similar. It is possible that the IgG₁-Fc in particular may not unfold cooperatively due to the extremely high apparent stability of the Cγ3 domain. However, similar thermal unfolding experiments with human IgG2, IgG3, and IgG4 also demonstrated separate unfolding transitions for the Cγ2 and Cγ3 domains of these isotypes that contained relatively less stable Cγ3 domains than

the Cγ3 domain of IgG₁.⁶ It should be noted that the Cγ2 and Cγ3 domains of mouse IgG_{2a} *do* appear to unfold at the same temperature under neutral pH conditions (31). This phenomenon alone does not indicate folding cooperativity between the domains; the domains could simply have relatively close T_M values. The Cε3 and Cε4 domains of Fcε appeared to unfold cooperatively under all pH and salt conditions tested.

Apparent cooperativity between Cε3 and Cε4 coincides with a recent study that describes the partially unfolded nature of an individual recombinant Cε3 domain (16, 18). We found that portions of the Cε3 domain proteolyzed more slowly than Cε4 at pH 4.5. It is possible that incomplete unfolding of the Cε3 domain hinders the rapid proteolysis of Cε3. When we removed Cε4 from Fcε using a recombinant fusion construct Fcγ-linker-Cε2Cε3, this protein demonstrated a strong tendency to aggregate and poor overall biophysical behavior. Although FcεRIα has been shown to bind the Cε3 domain, removal of Cε4 from Fcε abrogates the ability of Fcε to interact with cell surface FcεRI (37). Our results and those of others suggest Cε3 folding is dependent on Cε4 and explain why the presence of Cε4 is critical for high affinity FcεRI binding (16, 18, 37).

A cooperatively folded structure for Cε3 in the presence of Cε4 does not discount potential flexibility of various loops or flexibility at the Cε2/Cε3 interface important for the observed biphasic binding kinetics to FcεRIα (11) and the existence of closed *versus* open forms of the receptor-binding region (15, 38). In fact, we did observe changes in the CD spectrum of Fcε/Fcγ-FcεRI (Fig. 5) upon formation of the complex. This is indicative of stabilization of β-structures or limited structural changes resulting from the "locking down" of flexible loops similar to what was observed by Sechi and co-workers (34).

The pH stability profiles of Fcγ and Fcε were also very different. The pH-dependent unfolding of Cε3Cε4 occurred at pH 5.0 likely due to the protonation of one or more basic amino acids that subsequently destabilizes the native fold. pH-dependent protein stability is often observed for proteins whose folds either stabilize the burial of charged amino acids or are dependent on strong charge-charge interactions. In the pH range where Fcε appears to undergo its unfolding transition (pH 4.4–5.5), the most likely amino acid to undergo protonation would be histidine. Four of 9 histidines within Fcε (His⁴²², His⁴⁸⁰, His⁴⁹⁰, and His⁵²⁸) were found to be ≥90% buried (14). One is in the Cε3 domain and the remaining 3 are in the Cε4 domain. Of the four Fcε histidines, only His⁵²⁸ is conserved at the homologous position of Fcγ (36). The two histidines, His⁵²⁸ in IgE and His⁴²⁹ in IgG, also occupy nearly identical chemical environments suggesting that His⁵²⁸ of IgE is unlikely to play a role in the intermediate pH unfolding transition of Fcε. Alternatively, His⁴²², His⁴⁸⁰, and His⁴⁹⁰ of Fcε are all occupied by polar residues at their homologous positions within IgG (Asn³²⁵, Ser³⁸³, and Tyr³⁹¹, respectively) and more likely involved in the pH sensitivity of Fcε.

The stability of the Cε2 domain was relatively insensitive to pH. Our DSC experiments demonstrate that the domain is

natively folded between pH 2.5 and 9.0. Cε2 likely remains folded at even lower pH values considering that the acidic groups within the domain should all be protonated by pH 2.5 and incapable of inducing large electrostatic changes in domain stability. Cε2 contains significantly fewer charged groups than the Cε3 and Cε4 domains of Fcε. There are only 16 charged amino acids within Cε2 and only one, Glu²⁷⁰, is >80% buried within the protein fold (15, 19). In contrast, Cε3 and Cε4 contain 25 and 28 charged amino acids, respectively. In these two domains, 12 of the charged amino acids are >85% buried within the fold, and several additional charged amino acids within Cε4 are >80% buried (15). The different charged residue compositions of Cε2 and Cε3Cε4 as well as the extent to which they are buried within their folds very likely lead to the different pH stability behaviors.

pH Effects on the Thermodynamics of the Fcε/Fcγ-FcεRIα Interaction—The affinity between Fcε and the Fcγ-FcεRIα fusion protein was extremely high under all pH conditions where the Cε3Cε4 domains of Fcε remained folded. DSC is uniquely suited to the study of very strong binding interactions (29). There are a multitude of parameters that must be determined with reasonable accuracy to obtain the K_D by DSC including some that are estimated (*i.e.* $\Delta C_P^0_{\text{unfolding}}$ for both the isolated Cε3Cε4 domains and domains 1 and 2 of FcεRIα). Due to the combination of experimental errors from the many variables, the magnitude of the K_D represents the best that can be expected from the DSC measurements and likely includes an error in the range of 10–100-fold. Encouragingly, the change in the Fcε/Fcγ-FcεRIα DSC curves upon complex formation looked very similar to DSC simulations of a theoretical $K_D = 10^{-12}$ M interaction described by Brandts and Lin (29). The experiments described here were performed at protein concentrations 2 orders of magnitude lower than their simulated curves; therefore, the simulations would predict that the Fcε/Fcγ-FcεRIα K_D was close to 10^{-14} M. The existence of two binding sites per single Fcγ-FcεRIα fusion protein may improve the affinity of the construct for IgE over what has previously been observed for the monomeric soluble receptor. However, thermal unfolding of both Fcε and Fcγ-FcεRIα is irreversible and complicates the interpretation of the thermodynamic parameters as additional avidity could be induced during unfolding due to aggregation. Therefore, we simply state that the $K_D < 10^{-12}$ M.

Whereas the magnitude of the affinity between Fcε and Fcγ-FcεRIα at pH 7.4 and 6.0 was relatively unchanged, the thermodynamic parameters of the interaction changed remarkably. The unfavorable $-T\Delta S_A^0(25^\circ\text{C})$ entropy term at pH 7.4 (+0.7 kcal mol⁻¹) changed by −10 kcal mol⁻¹ upon titration to lower pH values ($-T\Delta S_A^0(25^\circ\text{C}) = -10$ kcal mol⁻¹ at both pH 6.0 and 5.5). These results suggest a number of possibilities that all relate to the presence of charged groups directly at the binding interface. First, *apparent* changes in enthalpy are likely to arise from differences in buffer ionization. The buffer was changed from phosphate at pH 7.4 to acetate at pH 6.0 and below. It is possible that uptake of one or more protons at the binding interface may account for much of the enthalpy change because phosphate has an intrinsically low enthalpy of ionization (39).

⁶ S. J. Demarest and F. Taylor, unpublished data.

The titration of one or more residues between pH 7.4 and 6.0 may also affect the overall thermodynamics of binding and potentially the binding mode between the two proteins as well. No secondary structure changes were observed for apo-Fcε or apo-Fcγ-FcεRIα by CD upon titration from 7.4 to 5.2; therefore, the significantly altered binding thermodynamics could be the result of changes in charge density at or near the binding interface, local changes in the loop structures, or perhaps dynamic changes in quaternary structure. Consistent with the latter possibility, "open" and "closed" states have been shown to exist for Fcε in crystal structures with FcεRIα bound and absent (40). Titration to pH 6.0 and below also resulted in anomalous Fcε binding to the carboxy dextran-coated surface of the SPR chips (not shown) suggesting a change in polarity on the surface of Fcε. Large changes in the heat capacity of the interaction also suggest a change in how the proteins are associating at the different pH values. Quaternary structure rearrangements at low pH that modify the surface exposure of apo-Fcε or apo-FcεRIα could explain the experimentally determined ΔC_p^0 changes. One could speculate that this is due to stabilization of the closed form at lower pH.

Biological Importance of the pH-dependent Fcε Unfolding Event—It seems likely that the unusual pH sensitivity of Fcε, which differs greatly from what was observed for Fcγ, plays a role in its biological function or regulation. We have found the same pH sensitivity in an independent construct containing mouse Fcε,⁷ suggesting that this pH sensitivity is a general property of IgE. Mildly acidic solutions of sodium acetate have been used to strip bound IgE from the surface of basophils suggesting that the pH unfolding/unbinding mechanism of Fcε abolishes the ability of IgE to remain bound to FcεRI (8, 41).

As the half-life of FcεRI-associated IgE is extremely long and differs from the short half-life of IgE in the serum (12), the regulation of mast cell surface-bound IgE is a key factor determining the duration of allergic sensitivity. High levels of monomeric IgE increase the amount of FcεRI found on the surface of mast cells and basophils and induce enhanced survival of these inflammatory cells (6, 10). Cross-linking of receptor bound IgE and subsequent endocytosis is one recognized method of antigen-specific cell surface IgE clearance (8). It has been shown that cross-linking leads to shuttling of endocytic vesicles containing IgE/FcεRI from prelysosomal to lysosomal compartments (8). Additionally, IgE/FcεRI cross-linking leads to the accumulation of IgE fragments in the lysosomal-like secretory granules (9). These mechanisms of antigen-specific IgE turnover appear to involve the introduction of IgE to moderately acidic lysosomal compartments. Under these conditions, we would predict that the Fcε portion of IgE unfolds and dissociates from its receptor. This release may lead to IgE proteolysis within lysosomal compartments, whereas the less pH-sensitive FcεRIα may recycle back to the cell surface. Thus, the pH sensitivity of Fcε may play a key role in the turnover of receptor-bound IgE.

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