The interaction between IgE-Fc (Fce) 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 Fce-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 Fce and IgG1-Fc (Fcy). Fce was found to be significantly less stable than Fcy under all pH and NaCl conditions tested. Additionally, the Ce3Ce4 domains of Fce were shown to become intrinsically unfolded at pH values below 5.0. The interaction between Fce and an Fcy-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 binding affinity, and titration to pH 5.5 only modestly attenuated affinity. At pH values below 5.0, the receptor binding domains of Fce unfolded, and interaction of Fce with the Fcy-FcεRIα fusion protein was abrogated. The unusual pH sensitivity of Fce 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 (Fce) is responsible for binding to its two receptors, FceRI and CD23 (also known as FceRII). FcεRI is expressed on the surface of mast cells, basophils, and some antigen presenting cells in humans (2, 3), whereas FceRII 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:FceRI 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:FceRI 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 (KD ≈ 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 Fce/FcεRIα interaction occurs in a structurally homologous manner to what has been observed for IgG1 constant domain (Fcy) binding to FcγRII-III (13–15). Domain 2 of the FcεRIα subunit interacts with the homodimeric interface of the Ce3 domains of Fce. Ce4 of Fce does not participate directly in the binding but is crucial for maintaining high affinity (16–18). The Ce2 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/FcεRI 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 Fce, particularly at an industrial scale, have not been established. In particular, it is not known whether Fce is similar to Fcy in terms of its thermostability and pH sensitivity. Exposure to destabilizing conditions could lead to denaturation or aggregation. Even low levels of Fce dimer or higher order aggregates could have profound effects on cellular or in vivo function.
functional assays by inadvertently cross-linking FcεRI in the absence of antigen (5, 7). Our initial studies revealed an unusual sensitivity of FcεRI to various buffer and pH conditions. This led us to characterize in detail the biophysical properties of FcεRI important for maintaining IgE-FcεRI interactions and for IgE half-life.

Here we describe detailed biophysical analyses of FcεRI focused on deriving domain-specific unfolding information for FcεRI and how buffer conditions, particularly pH, affect FcεRI binding. Using circular dichroism (CD) and differential scanning calorimetry (DSC), we demonstrate that FcεRI is much less thermostable than FcεRI. The native structure of FcεRI is also highly sensitive to intermediate pH levels (5.0 and below) and high ionic strength. Binding of FcεRI to an Fcy-FcεRIα fusion protein is extremely high affinity at all pH values where FcεRI remains folded. Unfolding of the Ce3 and Ce4 domains below pH 5.0 abolishes FcεRI binding to the Fcy-FcεRIα fusion. This pH-dependent unfolding property of FcεRI 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 FcεRI, Fcy, Fcy-Ce2, Fcy-Ce2Ce3, and Fcy-FcεRIα-FceRIα was subcloned from mRNA extracted from human blood B cells (CD20+ and CD20+ IgM−). Complementary DNA primers, GTACATGCGACCACATCGCTTGT and TTTACCGGATTACAGACACCGCCG, which amplify from the C terminus of the CeI domain to the C terminus of the Ce4 domain, were used to create an FcεRI 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 CGGCGCGCCCTCACCATGCGCGAGCTATCGTCGTTGGCCTGGCGTGACGAGCGAATCCTTCTCGCTTCTCGTGGCGTCGGGGACCGCGACCGCGACCGGCTGTGGAGCGAGCCAAATCTTGTGACAA and TTAATTGATCCTGCTGTTCCTGGTGGCCGTGGCCACCCGCGGATTTACAG. 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-20 (Biogen Idec). The theoretical subcloned sequence following signal processing was (in Kabat numbering)248F T P P...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 FcεRI 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, Fcy-FcεRIα, containing human Fcy connected at its C terminus (Nterm, 228EPK...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 CGGCGCGCCCTCACCATGCGCGAGCTATCGTCGTTGGCCTGGCGTGACGAGCGAATCCTTCTCGCTTCTCGTGGCGTCGGGGACCGCGACCGGCTGTGGAGCGAGCCAAATCTTGTGACAA and TTAATTGATCCTGCTGTTCCTGGTGGCCGTGGCCACCCGCGGATTTACAG. The resulting PCR product was subsequently digested with XbaI and SalI and subcloned into a modified INPEP4-Fc vector containing a T318A (EU299) mutation that abolishes glycosylation at Asn314(EU297). The C-terminal Lys on FcεRIα was not included because this residue was cleaved at >90% within the cell culture for Fcy (see above). The plasmid was transfected into dihydrofolate reductase-CCHO DG44 cells using electroporation (25) and cloned in medium containing CHO-S-SFMI (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 FceRI producing CHO cells.

The chimeric proteins, Fcy-Ce2 and Fcy-Ce2Ce3, were prepared by linking the Ce2 or Ce2Ce3 domains of IgE to the C terminus of Fcy via a 15-amino acid linker GSGGSG(GGGS)5. The C-terminal Lys on Fcy was also not included here. The sequences of the Fcy-Ce2 and Fcy-Ce2Ce3 protein domains began at the same N terminus as the above FceRI construct and

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3 The abbreviations used are: CD, circular dichroism; Fcy, IgG constant domain fragment; IgG, immunoglobulin G; Ce2, Ce3, and Ce4, second, third, and fourth constant region domains of the IgG heavy chain, respectively; DSC, differential scanning calorimetry; Cy2 and Cy3, 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; ΔH25(T), molar enthalpy of association; ΔCp(T), molar heat capacity of association; Tm(T), midpoint of thermal unfolding transition; ΔSG(T), molar entropy of association.
contained the following C termini: ...SNP^{364} for Fcγ-Cε2 and ...GPR^{699} for Fcγ-Cε2Ce3. CHO cell lines producing high titer of Fcγ-Cε2 and Fcγ-Cε2Ce3 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 Fce was titrated to pH 9.0 using 1 ml Tris base, pH 7.4, and loaded on to a Q Sepharose column equilibrated in 20 mm, Tris, pH 7.0, at 10 ml/min. The column was washed with running buffer, and Fce was eluted using a linear gradient to 200 mm NaCl. Fce 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 NaCl, pH 5.0, and further concentrated using an Amicon stir unit with a 10,000 Mₐ cut-off YM10 membrane, and passed over a 350-ml Superdex 200 column equilibrated with PBS to remove aggregates, multimers, and remaining contaminants. Purified Fce was concentrated using an Amicon Ultra-15 centrifugal filter device, 10,000 Mₐ (Millipore). Protein concentration was measured by AU280 nm using an extinction coefficient of 1.4 ml mg⁻¹ cm⁻¹.

CHO supernatants containing recombinant Fcγ, Fcγ-FceRα, or Fcγ-Cε2 were loaded onto a Protein A-Sepharose FF (Amer sham 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, diluted 10 times, passed over a 350-ml Superdex 200 column equilibrated with PBS, and eluted in a single step using a 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 Mₐ cut-off YM10 membrane, and passed over a 350-ml Superdex 200 column equilibrated with PBS to remove aggregates, multimers, and remaining contaminants. Purified Fce was concentrated using an Amicon Ultra-15 centrifugal filter device, 10,000 Mₐ (Millipore). Protein concentration was measured by AU280 nm using an extinction coefficient of 1.4 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 Fce 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 Fce 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 Fce 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 Fce 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. Fce, Fcγ, and Fcγ-FceRα 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. Fce, Fcγ, and Fcγ-FceRα samples were dialyzed against 10 mm sodium citrate, 10 mm NaCl at pH 4.5, 5.0, 5.5, and 6.0. Additional Fce, Fcγ, and Fcγ-FceRα samples at pH 7.4 were prepared by dialysis against PBS. To map the apparent unfolding transition of Fce in detail, additional Fce 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 dimeric molecular mass (71.8 kDa) used for Fce and the monomer molecular mass used for Fcγ (24.7 kDa) and the divalent Fcγ-FceRα fusion protein (49.8 kDa). Thermal melts of Fce 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**—Fce 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 Fce 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 Fce in PBS. Various concentrations of trypsin (30, 300, and 3000 units, Sigma T-1426) and endoproteinase Glu-C (10 and 100 units,
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Sigma P-2922) were added to individual pH 4.5 Fce 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 Fce were also prepared for identifying non-Fce 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 Fce 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 Fce and Fcγ were diluted to 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 Fce and Fcγ-Ce2 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 Fce and Fcγ-FceRIα, 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-Fce and the monomeric molecular weight for apo- and holo-Fcγ-FceRIα, 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 Fce, Fcγ, Fcγ-FceRIα, and Fce/Fcγ-FceRIα were deconvoluted using the multiplex fitting routine within the software.

Isothermal Titrination Calorimetry (ITC)—Fce and Fcγ-FceRIα 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 Fce and Fcγ-FceRIα against 1× PBS. Fce 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 Fce (15 µl) were injected into the reaction cell containing 5 µM solutions of Fcγ-FceRIα to obtain a final Fce/Fcγ-FceRIα ratio of ~2.1. Dialysates were used in the reference cell. A 4-min equilibration period was used between all Fce 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). ΔH°(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-FceRIα, was saturated with Fce. 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 Fce 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 Fce/Fcγ-FceRIα using DSC and ITC-K(T,M) values for the isolated Ce3Ce4 domains of Fce and the apodomain of FceRIα were extrapolated from the measured calorimetric enthalpies and T,M values of the protein domains (i.e. with Fce alone or Fcγ-FceRIα 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 and ΔH°(25 °C) values for the Fce/Fcγ-FceRIα 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 Fce—Concentrated Fce 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 Fce peak(s) was determined by static light scattering and refractive index analyses. Following incubations between pH 5.3 and 9.0, Fce eluted as a single peak with
FIGURE 1. A, SEC chromatograms of Fc samples incubated at various pH values (indicated next to each line) before injection over the SEC column. Chromatograms of Fc samples with multiple peaks in the elution profile are shown with dotted lines. B, fluorescence intensity of ANS in the presence of Fc (□) 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 Fce 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 Fcy or Fce. 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 Fcy nor Fce associated with ANS as judged by fluorescence (Fig. 1B). In samples containing Fcy, 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 Fce 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 Fcy. In 750 mM NaCl and pH values below 6, the dye bound to Fce (Fig. 1B, inset), suggesting that high ionic strengths may facilitate the unfolding event.

To more directly evaluate secondary structure, CD spectra of Fce were taken under buffer conditions ranging from pH 4.5 to 7.4. Between pH 5.2 and 7.4, the spectra of Fce 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 Fce 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 Fce, Ce3Ce4, Unfold Below pH 5—To determine whether Fce unfolding observed at pH values below 5.0 was limited to particular domains or occurred over the entire Fce 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 Ce4 domain and to a lesser extent the Ce3 domain. Several peptides from the Ce3 domain appeared at a moderately slower rate. After 5 h, peptides covering >80% of the sequences of the Ce3 and Ce4 domains were observed (Fig. 2A). After 24 h of proteolysis, several small peaks corresponding to initial Ce2 proteolytic events appeared (not shown).

To confirm that the Ce2 domain was stable at low pH values relative to the Ce3 and Ce4 domains, a recombinant Ce2 domain without Ce3 and Ce4 was created by fusing the domain to the C terminus of Fce. Both Fce and the Fcy-Ce2 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 Ce2 within the context of the multidomain proteins Fce and Fcy-Ce2. Both Fce and Fcy-Ce2 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 (Tm) between 60 and 70 °C corresponding to the denaturation
**pH Unfolding of IgE-Fc Abrogates Binding to FceRIα**

A.  

| pH | Cε2 | Cε3 | Cε4 |
|----|-----|-----|-----|
| 2   | -   | -   | -   |
| 3   | Cε2 |  -  | -   |
| 4   | Cε2 | Cε3 | -   |
| 5   | Cε2 | Cε3 |  -  |
| 6   | Cε2 | Cε3 | Cε4 |

**FIGURE 2.** A, schematic (rectangle) diagram of Fce 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 Fce (black line) and Fcy-Cε2 (gray line) performed using samples dialyzed against the same pH 2.5 phosphate buffer. Schematic diagrams of the Fce and Fcy-Cε2 proteins are shown above the DSC curves.

The pH-dependent stabilities of both Fce and Fcy were compared using DSC. The unfolding transitions of both Fce and Fcy 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 Fce and Fcy domains by measuring $T_M$ values under identical instrument conditions. The Cε2 domain of Fce was pH stable and exhibited an unfolding transition very similar to Cε2 measured within the Fcy-Cε2 fusion protein at pH values between 7.0 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 Fce or Fcy-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

of Cε2 within both Fce and Fcy-Cε2 was observed. Fce and the Fcy-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 Fce—**Based on the pH-dependent unfolding described above, we investigated whether Fce 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 Fce stability under the different conditions. Thermal denaturation of Fce 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 Fce 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 Fcy-Cε2 DSC analyses and the Fce limited proteolysis studies at pH 4.5.
cooperativity between these domains at pH 8.0. The Ce3Ce4 domains of Fcε were pH labile (Fig. 3B). The Ce3Ce4 domains unfolded cooperatively with a maximum TM of 57 °C at pH 8. As the pH was lowered, the single unfolding transition of the Ce3Ce4 domains shifted to lower temperatures, whereas the unfolding transition of the Ce2 domain remained relatively static. As the pH was lowered to pH 4.8, the Ce3Ce4 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 Cy2 and Cy3 domains of Fcγ were generally much more stable than the homologous Ce3Ce4 domains of Fcε (Fig. 3C). Unlike the cooperative unfolding observed for the Ce3Ce4 domains of Fcε, the Cy2 and Cy3 domains unfolded in two separate transitions. The Cy2 domain was significantly less thermostable than the Cy3 domain. The Cy2 transition was identified by the effect that deglycosylation had on its thermostability. The Cy3 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 Ce3Ce4 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 Cy2 and Cy3 domains of Fcγ and the Ce3 and Ce4 domains of Fcε were slightly destabilized. This was seen as a small shift in their TM 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 affect on the in vitro half-life of Fcγ within this pH range because the TM of both the Cy2 and Cy3 domains remained above 60 °C. Interestingly, in high salt, the Ce2 domain of Fcε appeared to be slightly more stable. Ce2 was especially stabilized at neutral pH and 750 mM NaCl with a TM more than 7 °C higher than the TM measured using 15 mM NaCl (Fig. 4B). In contrast, NaCl significantly destabilized the Ce3Ce4 domains between pH 5 and 6 (Fig. 4B). Ce3Ce4 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 Fce/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 FcεRIα is important for serum IgG recirculation and half-life (33). Because marked decreases in Fce 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, FceRIα. To facilitate the expression of soluble FceRIα, the receptor subunit was fused to Fcγ (26). The resulting protein was a dimeric chimera of Fcγ and FceRIα. A mutation within Fcγ (T299A) that abrogated glycosylation was utilized to prevent potential nonspecific binding of Fcγ to FceRIα.

We first investigated whether FceRIα alone exhibited pH-dependent unfolding similar to the Ce3Ce4 domains of IgE.

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4 S. J. Demarest, unpublished results.


**FIGURE 5. pH-dependent structural changes within Fce/Fcy-FceRIα upon complex formation.** A, difference CD spectra obtained by subtracting Fcy spectra from Fcy-FceRIα spectra at the indicated pH values. The difference spectra reflect the expected CD spectra of FceRIα alone. B–F, CD spectra of equimolar mixtures of Fce and Fcy-FceRIα (dotted lines) and the mathematical addition of the spectra of apo-Fce and apo-Fcy-FceRIα 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 Fce 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 Fce and Fcy-FceRIα 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 Fce/FceRIα Binding Thermodynamics**—Quantification of Fce-FceRIα 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 \(k_2 = 2.6 \times 10^{-10} \text{ m} \) 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 Fce to Fcy-FceRIα at various pH values using DSC. The DSC curves of the individual Fce and Fcy-FceRIα proteins at pH 7.4 are shown in Fig. 6. An Fcy(T299A) construct was also analyzed to differentiate the unfolding transitions of Fcy from the single transition observed for FceRIα in the Fcy-FceRIα fusion protein,\(^5\) as the two transitions could not be separated by deconvolution (not shown). In particular, the two domains of FceRIα were found to unfold at the same temperature as the Fcy(T299A) Cγ2 domain. The DSC curve of complexed Fce/Fcy-FceRIα was significantly different from the simple addition of the isolated Fce and Fcy-FceRIα curves (dotted lines in Fig. 6) indicative of a strong interaction between the two proteins. The transitions corresponding to the Ce3Cε4 domains of Fce and domains 1 and 2 of FceRIα coalesced into a single transition that occurred at a higher temperature (\(\sim 8^\circ \text{C} \) higher) than the unfolding transitions of Ce3Cε4 and FceRIα 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 Fce and Fcy-FceRIα 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, \(K_{eq}(T) = [U_{\text{FceRIα}}][U_{\text{Ce3Cε4}}]/[F_{\text{FceRIα}}C_{\text{Ce3Cε4}}] \), where \([U_{\text{FceRIα}}]\) and \([U_{\text{Ce3Cε4}}]\) are the concentrations of unfolded FceRIα and Ce3Cε4, respectively, and \([F_{\text{FceRIα}}C_{\text{Ce3Cε4}}]\) is the concentration of the complex that was folded at all temperatures. Although the unfolding was not two-state for Fce or FceRIα due to aggregation at temperatures where unfolded protein exists, the scans were performed at maximal rates (4 °C/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 (\(\Delta C_p^0\)) and the enthalpy of the interaction at 25 °C (\(\Delta H^0(25 \text{ °C})\)), the temperature where we report the affinity, needed to be determined by ITC. The \(\Delta C_p^0\) (–760 cal mol\(^{-1}\) K\(^{-1}\)) and \(\Delta H^0\) (25 °C, –20.4 kcal mol\(^{-1}\)) values for binding of Fce to Fcy-FceRIα 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 Fc 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 Fce and Fcy-FceRIα remained extremely strong at pH 6.0 and 5.5 (Table 2). Measurements of $\Delta C_p^0$ (−670 cal mol$^{-1}$ K$^{-1}$) and $\Delta H_0^0$ (25 °C, −8.9 kcal mol$^{-1}$) at pH 6.0 and $\Delta C_p^0$ (−520 cal mol$^{-1}$ K$^{-1}$) and $\Delta H_0^0$ (25 °C, −6.9 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 binding affinity by ITC (Table 2) and indicated a $K_D < 10^{-9}$ M, consistent with the values obtained using DSC.

DSC experiments with Fce, Fcy-FceRIα, and Fce-Fcy-FceRIα 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 Fce and Fcy-FceRIα at both pH 6.0 and 5.5 resulted in large increases in the apparent $T_m$ values of the Ce3Ce4 domains of Fce and domains 1 and 2 of FceRIα 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 Fce and Fcy-FceRIα. 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 Fce (Figs. 1C and 5E) appeared coincidently with a reduced stability of the Ce3Ce4 domains (Fig. 3B). Formation of the complex between Fce and Fcy-FceRIα at pH 5.0 resulted in a marginal increase in the $T_m$ of the Ce3Ce4 domains and the apodomains of FceRIα over what was observed for the two proteins alone (Fig. 8C). The integrated enthalpy under the DSC curve of the Fce-Fcy-FceRIα 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 the apo-Fce and apo-Fcy-FceRIα 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 Fce bound to FceRIα (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, Fce and Fcy-FceRIα 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. Fce was found to be completely

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**TABLE 1**

| Construct (pH) | Ce3Ce4 Fce $T_m$ | Ce2 Fce $T_m$ | FceRI $T_m$ | Cy2 Fcy $T_m$ | Cy3 Fcy $T_m$ |
|----------------|-----------------|----------------|--------------|----------------|----------------|
| Fce (7.4)      | 60.8 (225)*     | 67.2 (78)      | 63.7 (60-30/30)* | 63.7 (31)     | 82.7 (62)      |
| FceRlα (7.4)   | 70.3 (413)*     | 70.2 (78)      | 62.3 (67-33/34)* | 62.3 (34)     | 82.1 (40)      |
| Fce (6.0)      | 57.22 (156)     | 70.1 (78)      | 60.4 (80-40/40)* | 60.4 (40)     | 82.9 (45)      |
| FceRlα (6.0)   | 69.6 (279)*     | 71.8 (79)      | 59.5 (78-38/40)* | 59.5 (40)     | 81.4 (67)      |
| FceRlα/Fce (6.0) | 53.5 (95)     | 71.8 (79)      | 54.3 (44)      | 61.0 (27)      | 80.0 (48)      |
| Fce (5.5)      | 66.9 (200)*     | 73.0 (80)      | 54.0 (52)      | 62.3 (53)      | 81.1 (38)      |

* Calorimetrically determined unfolding enthalpy in kcal/mol.

a FceRlα $T_m$ determined by curve fitting the two peaks under the single DSC peak where Fcy-Cy2 and FceRlα unfold to a single transition. The integrated calorimetric enthalpy of the Fcy-Cy2 peak was held constant based on the independent measurement of the Fcy-Cy2 calorimetric enthalpy measured for the non-fusion Fcy construct. Values in parentheses are (total integrated enthalpy-FceRlα calorimetric enthalpy)/(Fcy-Cy2 calorimetric enthalpy).

b Ce3Ce4 domain unfolding transition merges with the unfolding transition of the FceRlα domains upon binding. Both domains demonstrate increases in their overall melting temperatures ($T_m$ values) upon binding.
**pH Unfolding of IgE-Fc Abrogates Binding to FcεRIα**

**TABLE 2**

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

*ITC curves all contained a maximum of 1 or 2 points in the transition region.
* $10^°$ data impossible to analyze due to the lack of enthalpy of denaturation.

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FIGURE 7. Examples of ITC measurement of $\Delta H^o(T)$ and $\Delta C_p$ of the interaction between Fce and Fcγ-FcεRIα. A and B, titration of Fce into solutions containing Fcγ-FcεRIα at pH 6.0 (A) and pH 5.5 (B). C, determination of $\Delta C_p$ of the interaction by linear regression of $\Delta H^o(T)$ measured between 10 and 37°C at pH 7.4 (A), 6.0 (B), and 5.5 (C).

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FIGURE 8. Sum of the DSC traces of the apo-Fce 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.

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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 Fce was localized to the Ce3Ce4 domains and abolished the ability of IgE to interact with its high affinity receptor, FcεRI, below pH 5.0. The thermostability of Ce3Ce4 decreased significantly as the pH was reduced from pH 7.4 to 5.0. Attenuated Fce thermostability did not significantly attenuate binding to FcεRI until intrinsically unfolded Fce became present at pH 5.0 and below.

**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 Fce was localized to the Ce3Ce4 domains and abolished the ability of IgE to interact with its high affinity receptor, FcεRI, below pH 5.0. The thermostability of Ce3Ce4 decreased significantly as the pH was reduced from pH 7.4 to 5.0. Attenuated Fce thermostability did not significantly attenuate binding to FcεRI until intrinsically unfolded Fce became present at pH 5.0 and below.

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the Cγ3 domain of IgG1. It should be noted that the Cγ2 and Cγ3 domains of mouse IgG2a 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 Fce appear 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 Fce using a recombinant fusion construct Fcγ-linker-Cε2Cε3, this protein demonstrated a strong tendency to aggregate and poor overall biophysical behavior. Although FceRIα has been shown to bind the Cε3 domain, removal of Cε4 from Fce abrogates the ability of Fce to interact with cell surface FceRI (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 FceRI 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 FceRIα (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 Fce/Fcγ-FceRI (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 Fce 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 Fce 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 Fce (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 Fce 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 Fce. Alternatively, His422, His480, and His490 of Fce 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 Fce.

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 Fce. 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 Cε3/Cε4 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 Fce/Fcγ-FceRIα Interaction**—The affinity between Fce and the Fcγ-FceRIα fusion protein was extremely high under all pH conditions where the Cε3Cε4 domains of Fce remain 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 $K_p$, DSC including some that are estimated ($\Delta C_p$ unfolding for both the isolated Cε3Cε4 domains and domains 1 and 2 of FceRIα). Due to the combination of experimental errors from the many variables, the magnitude of the $K_p$ 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 Fce/Fcγ-FceRIα DSC curves upon complex formation looked very similar to DSC simulations of a theoretical $K_p = 10^{-12}$ 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 Fce/Fcγ-FceRIα $K_p$ was close to $10^{-14}$ M. The existence of two binding sites per single Fcγ-FceRIα 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 Fce and Fcγ-FceRIα 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 $K_p < 10^{-12}$ M.

Whereas the magnitude of the affinity between Fce and Fcγ-FceRIα at pH 7.4 and 6.0 was relatively unchanged, the thermodynamic parameters of the interaction changed remarkably. The unfavorable $-\Delta S^{\circ}(25^\circ C)$ entropy term at pH 7.4 (+0.7 kcal mol$^{-1}$) changed by $-10$ kcal mol$^{-1}$ upon titration to lower pH values ($-\Delta S^{\circ}(25^\circ C) = -10$ 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).
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 ΔC⁰ 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, 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 mechanism of antigen-specific cell surface IgE clearance (8). It has been shown that cross-linking leads to shuffling 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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