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Crystallographic evidence for deviating C3b structure?

Arising from: A. A. Ajees *et al. Nature* 444, 221–225 (2006)

Activation of the protein C3 into C3b in the complement pathway is a crucial step in the complement immune response against pathogenic, immunogenic and apoptotic particles. Ajees *et al.*[1] describe a crystal structure for C3b that deviates from the one reported by Janssen *et al.*[2] and by Wiesmann *et al.*[3]. We have reanalysed the data deposited by Ajees *et al.*[1] and have discovered features that are inconsistent with the known physical properties of macromolecular structures and their diffraction data. Our findings therefore call into question the crystal structure for C3b reported by Ajees *et al.*[1].

Three structures of the 12-domain protein C3b have been reported[1–3]. In the structure of C3b reported by Ajees *et al.*[1], the complement C1r/C1s, Uegf, Bmp1 (CUB) domain adopts an unfolded conformation, and the thioester (TED) domain has a C3-like conformation, rather than an activated, C3d-like shape, and is positioned away from the main body of the molecule. The structure of the remaining ten domains is similar between the three structures[1–3] and resembles the C3c structure[4]. The conformation and location of the CUB and TED domains are of specific interest because they are crucial to the biological functions of this molecule that are central to the complement system.

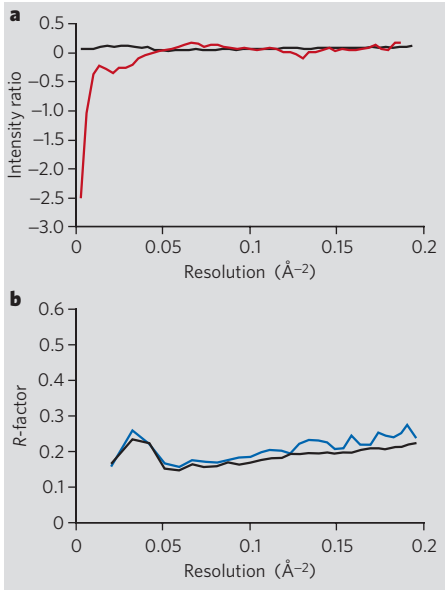

Figure 1 | Absence of bulk solvent in the deposited diffraction data of Ajees *et al.*[1]. **a**, Plot, as a function of resolution, of the logarithm of the ratio between the average observed intensities and those calculated from a model without a bulk-solvent contribution. Black line shows the results for the structure of Ajees *et al.* (Protein Data Bank entry 2HR0); red line shows results for a control using data from Protein Data Bank entry 1H18 (ref. 11), which has a similar size and resolution limits. The plot is expected to fall off at low resolution, as seen for 1H18, because the presence of disordered solvent reduces the contrast and hence the average diffraction intensity. **b**, R (black line) and R_{free} (blue line) plotted as a function of resolution, calculated with computer program CNS 1.2 (ref. 6) using the data and the 2HR0 structure of Ajees *et al.* without a bulk-solvent model. In the absence of a bulk-solvent model, a large R factor (beyond 0.5) is expected at low resolution because the model does not account for the contribution of the bulk solvent in the crystal to the diffraction data[8].

In an analysis of the structural differences between the three structures[1–3] reported for C3b, we noticed that the coordinates deposited by Ajees *et al.* (Protein Data Bank entry 2HR0) do not form a connected network of molecules in the crystal lattice. The crystal structure forms layers that are separated by a large void in the c-direction (a slab of about 30–40 Å thick that spans the entire unit cell). To investigate this highly unusual, and unreported, feature, we used two computer programs (REFMAC[5] and CNS 1.2 (ref. 6)) to reproduce the reported refinement statistics from the diffraction data[1] deposited in the Protein Data Bank. We found that the published statistics, the deposited coordinate model and diffraction data were consistent with each other.

We then tested whether the gap could be explained by a missing protein molecule. Re-determination by molecular replacement with the program PHASER[7] resulted in the same overall molecular arrangement as that seen by Ajees *et al.*[1]. We found no evidence for potentially absent protein molecules, either from features in the electron-density map or from increased scores in the log-likelihood function when searching for additional components.

In addition to the absence of crystal contacts in the c-direction, we noticed other physically implausible features. The diffraction data do not show the features that should arise from the presence of bulk solvent (Fig. 1), whereas the molecular arrangement indicates that large regions are not occupied by protein molecules. In other words, the diffraction data are consistent with protein molecules in a vacuum but not with those surrounded by disordered solvent, as is always seen for macromolecular crystals[8].

The B-factors of the model (both the deposited B-factors and those obtained by rerefinement) do not vary significantly throughout the molecule, even though long segments of the chain are almost completely exposed to solvent (Fig. 2). B-factors describe the size of displacements available to the atoms, so they are correlated with disorder for surface-exposed residues and rigid-body-like motion of domains[9]. The R_{free} (ref. 10) and R distributions are exceptionally low at low resolution, and the difference between R_{free} and R is unusually small for a structure refined at 2.3 Å resolution with an amplitude-based target function (Fig. 1b).

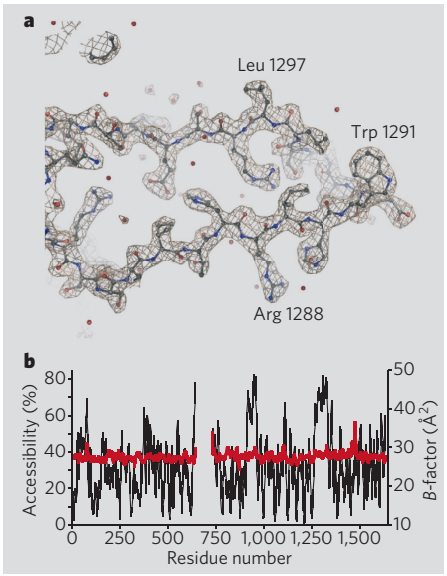

Figure 2 | Lack of correlation between surface exposure and disorder of residues in the C3b crystal structure of Ajees *et al.*[1]. **a**, The electron density (calculated with coefficients $2mF_{obs}-DF_{calc},\varphi_{calc}$; ref. 12) of a region in the unfolded CUB domain is contoured at 2.5σ (0.55 e Å^{-3}). It is very unusual to see such a well defined electron density at a high contour level for an unfolded domain that has no stabilizing contacts with other parts of the molecule or neighbouring symmetry-related molecules. **b**, Plot of surface accessibility (black, computed with NACCESS[13]) and atomic B factors after rerefinement (red), both averaged over a window of nine residues. Normally, the two measures would be highly correlated.

We think that these physically implausible features undermine the validity of the model presented by Ajees *et al.*[1] and the deposited diffraction data from which it derives. Only when the experimental diffraction images are made available can the deviating C3b model be either verified or falsified.

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Ajees et al. *reply*

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We concur with Janssen *et al.*[1] that the structure of C3b that we reported (Protein Data Bank entry 2HR0)[2] differs from the other two (2ICF (ref. 3) and 2I07 (ref. 4) in the conformation of the CUB and TED domains. The deviation of 2HR0 from 2I07 is probably relevant physiologically, considering that CUB eventually unfolds.

An electron microscopy study[5] reports a movement of about 75–100 Å for TED in C3b, which is closer to that found in 2HR0 (about 84 Å) than in 2I07 (about 65Å). Additionally, the only domain not seen in the electron micrographs is CUB; nine other domains of similar size and structure are all visible, indicating that C3b adopts conformations in which CUB is less compact than in 2I07. Furthermore, in 2I07 TED is not optimally positioned for interaction with some ligands[6], whereas it is in 2HR0. The 2.3Å-resolution structure of 2HR0 positions all C3b domains more accurately than is possible at 4 Å in 2I07. The unfolded, but well ordered, CUB has *B* factors comparable to the rest of the structure, indicating that we are observing a different, but physiologically interesting, conformation of C3b.

Statistical disorder[7] resulting in apparent 'gaps' in the lattice has been observed for other proteins (W. A. Hendrickson, personal communication). In the gap along the *c*-direction, PHASER[8] locates, with reasonable *Z*-scores (Table 1), fragments that are apparently from a protein of the RCA (for regulators of complement activation) family[9], which specifically bind C3d and C3dg (ref. 10). These fragments, which are probably contaminants, bridge two C3b molecules to form the lattice (Fig. 1a). Putative fragments here interact non-specifically with several domains of C3b (Fig. 1b). They are statistically disordered and are present in different orientations in different sets of unit cells, which would prevent construction of an atomic model for these regions. Stabilization of the protein lattice by adventitious entities is not unprecedented[11]. Figure 2a of Janssen *et al.*[1] seems not to be contoured at a level needed to display disordered entities, as shown in our Fig. 1. These observations unambiguously indicate the existence of a crystal lattice.

We have also determined and refined a structure of C3b in another crystal form, with a shorter *c*-axis, which reveals direct crystal contacts between C3b molecules. Notably, the rest of the structure is nearly identical to 2HR0 except for an unmodified glutamine residue at position 991. Changes in the length of the axis, accompanied by hydration adjustments, leaving the molecular conformation mostly unaltered, have been seen in other structures[12].

Figure 2b of Janssen *et al.*[1] ignores the fact that *B*-factors are much higher in 2I07 (*B*=<174>), within the model errors and uncertainties at 4 Å, than in 2HR0 (*B*=<27>), at 2.3 Å. When normalized for this difference, variation in *B*-factors as a function of solvent exposure is comparable for 2I07 and 2HR0 (Fig. 1 c, d). *R* and R_{free} values, and the difference between them, which are similar to those reported by us, are widespread (for example, see Protein Data Bank entries 1Q0D, 2BL2, 2BS3). Bulk-solvent modelling is contentious, making many refinements necessary to constrain parameters to obtain acceptable values[13]. Analysis of the few deposited values reveals no correlation of either parameter with

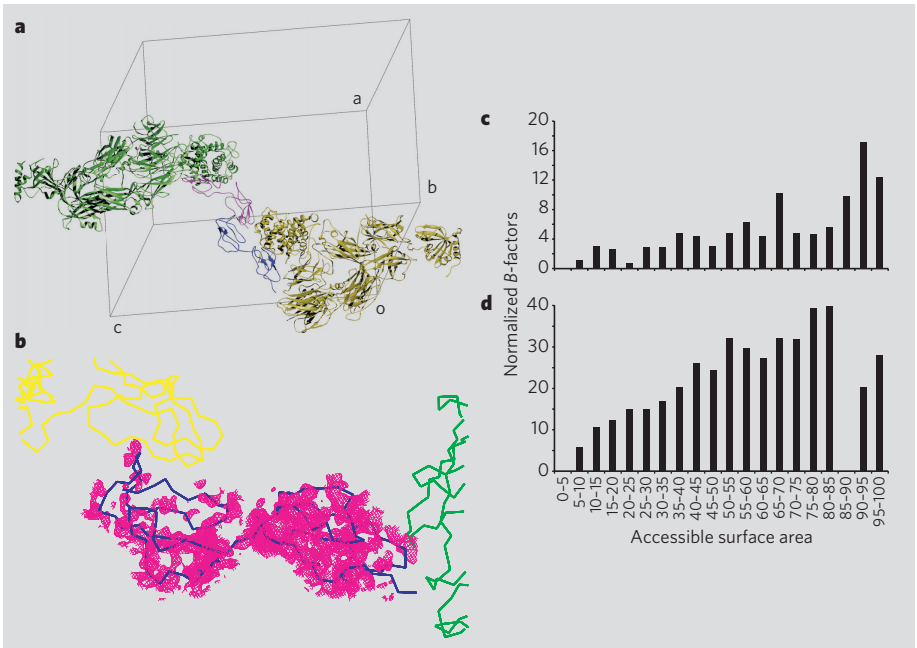

Figure 1 | Crystal lattice and normalized *B* factors. **a**, Packing diagram of C3b with the vaccinia virus complement-control protein VCP1-2, showing one symmetry-related molecule. The Cα 'worm' of C3b in the asymmetrical unit is coloured yellow and VCP1-2 is blue. Symmetry equivalents are green (C3b) and magenta (VCP1-2). **b**, A section of the 2*Fo*–*Fc* map (magenta), calculated using the published data from PDB entry 2HR0, contoured at 0.7σ around solution 6 (Table 1). C3b is shown in green and VCP1-2 in blue; the symmetry-related molecule is in yellow. **c**, **d**, Plots of normalized *B* factor versus accessible solvent area for **c**, 2HR0, and **d**, 2I07. Normalized *B*-factor calculated as $((B_{group}-B_{ref})/B_{group})\times100$, in which B_{ref} is the reference *B*-factor for residues in the 0–5% accessibility range, and B_{group} is the average *B*-factor for residues with other accessibility ranges.

solvent content or composition[13]. If our data had really represented a structure in a vacuum, we would not have been able to locate the RCA protein fragments.

Hence we rebut the arguments of Janssen *et al.*[1] and stand by our model, the underlying diffraction data and the functional implications we derive from the model.

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Table 1 | Crystal lattice gap

Molecule	*Z*-scores		Closest C3b domain to PHASER solution
	RFZ	*TFZ*	
C3b (PDB ID: 2HR0)	87.4	92.2	
C3b + VCP SCR 1 and 2 Ajees*			
Fragments bridging two C3b molecules in adjacent unit cells			
Solution 1	3.6	5.0	TED
Solution 3	3.1	5.1	MG5
Solution 5	3.6	4.7	TED
Solution 6	3.6	4.5	TED
Solution 11	3.6	4.2	TED
Solution 12	3.2	4.8	TED
Solution 13	3.1	4.6	TED
Solution 15	3.2	4.6	MG4
Solution 16	3.1	4.5	MG5
Solution 17	3.6	4.4	MG5
Solution 18	3.8	4.5	TED
Solution 19	3.2	4.6	TED
Fragments with symmetry clashes			
Solution 2			CUB
Solution 4			MG1
Solution 10			TED
Solution 20			TED
Other			
Solution 7			TED
Solution 8			MG5
Solution 9			MG8
Solution 14			CUB
Solution 21			MG4
Solution 22			TED
C3b + VCP_NMR SCR 1–2 (PDB ID: 1VVC)			
Fragments bridging two C3b molecules in adjacent unit cells			
Solution 3	3.8	4.9	TED
Solution 5	3.2	4.6	TED
Solution 6	3.8	4.7	MG5
Fragments with symmetry clashes			
Solution 1			MG4
Solution 2			TED
Solution 4			TED
C3b + CR2 (PDB ID: 1LY2)			
Fragments bridging two C3b molecules in adjacent unit cells			
Solution 1	4.0	4.2	TED
Solution 2	4.0	4.4	MG5
Solution 5	4.6	4.5	TED
Solution 10	4.0	4.0	TED
Fragments with symmetry clashes			
Solution 4			MG5
Solution 9			MG8
Other			
Solution 3			MG5
Solution 6			MG4
Solution 7			TED
Solution 8			MG1
Solution 11			MG4

Solutions were obtained from PHASER (using search option RMS 1.5) using our deposited F_{obs}, fragments from vaccinia virus complement-control protein (VCP), complement receptor-2 (CR2), C3 and C3b domains, as well as haemoglobin. Negative results from PHASER: using Protein Data Bank (PDB) ID code 2G7I, 1G40 (SCR1-4), 2A73 (MG1; MG2; MG8; TED and CUB domains) and 2HBF. *A. A. A., unpublished.