

## The Key Role of Protein Flexibility in Modulating IgE Interactions\*

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**The interaction between IgE and its high affinity receptor (FcεRI) is a critical step in the development of allergic responses. Detailed characterization of the IgE-FcεRI interaction may offer insights into possible modes of inhibiting the interaction, which could thereby act as a potential therapy for allergy. In this study, NMR, CD, and fluorescence spectroscopies have been used to characterize structurally the Cε3 domain of IgE and its interaction with other protein ligands, namely, Cε2, Cε4, sFcεRIα, and CD23. We have shown that the recombinant Cε3 domain exists alone in solution as a “molten globule.” On interaction with sFcεRIα, Cε3 adopts a folded tertiary structure, as shown by the release of the fluorescent probe 8-anilidonaphthalene-1-sulfonate and by characteristic changes in the <sup>1</sup>H, <sup>15</sup>N heteronuclear single quantum coherence NMR spectrum. However, the interactions between the Cε3 domain and Cε2, Cε4, or CD23 do not induce such folding and would therefore be expected to involve only local interaction surfaces. The conformational flexibility of the Cε3 domain of the whole IgE molecule may play a role in allowing fine tuning of the affinity and specificity of IgE for a variety of different physiological ligands and may be involved in the conformational change of IgE postulated to occur on interaction with FcεRI.**

IgE in mammals plays a crucial role in host defense against parasitic infections (1). However, overproduction of specific IgE antibodies in response to common, innocuous antigens underlies allergic reactions (2). Allergic diseases affect approximately one-third of the general population (3).

IgE is a heterotetramer of two identical heavy chains and two identical light chains, organized into discrete globular domains containing the immunoglobulin fold (reviewed in Refs. 1 and 4). Each of these immunoglobulin domains is comprised of about 110 amino acids, folded into a β-sheet “sandwich” in the C-type topology. IgE contains an additional constant domain, Cε2, in place of the flexible hinge region found in IgG. IgE-Fc is a dimeric fragment of Cε2-Cε4 and retains full binding activity for the high affinity IgE receptor (FcεRI) (5). The recently determined crystal structure of IgE-Fc (6) indicates that the presence of the Cε2 domains causes IgE-Fc to assume a highly bent conformation, confirming previous scattering (7) and fluorescence (8) studies. This structural information, taken together with the effect of Cε2 domains on the kinetics of binding

to FcεRI (9), may account for the distinct features of the kinetics of the interaction of IgE with its receptor compared with those of IgG and its receptor (10).

In order for IgE to trigger cellular responses, it must function in combination with a membrane-bound receptor. The human FcεRI can form either a heterotetramer composed of three different transmembrane polypeptides, αβγ<sub>2</sub>, or a heterotrimer, αγ<sub>2</sub> (11). The extracellular portion of the α chain is necessary for binding of the Fc region of IgE and consists of two globular domains, D1 and D2. FcεRI is present constitutively on mast cells and basophils and is also expressed on activated eosinophils. The high binding affinity of FcεRI for IgE ( $K_a \sim 10^{10} \text{ M}^{-1}$ ) means that most IgE is found fixed to the cells that bear FcεRI even in the absence of bound antigen (12). IgE also has a second, lower affinity cell surface receptor, CD23 (FcεRII). CD23 is unlike other immunoglobulin receptors in that it is not a member of the immunoglobulin superfamily; instead, it belongs to the family of C-type lectins (13).

The interaction of IgE with FcεRI is a critical step in the initiation of an allergic response (2). The crystal structure of the complex sFcεRIα/Cε3–4 has been determined and reveals two distinct binding sites on the extracellular portion of the α chain of sFcεRIα (14) (Fig. 1). Comparison of the sFcεRIα/Cε3–4 complex structure and the recent crystal structure of the whole IgE-Fc (Cε2–4) indicates that a substantial conformational change involving Cε2 may occur on binding to sFcεRIα (6, 15).

The binding of IgE to both FcεRI and CD23 has been localized to the Cε3 domains (16), suggesting this domain might be an attractive target for structural and functional studies. However, Cε3 has proven a difficult protein to produce; attempts to express it in mammalian and yeast systems have been unsuccessful (data not shown). In hindsight, this may be due to the inherent instability of the Cε3 domain alone. Recombinant Cε3 has been expressed in bacteria and purified, and although it appears to have little recognizable secondary structure or hydrophobic core, it is able to bind to FcεRIα (17, 18). It has been postulated that this “unfolded” Cε3 domain could undergo a conformational change to a more ordered state on binding to FcεRIα; however, initial far-UV CD studies on the individual proteins and mixtures were unable to confirm this (17).

“Molten globules,” initially described in 1983, were proposed as intermediates in protein folding pathways (19). These compact intermediates are characterized by the presence of secondary structure without rigid tertiary structure (reviewed in Ref. 20). Because molten globules lack rigid tertiary structure, their amino acid side-chains have high internal mobility, leading to the exposure of some clusters of internal nonpolar groups. This allows them to bind hydrophobic molecules, such as 8-anilino-

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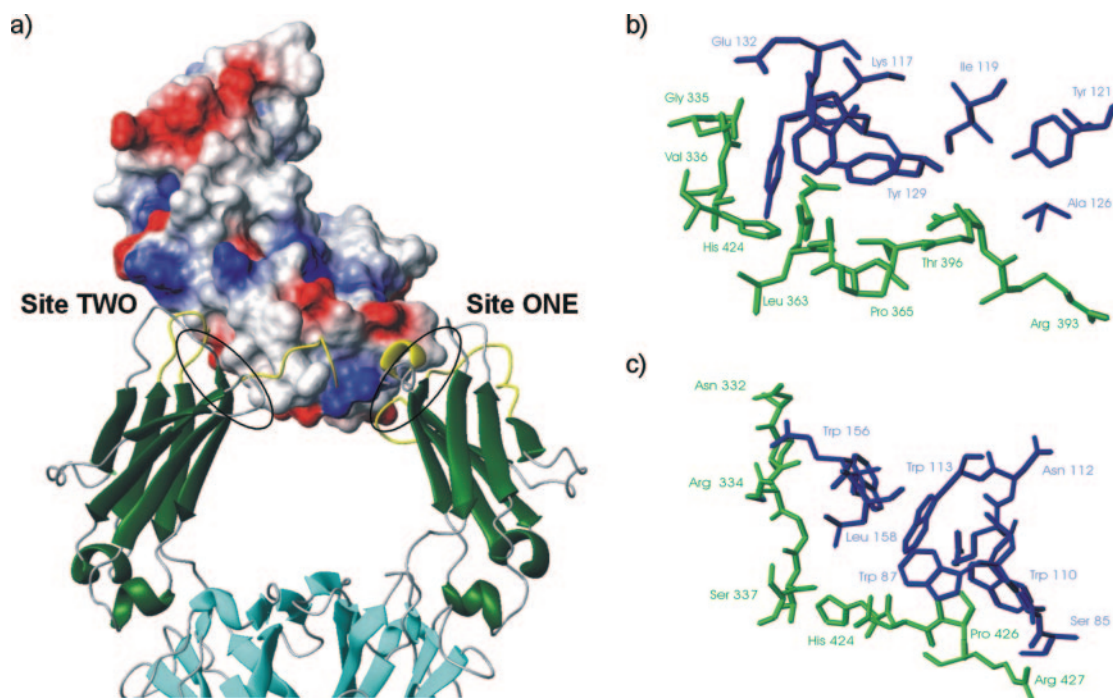


FIG. 1. *a*, the structure of sFcεRIα bound to Cε3-4 dimer. The structural data are taken from Garman *et al.* (14) (Protein Data Bank code 1F6A). sFcεRIα is shown in *surface representation*, and Cε3-4 is shown in *ribbon representation*, with Cε3 domains in *green* and Cε4 domains in *cyan*. *b*, binding site 1. Cε3 residues (*green*) are contributed from the Cε2-3 linker and BC, DE, and FG loops. sFcεRIα residues (*blue*) are contributed from the C-C' region of the D2 domain. *c*, binding site 2. Cε3 residues (*green*) are contributed from the Cε2-3 linker and FG loop. sFcεRIα residues (*blue*) are contributed from the D1-D2 interface.

naphthalene-1-sulfonate (ANS),<sup>1</sup> much more strongly than either the native or fully unfolded states.

Over the last two decades, the native state of proteins has been increasingly viewed as consisting of a dynamic ensemble of conformational states. It has been shown that there is an uneven distribution of structural stability throughout proteins, with sites involved in mediating protein-protein interactions often characterized by the presence of regions of low structural stability (21). Such an arrangement may facilitate ligand-induced conformational changes and can allow for fine-tuning of specificity and affinity between ligands and proteins (22). It has been estimated that 36–63% of cellular proteins or protein domains exist in a partially disordered state (23). When such a protein, existing as an ensemble of conformers, binds to a ligand, it may stabilize one protein conformer, shifting the equilibrium and causing a population shift in favor of this conformer (24, 25). These changes in protein conformation can be monitored spectroscopically, using fluorescence, CD, and NMR.

The melting temperature ( $T_m$ ) of a protein, determined by differential scanning calorimetry, can provide a global measure of its stability (reviewed in Ref. 26). A comparison of the  $T_m$  obtained for IgE and that for IgG (27, 28) indicates that IgE is less stable than IgG. Thermal denaturation of proteolytic fragments of IgE indicates the determinants of the lower  $T_m$  are localized to the region that interacts with FcεRI (27).

The aim of the work described in this study is to characterize how structural and energetic contributions define the pathway by which IgE and FcεRI interact. We have used spectroscopic techniques to observe the conformation of the isolated Cε3 domain in solution. The Cε3 domain alone exists as a molten globule, lacking stable tertiary structure. The effect of addition of a number of ligands on the conformation of Cε3 has been observed. Addition of sFcεRIα to Cε3 causes the domain to

adopt a folded structure. The folding of Cε3 can be monitored simply by the binding of a fluorescent hydrophobic ligand, such as ANS.

#### EXPERIMENTAL PROCEDURES

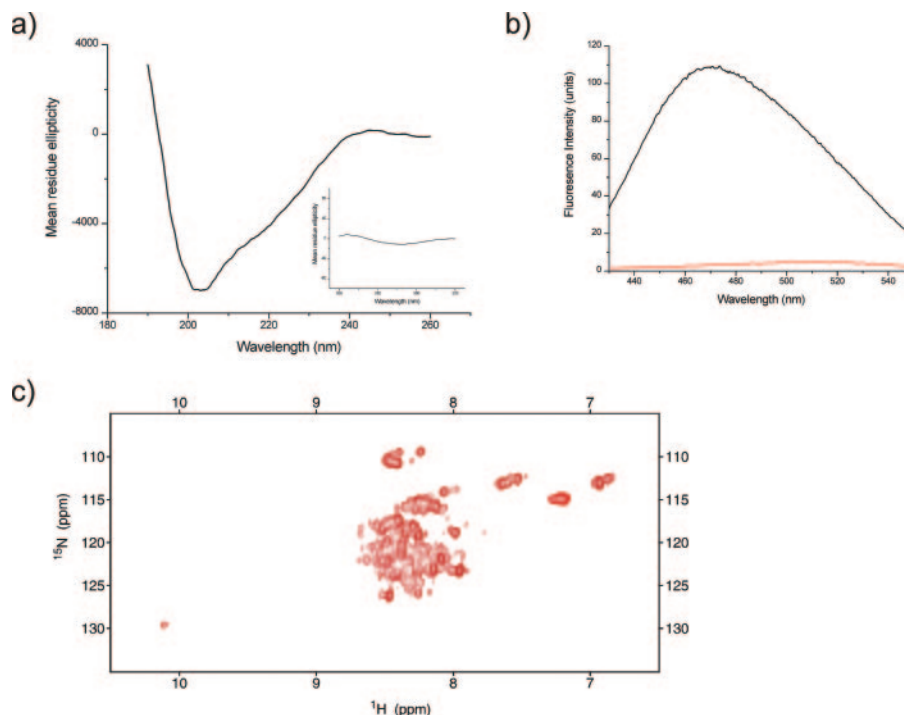
**Expression and Purification of sFcεRIα**—The production of a soluble fragment of human sFcεRIα (Val<sup>1</sup> to Lys<sup>176</sup>) for mammalian expression in the mouse myeloma cell line NSO (29) and subsequent cloning and expression of a shorter construct (Val<sup>1</sup> to Ala<sup>172</sup>) in yeast *Pichia pastoris* have been detailed previously (29, 30). The pPICZα vector (Invitrogen) containing the shorter sFcεRIα construct with an N-terminal polyhistidine tag was transformed into *P. pastoris* strain SMD1168H. Fermentative growth of the *P. pastoris* clone was performed according to the manufacturer's guidelines (Invitrogen). Yeast supernatant was filtered and concentrated by flowing across a Viva-Flow 200 membrane (VivaScience) and then dialyzed extensively against phosphate-buffered saline (PBS) at pH 7.4. Protein was purified on a phenyl-Sepharose column (Amersham Biosciences) and a Chelating Sepharose Fast Flow column (Amersham Biosciences) charged with nickel. Purified recombinant sFcεRIα was concentrated and buffer exchanged into PBS, pH 5.0. The protein concentration was spectrophotometrically determined using an extinction coefficient at 280 nm of 2.56 for a 1 mg/ml solution (30).

**Cloning of Recombinant Cε3**—The Cε3 fragment (Cys<sup>328</sup>-Ser<sup>437</sup>), with C328S mutation, produced by PCR using an IgE-Fc cDNA clone isolated from U266 cells, was subcloned into the NdeI and BamHI cloning sites of pET5a expression vector (Novagen) as described previously (17). The Cε3 fragment was isolated from the pET5a clone by PCR using primers TAATACGACTCACTATAGGG and TATCACGAGGC-CCTTT and subcloned into the NdeI and BamHI cloning sites of pET28a expression vector (Novagen). The integrity of the clone was confirmed by DNA sequencing (Department of Biochemistry, University of Oxford, Oxford, United Kingdom).

**Expression and Purification of Recombinant Cε2, Cε3, Cε4, and CD23**—Previously, the Cε2 construct (Ser<sup>225</sup>-Asp<sup>330</sup>), with C241S and C328S mutations, and the Cε4 construct (Ser<sup>437</sup>-Asn<sup>544</sup>) were produced by PCR using an IgE-Fc cDNA clone isolated from U266 cells. The Cε2 fragment was subcloned into the NdeI and BamHI cloning sites of pET5a expression vector (Novagen) (9). The Cε4 fragment was subcloned into the NdeI and BamHI cloning sites of pET15b expression vector (Novagen). Previously, the derCD23 construct (Ser<sup>156</sup>-Glu<sup>298</sup>) was subcloned from CD23 cDNA by PCR (31).

<sup>1</sup> The abbreviations used are: ANS, 8-anilino-1-naphthalene-sulfonate; HSQC, heteronuclear single quantum coherence; PBS, phosphate-buffered saline.

**FIG. 2. Spectroscopic properties of Ce3.** *a*, far-UV CD spectrum (Ce3 at 0.2 mg/ml in PBS, pH 5.0; cuvette path length, 0.2 mm). The near-UV spectrum (Ce3 at 3 mg/ml in PBS, pH 5.0; cuvette path length, 5 mm) is shown (*inset*). *b*, the emission spectra of ANS (17.3  $\mu$ M) in the presence of Ce3 (0.2 mg/ml) or in PBS, pH 5.0, alone are shown in *black* and *red*, respectively. The excitation wavelength was 370 nm. *c*,  $^{15}$ N HSQC of 0.2 mM  $^{15}$ N-Ce3 in PBS, pH 5.0 (*red*).



Recombinant Ce2, Ce3, Ce4, and CD23 were expressed in *Escherichia coli* host strain BL21(DE3).  $^{15}$ N- and  $^{13}$ C,  $^{15}$ N-labeled samples were prepared in M9 media with the addition of 1.0 g/liter  $^{15}\text{NH}_4\text{Cl}$  or 3 g/liter  $^{13}\text{C}$  glucose and 1.0 g/liter  $^{15}\text{NH}_4\text{Cl}$ . Recombinant protein expression was induced by addition of 1 mM isopropyl  $\beta$ -D-thiogalactopyranoside. Rifampicin was added to the cultures 40 min after induction had been started, to a final concentration of 50  $\mu$ g/ml as described previously (32). Cell pellets were resuspended in 10 ml of lysis buffer (100 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, and 50 mM  $\text{MgCl}_2$  in PBS, pH 7.4). After sonication, 5  $\mu$ l of DNase was added to the solution and incubated for 1 h. Cells were then washed by centrifugation at  $10,000 \times g$  for 12 min and resuspended in 10 ml of lysis buffer. Washing was repeated at least three times.

Ce2 and CD23 proteins were refolded and purified as described previously (9, 33). For Ce3 preparations, the inclusion bodies were isolated by solubilizing the final pellet in 10 ml of 6 M guanidinium chloride, 0.02 M  $\text{Na}_2\text{HPO}_4$ , 0.5 M NaCl, and 1 mM dithiothreitol at pH 7.4. This solution was then loaded onto a Chelating Sepharose Fast Flow column (Amersham Biosciences) charged with nickel. Refolding of Ce3 was carried out while it was bound to the column. After washing of the immobilized Ce3 with 6 M guanidinium chloride, 0.02 M  $\text{Na}_2\text{HPO}_4$ , and 0.5 M NaCl at pH 7.4, the guanidinium chloride concentration was reduced to 0 M. Bound protein was then eluted by decreasing pH with a linear gradient to pH 1.5. The procedure for Ce4 was nearly identical, except that refolding was carried out in solution, according to the method of Taylor *et al.* (34), and purification was performed on a Talon column (Clontech) charged with cobalt.

Purified recombinant Ce2, Ce3, Ce4, and CD23 were each concentrated and buffer exchanged into PBS, pH 5.0. The protein concentration was determined spectrophotometrically using an extinction coefficient at 280 nm: 1.32 for a 1 mg/ml solution of Ce2 (9), 1.15 for Ce3 (17), 1.51 for Ce4, and 2.81 for derCD23. There was no reaction between refolded Ce3 and 5,5'-dithiobis(2-nitrobenzoic acid) (35), showing that no free sulfhydryl groups were present.

**CD Spectroscopy**—The CD spectra of native and denatured Ce3 were recorded in either a Jasco J-600 or a Jasco J-810 spectropolarimeter at 20  $^\circ\text{C}$ , calibrated with reference to 1S-(+)-10-camphorsulfonic acid. Spectra in the far-UV region (260–195 nm) were recorded in cylindrical cells of 0.2-mm path length using a protein concentration of 0.2 mg/ml in PBS, pH 5.0. Spectra in the near-UV region (320–260 nm) were recorded in cylindrical cells of 5-mm path length, using a protein concentration of 3 mg/ml. In each case, three scans (recorded at scan rate of 10 nm/min with a time constant of 2 s) were averaged and corrected by subtraction of the spectrum of buffer alone. Mean residue ellipticities were calculated using a value of 112 for the mean residue weight of the protein. The far-UV spectrum was ana-

lyzed by CONTIN to give an estimate of the contribution of elements of regular secondary structure (36).

**Fluorescence**—The fluorescence spectra of native and denatured Ce3 were recorded on a Perkin Elmer LS 50 spectrofluorometer at 20  $^\circ\text{C}$ , using a protein concentration of 0.2 mg/ml in PBS, pH 5.0. Protein fluorescence emission was measured between 300 and 400 nm, after excitation at 290 nm. Ce3 was incubated for 1 h with various concentrations of guanidinium chloride. The concentration of the 6 M stock of guanidinium chloride was checked by refractive index measurements (37). Spectra were corrected for Raman scattering by the solvent. ANS binding to Ce3 was studied using an ANS concentration of 17.3  $\mu$ M. ANS fluorescence emission was measured between 430 and 550 nm, after excitation at 370 nm. ANS fluorescence emission was measured both for native and denatured Ce3 and for Ce3 after addition of protein ligands (sFceRI $\alpha$ , Ce2, Ce4, and CD23) at equimolar concentrations. Three of the protein ligands (sFceRI $\alpha$ , Ce2, and CD23) do not alter ANS fluorescence emission when added separately to ANS solutions. However, Ce4 alone does appear to bind weakly to ANS, with a <3-fold increase in fluorescence and a modest blue shift (20 nm) in the  $\lambda_{\text{max}}$  of relative to free ANS. This is probably due to exposure of hydrophobic surfaces that would not otherwise be exposed in the context of the IgE molecule. Emission spectra were corrected for the effect of dilution by performing control experiments in which buffer alone was added.

**Nuclear Magnetic Resonance**—NMR samples of 270–300  $\mu$ l, with 5%  $\text{D}_2\text{O}$  and 0.02% sodium azide, were transferred into Shigemi tubes (BM-3). All spectra were recorded on home-built NMR spectrometers with Oxford Instrument magnets, at operating proton frequencies of 500 or 600 MHz. Spectra were collected over a range of temperatures from 5  $^\circ\text{C}$  to 35  $^\circ\text{C}$ ; only spectra recorded at 25  $^\circ\text{C}$  are shown here. The proton carrier frequency was set on water at 4.74 ppm. One-dimensional NMR spectra were obtained using a sweep width of 16,000 Hz, 2048 points, and an acquisition time of 128 ms. Gradient enhanced  $^1\text{H}$ ,  $^{15}\text{N}$  heteronuclear single quantum coherence (HSQC) experiments were performed at a proton frequency of 600 MHz. The spectral width in the direct  $^1\text{H}$  dimension was 20,000 Hz, collected over 2048 points, with an acquisition time ( $t_2$ ) of 102.4 ms. The spectral width in the indirect  $^{15}\text{N}$  dimension was 1700 Hz, collected over 100 points, with an acquisition time ( $t_1$ ) of 58.8 ms. In each case, 64 scans were collected. The carrier frequency was set at 120 ppm. All NMR titrations were carried out in PBS, pH 5.0.

## RESULTS AND DISCUSSION

**Characterization of Recombinant Ce3**—Refolded Ce3 containing the correct intradomain disulfide bond (Cys<sup>358</sup>-Cys<sup>418</sup>) was shown to be predominantly monomeric by nonreducing SDS-PAGE and by electrospray ionization mass spectrometry.

In addition, NMR hydrodynamic relaxation studies can be used to give an estimate of the molecular mass of a sample relative to a series of protein standards. In this case, C $\epsilon$ 3 was shown to be monomeric, having a molecular mass of  $\sim$ 12,000 Da.

The far-UV CD spectrum of refolded C $\epsilon$ 3 is shown in Fig. 2a.

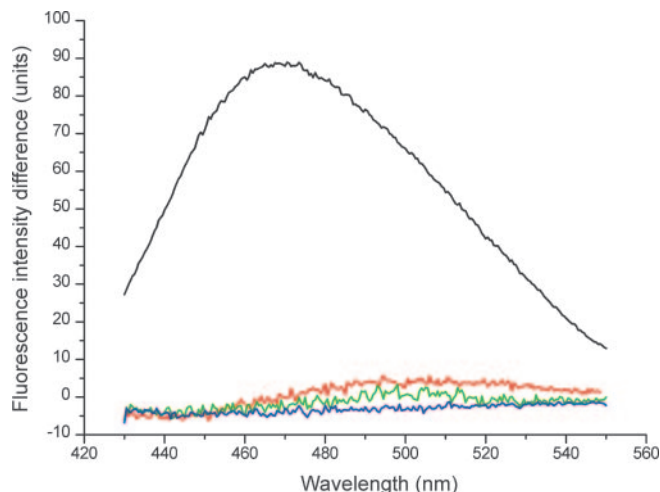


FIG. 3. **Fluorescence emission difference spectra for C $\epsilon$ 3 and ANS on addition of various ligands.** Difference spectra are calculated by subtraction from the (C $\epsilon$ 3 and ANS) spectrum alone. (C $\epsilon$ 3 and ANS) with sFceRI $\alpha$  added is shown in black, (C $\epsilon$ 3 and ANS) with CD23 added is shown in red, (C $\epsilon$ 3 and ANS) with Ce2 added is shown in green, and (C $\epsilon$ 3 and ANS) with Ce4 added is shown in blue. Each ligand is added in a 1:1 molar ratio. Each line represents an average of three separate experiments, and for each of those, an average of three fluorescence emission scans. A positive difference shows that ANS is dissociating from C $\epsilon$ 3.

Analysis of the spectrum by CONTIN (36) indicates that C $\epsilon$ 3 contains  $\sim$ 35%  $\beta$ -sheet structure. This value is in good agreement with the  $\sim$ 40%  $\beta$ -sheet structure present in the fully folded C $\epsilon$ 3 domain within IgE-Fc (6). However, the near-UV spectrum of refolded C $\epsilon$ 3 exhibited only very small ellipticities, indicating little tight packing of side-chains. The solvent accessibility of the two tryptophan residues of C $\epsilon$ 3 was measured by protein fluorescence spectroscopy. Refolded C $\epsilon$ 3 emits fluorescence with  $\lambda_{\text{max}}$  of 350 nm. As C $\epsilon$ 3 is exposed to increasing concentrations of guanidinium chloride, the intensity of fluorescence emission increases, and the  $\lambda_{\text{max}}$  shifts to 358 nm. A plot of percentage maximum change in fluorescence at 360 nm against guanidinium chloride concentration for C $\epsilon$ 3 is linear. The unfolding of C $\epsilon$ 3 is therefore not a cooperative process; this would be expected for a protein that lacks stable tertiary structure. ANS is a fluorescent hydrophobic probe that shows a stronger affinity for the molten globule state than for either the fully folded or unfolded states (38). Free ANS in aqueous solution shows weak fluorescence with a  $\lambda_{\text{max}}$  of 515 nm. Binding of ANS to the molten globule state causes a large increase in its fluorescence. ANS free in solution emits fluorescence with a  $\lambda_{\text{max}}$  of 515 nm. Incubation of ANS with C $\epsilon$ 3 causes a dramatic increase in the fluorescence intensity and shifts the  $\lambda_{\text{max}}$  to 470 nm (Fig. 2b). As C $\epsilon$ 3 is unfolded by increasing guanidinium chloride concentrations, ANS becomes displaced, and its fluorescence emission returns to that characteristic for ANS alone. The  $^{15}\text{N}$  HSQC NMR spectrum of refolded C $\epsilon$ 3 is shown in Fig. 2c. The lack of chemical shift dispersion in both dimensions is characteristic of a protein that lacks native stable tertiary structure and is sampling a series of different conformations during the course of the NMR experiment. Monomeric recombinant C $\epsilon$ 3 alone in solution has native-like secondary struc-

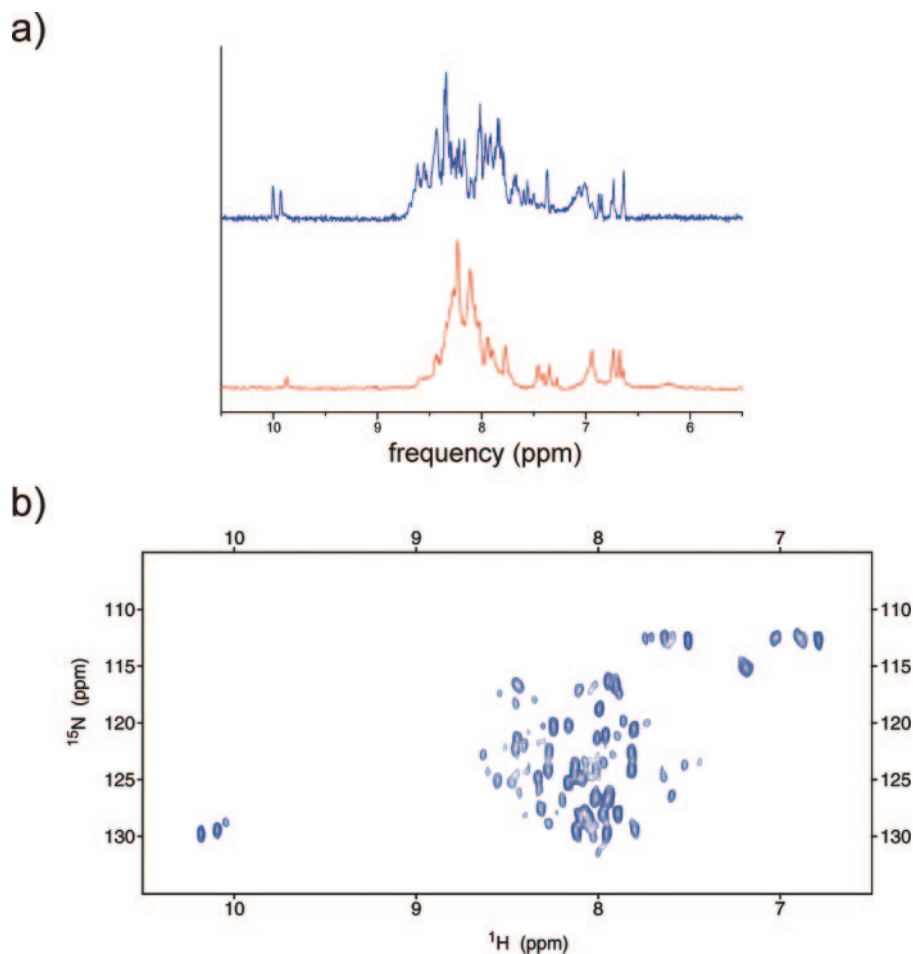


FIG. 4. **NMR of  $^{15}\text{N}$ -C $\epsilon$ 3.** a, a one-dimensional  $^{15}\text{N}$ -filtered proton spectrum for 0.5 mM C $\epsilon$ 3 before (red) and after (blue) addition of 0.3 mM sFceRI $\alpha$ . b, a two-dimensional  $^{15}\text{N}$  HSQC spectrum for 0.5 mM C $\epsilon$ 3 after addition of 0.3 mM sFceRI $\alpha$  (blue).

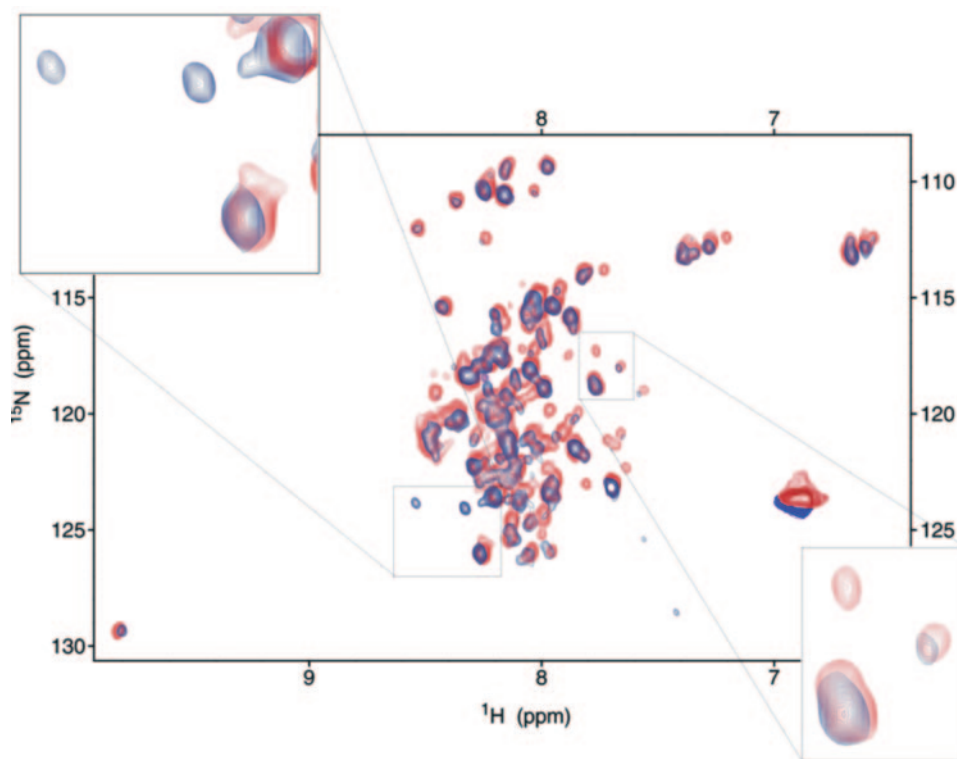


FIG. 5.  $^{15}\text{N}$ -C $\epsilon$ 3 HSQC. 0.2 mM  $^{15}\text{N}$ -C $\epsilon$ 3 before and after addition of 0.2 mM unlabeled C $\epsilon$ 2. C $\epsilon$ 3 alone is shown in red; C $\epsilon$ 3 with C $\epsilon$ 2 added is shown in blue. Enlarged regions highlight some obvious regions of change.

ture and lacks rigid tertiary structure and is therefore typical of the molten globule state (reviewed in Ref. 20).

**Interaction of C $\epsilon$ 3 with Protein Ligands**—The effect of addition of the ligands sFc $\epsilon$ RI $\alpha$ , C $\epsilon$ 2, C $\epsilon$ 3, and CD23 on the conformation of C $\epsilon$ 3 was studied using ANS fluorescence and NMR spectroscopies. Each of these ligands has been shown previously to bind to C $\epsilon$ 3, using surface plasmon resonance and/or NMR (17).

**Fluorescence (sFc $\epsilon$ RI $\alpha$ , C $\epsilon$ 2, C $\epsilon$ 4, and CD23 as Ligands)**—C $\epsilon$ 3 alone in solution binds to ANS, causing an increase in fluorescence emission and a shift in the  $\lambda_{\text{max}}$  compared with free ANS. Any alteration in the fluorescence emission properties of ANS after equimolar addition of ligands to the C $\epsilon$ 3/ANS mixture will result from changes in the conformation of C $\epsilon$ 3. sFc $\epsilon$ RI $\alpha$ , C $\epsilon$ 2, and CD23 were shown not to bind to ANS, and although C $\epsilon$ 4 binds weakly to ANS, the changes in intensity and  $\lambda_{\text{max}}$  are small relative to those of C $\epsilon$ 3 (less than a 3-fold increase in fluorescence intensity and a 20 nm blue shift in  $\lambda_{\text{max}}$ ). Addition of sFc $\epsilon$ RI $\alpha$  to C $\epsilon$ 3 causes ANS to dissociate from C $\epsilon$ 3, reducing the intensity and increasing the  $\lambda_{\text{max}}$  of ANS fluorescence emission. This reduction in binding of ANS to C $\epsilon$ 3 is an indication of the loss of the molten globule character, as would be expected if C $\epsilon$ 3 adopted a more stable tertiary structure on binding to sFc $\epsilon$ RI $\alpha$  (Fig. 3). The fluorescence emission spectrum represents the average emission over all the conformations present in the sample. Unlike sFc $\epsilon$ RI $\alpha$ , addition of C $\epsilon$ 2, C $\epsilon$ 4, or CD23 to C $\epsilon$ 3 does not cause ANS to dissociate from C $\epsilon$ 3 (Fig. 3) because there is no change in the intensity or  $\lambda_{\text{max}}$  of ANS fluorescence emission compared with that for C $\epsilon$ 3 alone. This indicates that C $\epsilon$ 3 does not adopt a stable tertiary structure on binding to C $\epsilon$ 2, C $\epsilon$ 4, or CD23.

**NMR (sFc $\epsilon$ RI $\alpha$  and C $\epsilon$ 2 as Ligands)**—The one-dimensional,  $^{15}\text{N}$ -filtered, proton NMR spectra of C $\epsilon$ 3 before and after addition of sFc $\epsilon$ RI $\alpha$  are shown in Fig. 4a. On binding of sFc $\epsilon$ RI $\alpha$ , signal dispersion beyond 8.5 ppm is observed, indicating the formation of stable tertiary interactions within C $\epsilon$ 3. The loss of

the large broad signal at  $\sim$ 8.3 ppm on binding sFc $\epsilon$ RI $\alpha$  indicates that the backbone amides of C $\epsilon$ 3 are no longer in a random coil conformation. It can therefore be seen even by the simple one-dimensional proton NMR spectrum that C $\epsilon$ 3 adopts a folded conformation on binding to sFc $\epsilon$ RI $\alpha$ .

$^{15}\text{N}$  HSQC experiments allow separation of the NMR signal into a second dimension, mapping the backbone amide groups of a protein according to their proton and nitrogen frequencies. C $\epsilon$ 3 alone shows little chemical shift dispersion in both dimensions,  $\sim$ 8.3 ppm in the  $^1\text{H}$  dimension (Fig. 2c). A reduction in temperature causes a decrease in the rate at which these conformations are sampled, reducing the line broadening, but even at lower temperatures, there is still little chemical shift dispersion.

On addition of sFc $\epsilon$ RI $\alpha$ , the  $^{15}\text{N}$  HSQC for C $\epsilon$ 3 shows large signal dispersion in both dimensions (Fig. 4b). The number of peaks observed in the  $^{15}\text{N}$  HSQC corresponds approximately to the number of residues in C $\epsilon$ 3. Binding to sFc $\epsilon$ RI $\alpha$  clearly causes C $\epsilon$ 3 to adopt a folded conformation with stable tertiary structure, rather than sampling a number of related conformations. A similar transition from molten globule state to fully folded conformation on interaction with a binding partner has been noted for a number of other proteins (24, 25). In contrast, addition of C $\epsilon$ 2 to  $^{15}\text{N}$ -C $\epsilon$ 3 does not cause the same large change in chemical shift dispersion in C $\epsilon$ 3 (Fig. 5). C $\epsilon$ 3 does not appear to undergo the same transition from molten globule state to fully folded conformation on interaction with C $\epsilon$ 2.

HSQC spectra can be used to map protein-protein interaction surfaces at atomic resolution, by monitoring the perturbation in the position or intensity of the chemical shift on titration of a binding partner. Although the transition to folded conformation of C $\epsilon$ 3 does not occur on incubation with C $\epsilon$ 2, a number of cross-peaks in the HSQC spectrum of C $\epsilon$ 3 apparently shift, appear or disappear on addition of C $\epsilon$ 2. This indicates that C $\epsilon$ 2 makes a limited number of local interactions on binding to C $\epsilon$ 3. In order to investigate this interaction further, the correspond-

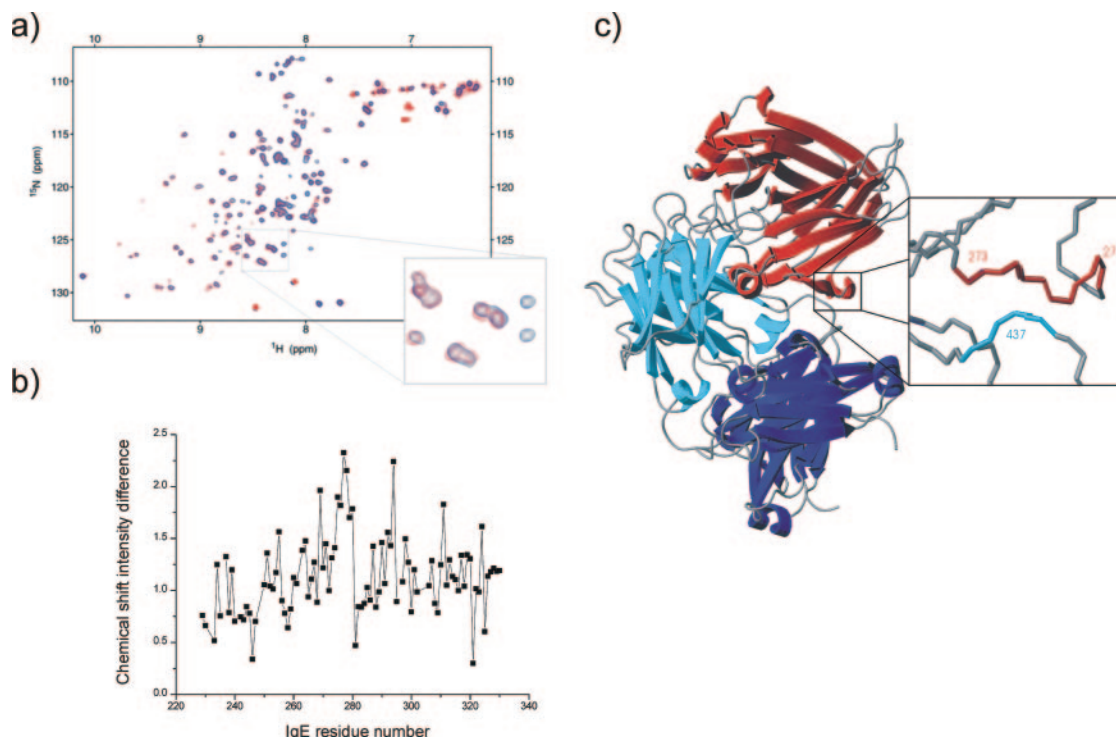


FIG. 6. *a*, 0.33 mM  $^{15}\text{N}$ -Ce2 with 0.19 mM unlabeled Ce3 added. Ce2 alone is shown in red; Ce2 with Ce3 added is shown in blue. *b*, plot of chemical shift intensity difference for Ce2 backbone amides on addition of 0.19 mM Ce3. *c*, the x-ray crystal structure of IgE-Fc taken from Wan *et al.* (6) (Protein Data Bank code 1LS0). Ce2, Ce3, and Ce4 domains are shown in red, cyan, and blue, respectively. The interaction region between Ce2 and Ce3, as shown by the chemical shift intensity difference, is boxed and magnified.

ing NMR experiment was carried out, namely, addition of Ce3 to  $^{15}\text{N}$ -Ce2. The effect of adding Ce3 into  $^{15}\text{N}$ -Ce2 can be seen to cause only small perturbations of the HSQC compared with Ce2 alone (Fig. 6*a*). Such an observation indicates there is only a local interaction surface on the Ce2 when interacting with Ce3 present as a molten globule. Because the structure of Ce2 has been solved by NMR (9), the backbone amides represented in the HSQC have been assigned. Hence, it is possible to determine where the interaction with Ce3 occurs by analysis of the change in intensity of each of the peaks (Fig. 6*b*). It can be seen that the plot of change in intensity appears relatively noisy; this may reflect some nonspecific aggregation occurring due to exposed hydrophobic residues of Ce3 and Ce2 that would normally be buried in the IgE. This aggregation is also seen in the difficulty of sample handling, with protein precipitating out of solution through the course of the Ce3/Ce2 titration. However, the area of greatest intensity change occurs between Gln<sup>273</sup> and Asp<sup>278</sup> of IgE. From the crystal structure of IgE-Fc (6), it can be seen that one of the Ce2 domains interacts with an unstructured loop at the C-terminal region of Ce3 of the other chain (Fig. 6*c*). Because this interaction site is not dependent on a rigid tertiary structure of Ce3, it is likely to occur when Ce3 is present as a molten globule.

Previously, recombinant Ce3 has been shown to bind to sFcεRIα (17), although no conformational change of Ce3 on binding to sFcεRIα was observed using far-UV CD spectroscopy. We have shown through ANS fluorescence and NMR spectroscopies that Ce3 does indeed form a stable tertiary structure on binding to sFcεRIα. However, this stable Ce3 tertiary structure is not formed on interaction with Ce2, Ce4, or CD23. The observation of this folded state will allow the use of NMR to map interactions of Ce3 with a variety of ligands at atomic resolution. In addition, we have validated the use of the ANS fluorescence assay using NMR. Such an assay could form the basis of a rapid method for screening additional ligands in order to identify those that cause the same changes in the

tertiary structure of Ce3; the effect of candidate ligands could then be explored in more detail by NMR. When considered in the context of the whole IgE molecule, it is probable that the Ce3 domains do indeed retain a degree of flexibility, with their structural integrity dependent on interaction with other domains of IgE. The existence of a less structured region within IgE mapping to the interaction site with FcεRIα has been known for some time (27) and could offer IgE a mechanism by which it can fine-tune its specificity and affinity according to the protein ligands present physiologically.

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