Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Structure of an isolated unglycosylated antibody C_H2 domain

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Received 14 June 2008
Accepted 5 August 2008

PDB Reference: antibody C_H2 domain, 3dj9, r3dj9sf.

The C_H2 (C_H3 for IgM and IgE) domain of an antibody plays an important role in mediating effector functions and preserving antibody stability. It is the only domain in human immunoglobulins (Igs) which is involved in weak interchain protein–protein interactions with another C_H2 domain solely through sugar moieties. The N-linked glycosylation at Asn297 is conserved in mammalian IgGs as well as in homologous regions of other antibody isotypes. To examine the structural details of the C_H2 domain in the absence of glycosylation and other antibody domains, the crystal structure of an isolated unglycosylated antibody $\gamma1$ C_H2 domain was determined at 1.7 Å resolution and compared with corresponding C_H2 structures from intact Fc, IgG and Fc receptor complexes. Furthermore, the oligomeric state of the protein in solution was studied using size-exclusion chromatography. The results suggested that the unglycosylated human antibody C_H2 domain is a monomer and that its structure is similar to that found in the intact Fc, IgG and Fc receptor complex structures. However, certain structural variations were observed in the Fc receptor-binding sites. Owing to its small size, stability and non-immunogenic Ig template, the C_H2-domain structure could be useful for the development by protein design of antibody domains exerting effector functions and/or antigen specificity and as a robust scaffold in protein-engineering applications.

1. Introduction

Antibodies, also called immunoglobulins (Igs), comprise two identical light chains and heavy chains linked by disulfide bonds. The light chain contains a variable (V_L) domain and a constant (C_L) domain. The heavy chain has four to five domains, depending on the isotype, including a variable (V_H) domain and several constant (C_H) domains: three C_H domains in IgG, IgA and IgD and four C_H domains in IgM and IgE. The antigen-binding fragment (Fab) consists of the light chain (V_L and C_L) and the first two domains of the heavy chain (V_H and C_{H1}) and is specifically involved in antigen binding. The Ig Fc (fragment crystallizable) portion consists of two constant domains, namely C_H2 and C_H3, from each heavy chain and binds to effector molecules in order to elicit host responses. C_H2 is the only antibody domain that exhibits very weak carbohydrate-mediated interchain protein–protein interactions, in contrast to the extensive interactions that occur between V_H–V_L, C_L–C_H1 and C_H3–C_H3 domains as seen in

intact antibody structures. The crystal structures of intact IgG (Larson *et al.*, 1991; Harris *et al.*, 1992, 1998; Saphire *et al.*, 2001) and Fc (Krapp *et al.*, 2003) reveal that the C_H2 domain interacts with the other C_H2 domain through sugar moieties only. The N-linked glycosylation at Asn297 of the C_H2 domain is conserved in all mammalian IgG molecules and the homologous regions of IgM, IgD and IgE. From a functional point of view, the C_H2 domain contains large portions of the entire binding sites for complement and Fc receptors that are critical for the effector function of antibodies (Vidarsson & van de Winkel, 1998; Woof & Burton, 2004) as well as for binding to the neonatal Fc receptor (Martin *et al.*, 2001), which is important for the preservation of antibody stability. The isolation and characterization of a C_H2 domain from myeloma IgG was reported quite early on as this domain is involved in complement activity (Seon & Pressman, 1975). Previously, a series of amino-acid substitutions in the C_H2 domains of various IgG subclasses was carried out to enhance the differential affinity for the Fc receptor (Canfield & Morrison, 1991). In the crystal structures of IgG1 Fc–FcγIII receptor complexes, Fc receptor (FcγR) exclusively interacts through the C_H2 domains of IgG1 Fc, in which the binding site includes the Asn297 residue of the C_H2 domain (Sondermann *et al.*, 2000; Radaev *et al.*, 2001). Several recent studies have also been focused on the characterization of the antibody constant domains, C_H2 and C_H3, in order to understand the folding and stability mechanisms of these domains (Demarest *et al.*, 2004; Feige *et al.*, 2004; McAuley *et al.*, 2008). Of these, kinetic studies exploring the folding mechanism of the C_H2 domain suggested that an unglycosylated murine C_H2 domain is a monomer with relatively low stability (Feige *et al.*, 2004). Although several crystal structures are available of intact IgG (Larson *et al.*, 1991; Harris *et al.*, 1992, 1998; Saphire *et al.*, 2001), Fc (Krapp *et al.*, 2003; Matsumiya *et al.*, 2007), Fab fragments (Stanfield *et al.*, 2006) and variable antibody domains V_H or V_L (Dottorini *et al.*, 2004; Park *et al.*, 2008), there are no structures of the C_H2 domain. Such a structure would allow us to determine the structural details in the absence of glycosylation and other antibody domains. Here, we present the crystal structure of an isolated unglycosylated C_H2 antibody domain. The isolated C_H2 domain is a monomer and is similar to the C_H2-domain structures embedded in the intact Fc, IgG and Fc receptor complex despite the absence of extensive interactions with sugar moieties and its isolation from other antibody domains. However, certain differences do exist which could be important in the use of such domains as scaffolds for high-affinity binders.

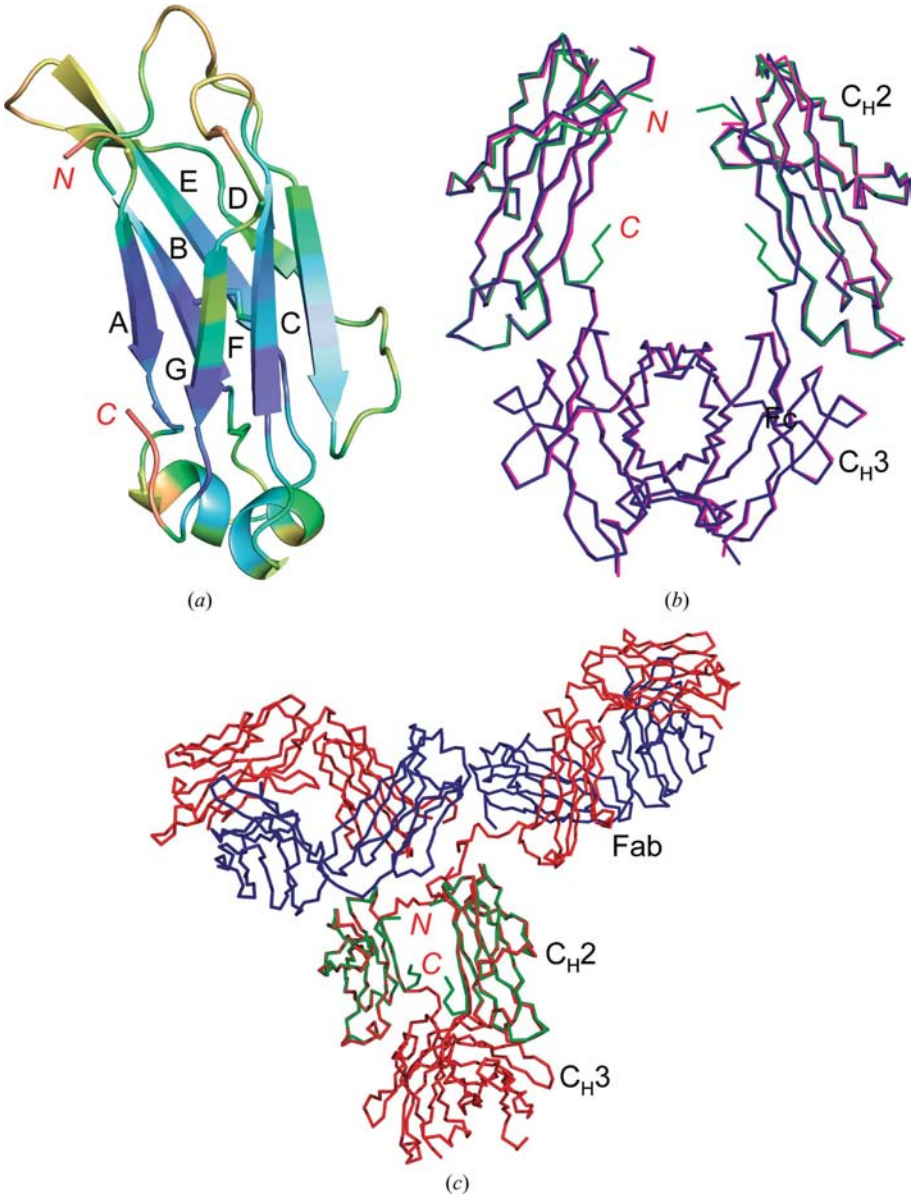


Figure 1
Structure of the C_H2 antibody domain and structural comparison with the corresponding region in the Fc and IgG structures. (*a*) Ribbon diagram of the isolated unglycosylated C_H2 domain from IgG γ1 is shown with a gradient ramp of colors according to the temperature factors (*B* factors): blue for lower (∼11 Å^2), green for medium (∼23 Å^2) and red for higher (∼48 Å^2) values. The N- and C-termini as well as strands A–G are marked. (*b*) The isolated C_H2 domain (green) was superimposed with a least-squares algorithm using the C^α traces of the C_H2 domains of fucosylated (blue; PDB code 2dtq) and nonfucosylated (purple; PDB code 2dts) Fc structures. (*c*) Superposition of the isolated C_H2 structure (green) with that of C_H2 portions of an intact IgG (PDB code 1hzh) using the C^α-trace alignment. The heavy and light chains of IgG are shown in red and blue, respectively. The carbohydrate moieties between the C_H2 domains of the Fc and IgG structures in (*b*) and (*c*) are omitted for clarity.

Table 1
X-ray data-collection and refinement statistics for the antibody C_H2 domain.

Data collection	
Wavelength (Å)	1.0
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 36.14, b = 40.68, c = 39.13, β = 106.7
Resolution range (Å)	26.36–1.75 (1.82–1.75)
Observations	42457
Unique reflections	10493
Redundancy	4.0 (3.0)
Completeness (%)	94.4 (72.0)
$I/\sigma(I)$	27.2 (7.0)
R_{merge}† (%)	0.040 (0.129)
Refinement statistics	
R factor‡ (%)	20.1 (23.6)
R_{free}‡ (%)	22.7 (31.1)
No. of atoms: protein/water	866/111
R.m.s.d. bond distances (Å)	0.006
R.m.s.d. bond angles (°)	1.4
Wilson B value (Å^2)	17.0
Average B values (Å^2)	
Protein atoms	20.8
Water O atoms	28.7
Ramachandran plot	
Most favored φ and ψ angles (%)	95.7
Additional allowed φ and ψ angles (%)	4.3

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. ‡ R factor and $R_{free} = \sum ||F_{obs}| - |F_{calc}|| / \sum |F_{obs}|$, where R_{free} was calculated over 5% of the amplitudes chosen at random and not used in the refinement.

High-affinity binders based on variable domains (also termed domain antibodies; dAbs; Holt *et al.*, 2003) have attracted much attention in recent years owing to their small size and stability, which are suitable for targeting the sterically confined binding sites on antigens or other protein surfaces. Since the unglycosylated antibody C_H2 domain appears to be a stable monomer and to be structurally independent, it might be useful as an alternative to domain antibodies for the generation of high-affinity binders. Therefore, the structure may be useful for optimization of the antibody C_H2-domain stability, creation of alternative antibody scaffolds based on the C_H2 domain (nanoantibodies) and as a small structural scaffold in protein design. Also, the use of C_H2 as a scaffold is much easier and cost-effective if expressed in bacteria that do not support glycosylation.

2. Materials and methods

2.1. Cloning, protein expression and purification

The gene encoding the C_H2 antibody domain was obtained by PCR amplification using the cDNA of the MAK33 γ_1 heavy chain as a template. Protein preparation and purification were carried out as previously described (Zhang *et al.*, 2004).

2.2. Size-exclusion chromatography

Purified C_H2 was loaded onto a Superdex75 10/300 GL column that had been pre-equilibrated with phosphate-buffered saline (PBS). The protein was eluted with PBS at 0.5 ml min^{-1}. The Superdex75 column was calibrated with protein molecular-weight standards of 669.0, 440.0, 232.0, 158.0, 67.0, 44.0, 25.0 and 13.7 kDa.

2.3. Crystallization, data collection and structure determination

High-throughput screening of crystallization conditions was carried out with a Hydra II Plus crystallization robot (Matrix Technologies, Hudson, New Hampshire, USA) using the Crystal Screen kit from Hampton Research (Laguna Niguel, California, USA). Thin plate-shaped crystals were grown from a crystallization condition consisting of 30% PEG 1500 with equal volumes of protein and well solutions. The crystals were cryoprotected with the well solution after 25% glycerol had been introduced. The diffraction data were collected on the SER-CAT 22-ID beamline of the Advanced Photon Source (APS), Argonne National Laboratory. Data were processed and scaled with the *HKL*-2000 program suite (Otwinowski & Minor, 1997). Data-collection statistics are given in Table 1. The structure of the C_H2 antibody domain was solved by molecular replacement with the *CCP*4 version of *AMoRe* (Navaza, 2001) using the C_H2-domain structure extracted from the intact antibody IgG b12 structure (Saphire *et al.*, 2001; PDB code 1hzh) as a search model. The initial model obtained from molecular replacement was iteratively refined using *CNS* (Brünger *et al.*, 1997) and rebuilt with *Coot* (Emsley & Cowtan, 2004) and *O* (Jones *et al.*, 1991). Water molecules were added automatically using *CNS* followed by visual inspection and refinement. The refinement statistics are presented in Table 1. Figures were prepared with *PyMOL* (DeLano, 2002).

3. Results and discussion

3.1. Structure of the isolated unglycosylated C_H2 domain

We used recombinant DNA techniques to express the C_H2 domain of an antibody (IgG) in *Escherichia coli* and purified the protein, which resulted in the production of the isolated unglycosylated C_H2 domain with a molecular weight of 12 kDa. Crystals appeared in 30% PEG 1500 within a week

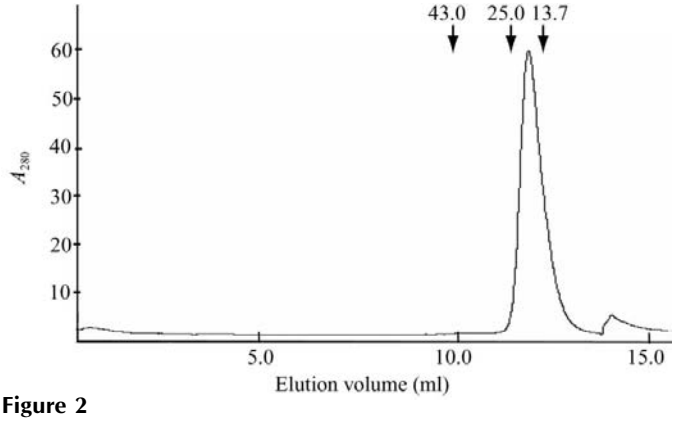
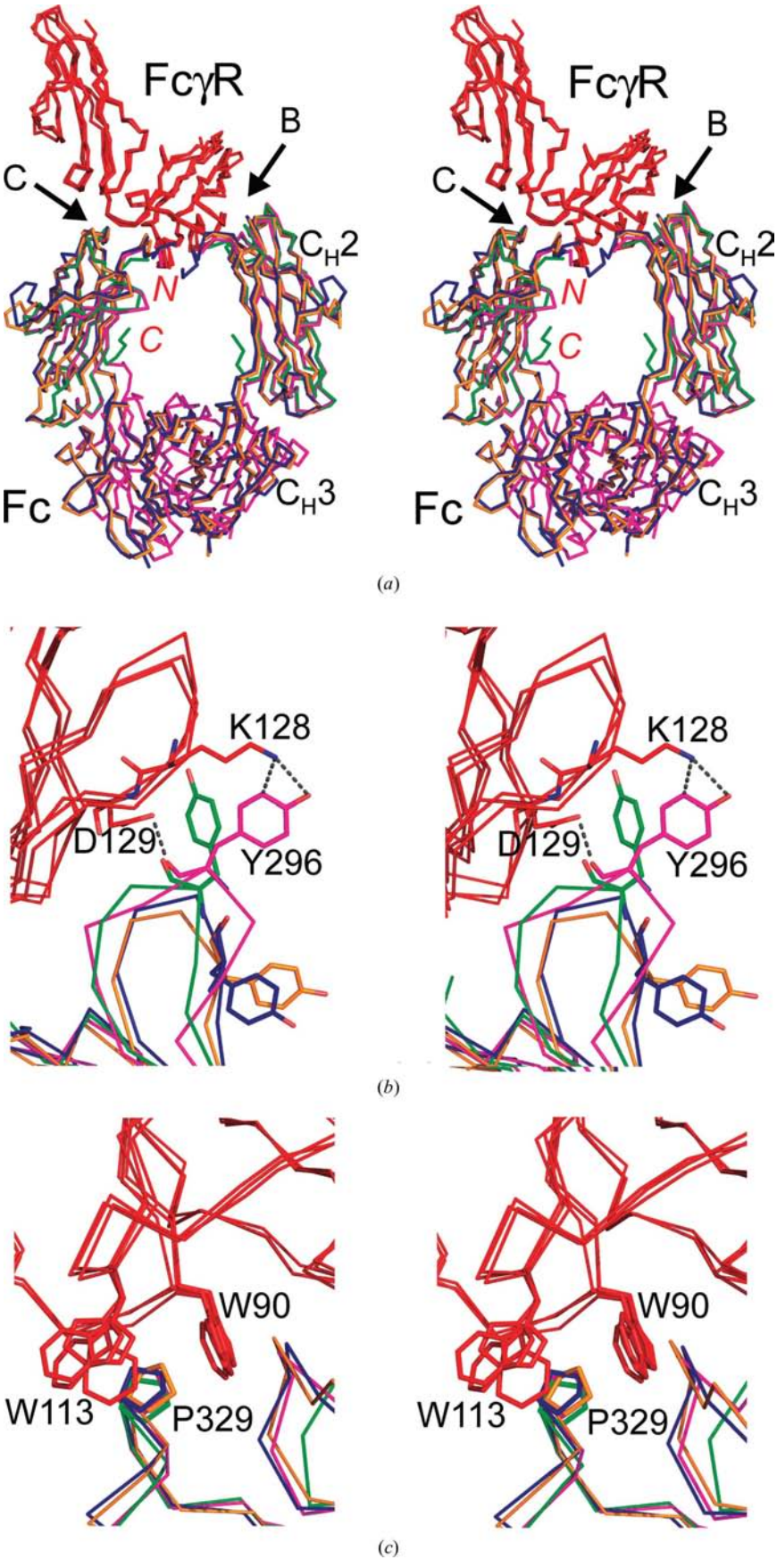


Figure 2
A sample of purified C_H2 was analyzed on Superdex75 10/300 GL column calibrated with molecular-weight standards. The arrows indicate the positions where the 43.0, 25.0 and 13.7 kDa molecular-weight standards eluted.



and grew as large plates that were suitable for X-ray diffraction. The crystal structure of the C_H2 domain was determined at 1.7 Å resolution by molecular replacement using the glycosylated C_H2 domain from the structure of an intact antibody IgG b12 determined previously at 2.7 Å resolution (Saphire *et al.*, 2001). A summary of X-ray data-collection and refinement statistics is presented in Table 1. The asymmetric unit contained one C_H2 domain and the final model included a total of 107 amino-acid residues, with more than 95% of residues in the most favored region of the Ramachandran plot (Ramachandran & Sasisekharan, 1968). A ribbon diagram of the unglycosylated C_H2 antibody domain is shown in Fig. 1(*a*), with a color coding corresponding to the *B*-factor values: blue for lower (∼11 Å^2), green for medium (∼23 Å^2) and red for higher (∼48 Å^2) values. The overall structure is similar to the intact glycosylated C_H2 domain as found in the Fc and IgG antibody structures (Figs. 1*b* and 1*c*), displaying a stable immunoglobulin fold with minor differences in the loop regions, the termini and the orientations of side chains of the binding site or surface-exposed residues. No significant intermolecular interactions that warrant the consideration of oligomerization are observed in the crystal lattice, which is in agreement with our size-exclusion chromatography data suggesting that the protein exists as a monomer in solution (Fig. 2). Our current data combined with the previous results from thermodynamic studies on the C_H2 domain of an IgG antibody (Feige *et al.*, 2004) have confirmed that the isolated unglycosylated

Figure 3
Stereoviews showing structural comparisons between the isolated C_H2 domain and similar C_H2 regions in Fc receptor complexes. (*a*) C^{α}-trace superposition of the isolated C_H2 domain (green) and the C_H2 domains of FcγRIII–Fc complex structures in various crystal forms: hexagonal forms (PDB codes 1e4k and 1t89, in blue and orange, respectively) and an orthorhombic form (PDB code 1t83, magenta). The FcγRIII receptor molecules in the complexes are shown in red. Arrows point to the Fc receptor-binding sites of the C_H2 domains. The carbohydrate moieties between the C_H2 domains of the FcγRIII–Fc complexes are omitted for clarity. (*b*) A close-up view of one of the binding sites from the D/E loop of the C_H2 domain, highlighting the orientation of the Tyr296 residue. (*c*) A close-up view of another binding site from the F/G loop of the C_H2 domain is shown. Amino-acid side chains are labeled according to the orthorhombic structure (PDB code 1t83).

C_H2 domain is a stable monomer in the absence of glycosylation and other antibody domains.

3.2. Structural comparisons of the C_H2 domain with intact Fc, IgG and Fc receptor complex structures

To analyze the conformational features of the isolated C_H2 domain, we compared it with recent crystal structures of human IgG Fc fragments with and without a fucose residue attached to the sugar moieties at Asn297 (Matsumiya *et al.*, 2007; PDB codes 2dts and 2dtq), intact IgG b12 (Saphire *et al.*, 2001; PDB code 1hzh) and Fc receptor complexes (Sondermann *et al.*, 2000; Radaev *et al.*, 2001; PDB codes 1e4k, 1t83 and 1t89). The two C_H2 domains in these antibody structures interact with each other through sugar moieties. Superposition of the unglycosylated C_H2 domain on the corresponding C_H2 domains of Fc and IgG yielded root-mean-square deviations (r.m.s.d.s) of 0.5 and 0.6 Å, respectively (Figs. 1*b* and 1*c*). This clearly indicates that glycan removal as well as isolation of the domain does not affect the structural integrity of the monomeric C_H2 antibody domain. Furthermore, we superimposed the C_H2 antibody domain with the C_H2 regions of FcγRIII–Fc complex structures available in three different crystal forms and found that the r.m.s.d.s ranged from 0.5 to 1.0 Å (Fig. 3*a*). In all three reported FcγRIII–Fc complexes, the D/E loop (residues 296–299 between β-strands D and E) of the C_H2 domain in Fc directly makes critical intermolecular interactions with the FcγRIII receptor. However, in the orthorhombic form only (PDB code 1t83), Tyr296 of the D/E loop in the C_H2 protrudes out at the tip and makes contacts with Lys128 and Asp129 of FcγRIII (in magenta; Fig. 3*b*). In the two hexagonal forms of the FcγRIII–Fc complexes, Tyr296 in the D/E loop has a different conformation (blue and orange in Fig. 3*b*), which suggests a requirement for conformational flexibility of Tyr296 for the binding of C_H2 to FcγRIII. When we overlaid the isolated C_H2 domain on these complex structures (in green; Fig. 3*b*), we observed that Tyr296 of the isolated C_H2 domain exhibited an upright conformation with the hydroxyphenyl side chain pointing out of the D/E loop at the tip as found in the Fc receptor complex structure of the orthorhombic form. The other C_H2 domain of Fc region in the complex also makes interactions with the FcγRIII receptor through its F/G loop (residues 325–331 between β-strands F and G), where Pro329 of the C_H2 domain is sandwiched by Trp90 and Trp113 of the receptor, which is also observed in the orthorhombic structure (Fig. 3*c*). This Fc receptor-binding site on the C_H2 domain is structurally well conserved, in contrast to the other binding site where Tyr296 of the D/E loop exhibits significant conformational flexibility. From these structural analyses, we found that the isolated unglycosylated C_H2 domain has a similar conformation to that embedded in the intact Fc and IgG structures. However, a significant variation was noted in the Fc receptor-binding sites when compared with the FcγRIII–Fc complexes, particularly at residue Tyr296 of the C_H2 domain.

Isolated antibody fragments can be used as scaffolds for binders. The smallest functional antigen-binding fragment of an antibody, a variable domain, either V_H or V_L, has been used successfully. From the structural point of view, the major difference between the variable and constant domains is the connecting loops between the β-strands. The complementarity-determining region (CDR) loops in the variable domains that make contacts with the antigen are longer than those found in the constant domains which interact with effector molecules. Using the structural details together with *in vitro* phage-display selection (Weiss & Penner, 2008; Dimitrov & Marks, 2008) and computational protein-loop design (Hu *et al.*, 2007), the C_H2 domain could be engineered to have predetermined specificities for various antigens and proteins. In general, the Ig fold is shared by many evolutionarily unrelated or distantly related proteins (Halaby *et al.*, 1999). A DALI database search (Holm & Sander, 1998) using the C_H2-domain fragment resulted in more than 470 different protein structures with a *Z* score greater than 2, mainly of immune-system and cell-adhesion molecules. Therefore, the scope for protein design using the antibody C_H2-domain template may have wider applications in addition to therapeutic high-affinity binders and stable structural scaffolds.

We thank the Advanced Biomedical Computing Center, NCI-Frederick for computing facilities. X-ray diffraction data were collected on the Southeast Regional Collaborative Access Team 22-ID beamline of the Advanced Photon Source, Argonne National Laboratory. Supporting institutions may be found at http://www.ser-cat.org/members.html. This work was supported by the Intramural AIDS Targeted Antiviral Program (IATAP), National Institutes of Health (NIH) to DSD and by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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