Hinge disulfides in human IgG2 CD40 antibodies modulate activity by regulation of conformation and flexibility

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1. Background & biological characterisation

Monoclonal antibodies (mAbs) are an increasingly important therapeutic modality, representing ~80% of therapeutic biologics (1). They continue to translate into the clinic, and by May 2021, the U.S. Food and Drug Administration had approved 100 antibody drugs, with a strong upward trend (2).

We previously demonstrated with two clinically relevant anti-CD40 mAb, SGN-40 and ChiLob7/4, that for mAb targeting CD40 the *h*IgG2 isotype afforded the strongest agonism in an Fc-independent manner but dependent upon the hinge (3). Uniquely, the *h*IgG2 hinge can undergo disulfide switching resulting from natural redox processes in the blood (4). During this process, disulfide bonds between two cysteines can be broken and reformed with an alternative cysteine at a different position. Although disulfide connectivity has been investigated before (5), the exact topology and relationship to biological properties remained unknown. Two isoforms, described as the A-form (*h*IgG2A) and the B-form (*h*IgG2B), are thought to represent the two extremes of this process. For CD40-targeting mAb, the two isoforms have starkly contrasting immunostimulatory activity: *h*IgG2A is inert, devoid of stimulatory activity, whereas *h*IgG2B is strongly agonistic.

To more precisely investigate the effect of the hinge disulfides on mAb agonistic activity, we generated a series of cysteine/serine (C/S) mutations, producing a total of six variants of the anti-CD40 antibody ChiLob7/4 (6). Multiple biological assays were then performed with $F(ab)_2$ fragments of these variants (Fig. 1) revealing an ordering of variants by increasing agonistic activity as C232S + C233S ≅ C233S < C232S < C233SκC214S < C239S ≅ C232SκC214S.

All ChiLob7/4 C/S variants had identical variable regions, and so, any change in activity would solely depend on their ability to form disulfides at the hinge region with potential impacts on structure. To evaluate this, we sought to determine the structure of their $F(ab)_2$ fragments using x-ray crystallography. 5 of the 6 variants generated crystals amenable to diffraction analysis (Figure 2).

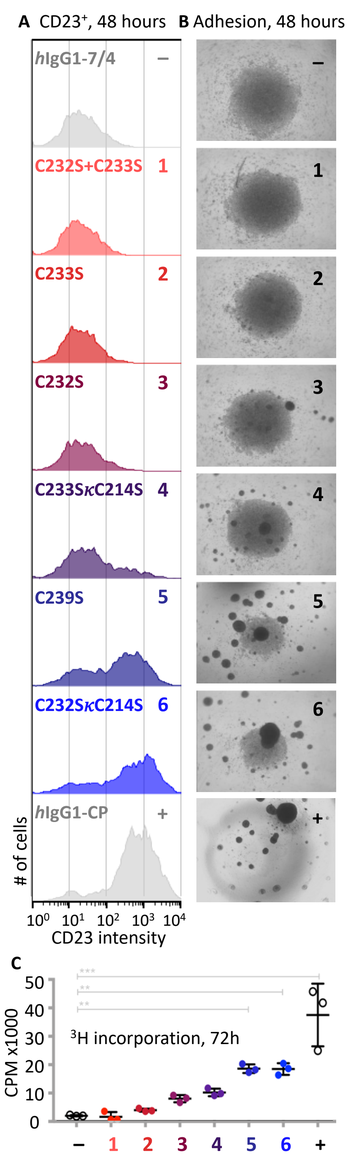


Figure 1 | Determination of agonistic activity of ChiLob7/4 *h*IgG2 $F(ab)_2$ variants. (**A**) Flow cytometry plots of *h*CD40 transgenic B cells 48 hours after addition of anti-*h*CD40 mAb. Fluorescence intensity (arbitrary units) arising from PE-labelled anti-CD23 mAb plotted against cell count. Plots are representative from *n* = 3. (**B**) Homotypic adhesion of *h*CD40 transgenic B cells measured 48 hours after addition of anti-*h*CD40 mAb. Higher levels of cell-cell clustering (densely packed dark regions) indicate greater B cell activation. Images are a representative from *n* = 3. Scale bar, 200 µm. (**C**) *h*CD40 transgenic B cell proliferation assessed by determining 3H-thymidine incorporation 72 hours after addition of anti-*h*CD40 mAb; CPM, counts per minute; means ± SD; *n* = 3; $^{**}P < 0.01$ and $^{****}P < 0.0001$ using a one-way ANOVA. mAb variants are numbered from 1 to 6 in all panels. The colour scheme in (A) and (C) indicates increasing agonistic activity from red to blue, with controls in grey; negative control, ChiLob7/4 *h*IgG1 (*h*IgG1-7/4); positive control, superagonist CP870,893 *h*IgG1 (*h*IgG1-CP).

2. Determination of hinge region disulfide patterns

Typically, disulfides are expected to occur in the hinge region connecting the two heavy chains at C232, C233, C239, and C242; in addition, heavy and κ light chains are linked by a disulfide between heavy chain C127 and κ light chain C214. As antibody hinge regions are difficult to observe in crystal structures due to their natural flexibility, an anomalous scattering approach was used to reveal the disulfide connectivity of the $F(ab)_2$ fragments. Performing structure determination at an x-ray wavelength where significant anomalous scattering for sulfur atoms was observed enabled the determination of sulfur position and thus enabled us to resolve hinge disulfide topology for the 5 variants.

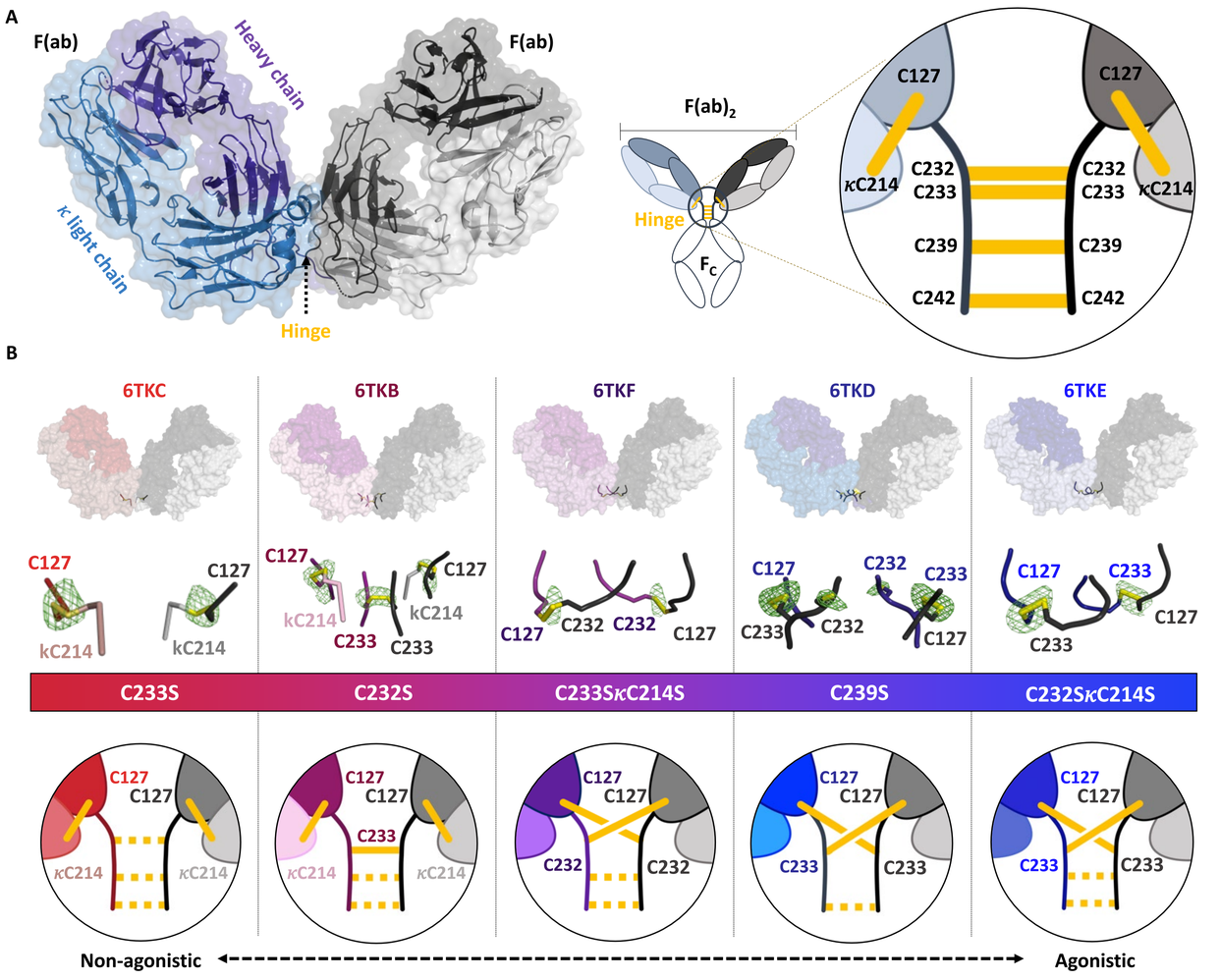


Figure 2 | Crystallographic analysis revealing the topology of hinge disulfides in ChiLob7/4 *h*IgG2 $F(ab)_2$ variants. (**A**) Left: The $F(ab)_2$ structure is formed by two heavy/light chain pairs, with the hinge region located between the two arms, shown as cartoon and molecular surface for 6KTD. Right: Scheme for the full *h*IgG2, where the expanded hinge highlights the predicted "paired ladder" topology of the native *h*IgG2 A-isoform; the Fc region, shown by open ovals, is not represented. (**B**) Surface representation of structures determined, with differences in the hinge expanded in stick representation (backbone colours matched to chain, disulfide bonds in yellow); the observed electron density is shown as green mesh (anomalous difference Fourier map, contoured at 4.5σ). Representations at the bottom show experimentally determined disulfides as solid lines (proposed disulfides, not resolved in electron density, are dashed). Increasing agonistic activity from red to blue.

3. SAXS, simulation & ensemble analysis

While crystallographic structure determination of the multiple variants revealed novel and differing hinge region disulfide bonding patterns, the global conformation of the five variants were analogous. This was hypothesised to be due to restrictions imposed by the crystal lattice. Therefore, structural information free of crystal lattice restraints was desired to investigate variability in global conformation. Small-angle X-ray scattering (SAXS) was used to perform these studies, revealing differences in flexibility between the variants.

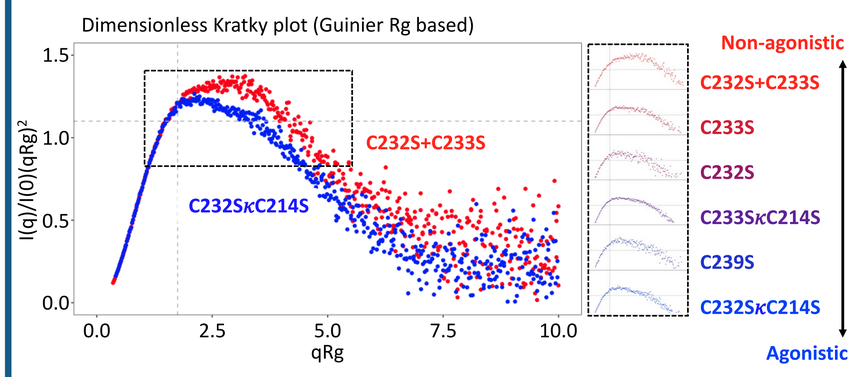


Figure 3 | SAXS for the six ChiLob7/4 *h*IgG2 $F(ab)_2$ C/S variants, Kratky analysis. The deviation from a Gaussian distribution in the dimensionless Kratky plot with a peak at the Guinier-Kratky point (grey crosshairs) indicates a departure from a rigid, globular particle for all six variants. The displacement of the peak up and to the right indicates varying degrees of flexibility and elongation of the samples. The extent of this deviation corresponds with activity (black dashed box), with the less agonistic variants (red) suggesting greater flexibility than the more agonistic variants (blue). Increasing agonistic activity from red to blue. SAXS data were collected at BM29, ESRF.

In order to combine the atomic level detail of the hinge disulfides gained from the crystal structures with the solution state information gained from SAXS, molecular dynamics simulation followed by SAXS based reweighting was performed. A trend of reduced conformational freedom that correlated with increased agonism was observed.

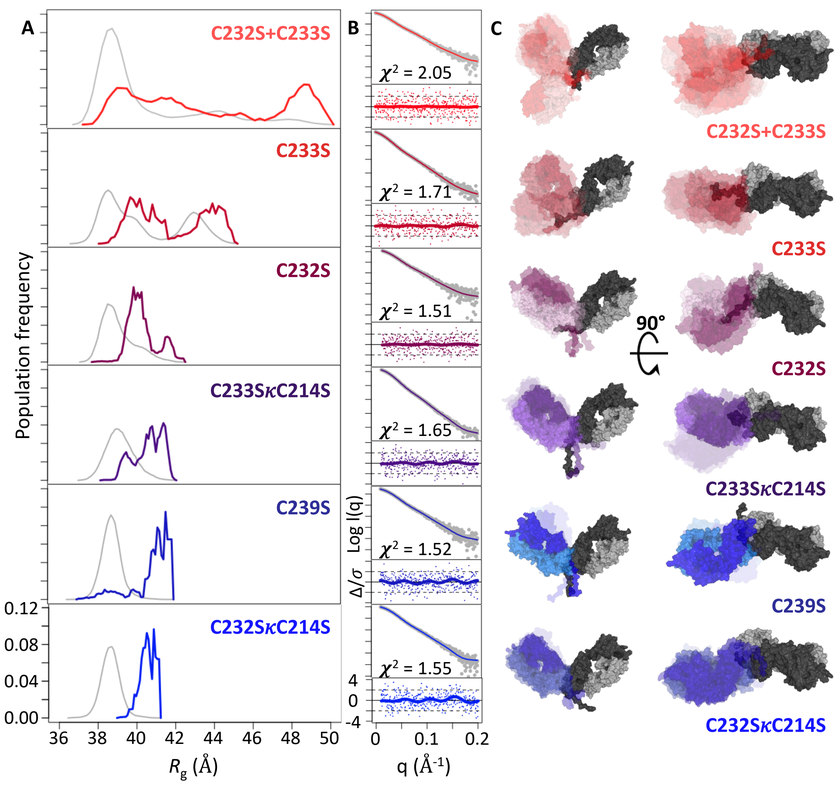


Figure 4 | Conformational states of ChiLob7/4 *h*IgG2 $F(ab)_2$ fragments revealed by combined SAXS/MD analysis.
(A) Distribution of structures for all variants plotted against their calculated *R*g values, where the MD pool is shown in grey, and the SAXS reweighted ensemble pool selected by GAJOE is shown in colour; increasing agonistic activity from red to blue as in Fig. 1. (B) Agreement of the calculated scattering curve from the reweighted ensemble with experimental SAXS data (grey dots) as indicated by χ^2 fit; the error-weighted residual plot below. (C) Representative conformational states are shown with one of the F(ab) arms aligned (grey) and the second F(ab) arm coloured; colour saturation indicates contribution to the ensemble.

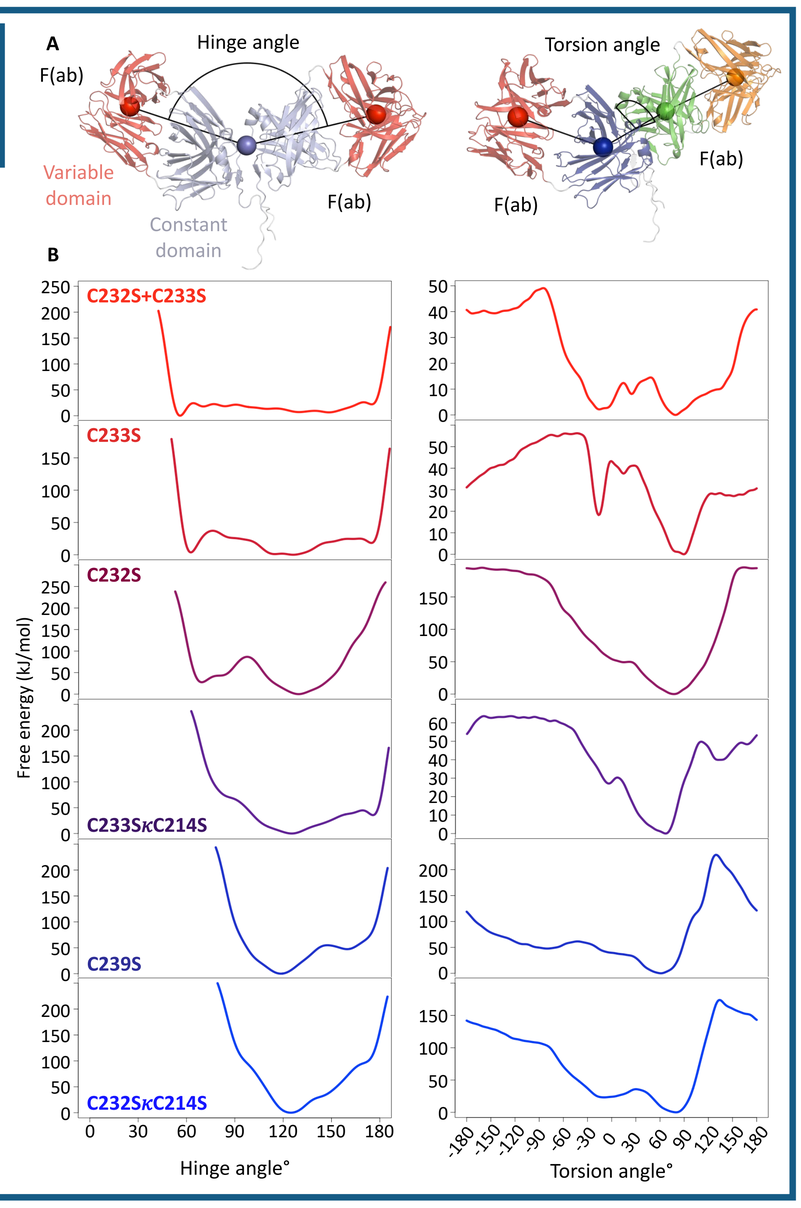
4. Enhanced sampling - Metadynamics

Analysis of the representative structures from SAXS based reweighting of the MD stimulations revealed that the degree of variability in $F(ab)_2$ hinge and torsion angles broadly correlated with activity.

To further investigate these motions, enhanced sampling simulations using metadynamics were performed, using hinge and torsion angles as collective variables (CVs). Metadynamics works to expand the conformational space sampled by applying a history dependent bias to the simulation along the CV being sampled.

A key feature of metadynamics is the ability to reconstruct free energy surfaces for the CVs sampled, allowing for analysis of the contributions of the CVs to the global conformation.

Figure 5 | Free energy surfaces for the hinge and torsion angles of ChiLob7/4 *h*IgG2 $F(ab)_2$ variants.
(A) Definition of the hinge and torsion angles of the $F(ab)_2$ variants. The hinge angle was defined as the angle between the centres of mass (COM) of the variable domains (red spheres) and the COM of the two constant domains (blue sphere). The torsion angle was defined as the rotation between the two F(ab) arms, as measured using the COMs of the variable domains (red and orange spheres) and the constant domains (blue and green spheres). (B) Free energy surfaces for the hinge and torsion angles of the $F(ab)_2$ variants. A constriction in the hinge angle free energy surface that correlates with increasing agonism can be observed. The torsion angle free energy surfaces are more complex with multiple features present and no clear trend reflecting activity.



Conclusion

Monoclonal antibodies (mAbs) are powerful therapeutic agents. The agonistic activity of mAbs is dependent on the epitope recognized as well as the isotype. For human IgG2 mAbs, the structure of the hinge region is important for modulating agonistic activity, but the underlying mechanism was unclear. We studied the structure and agonistic activity of a series of hIgG2 anti-CD40 cysteine to serine exchange variants targeting the hinge region. Agonistic activity was found to vary depending on the pattern of disulfide bonds within the IgG2 hinge region, with more agonistic antibodies featuring a disulfide cross-over as identified using an anomalous scattering approach. These cross-overs directly affect the flexibility and the conformation of the antibody. SAXS experiments revealed a trend of reduced flexibility that correlated with increasing agonism. Atomistic molecular dynamics simulations were then used to generate conformational pools of models which were reweighted using the SAXS data. A restriction in accessible conformational space correlated with increasing agonism. Enhanced sampling simulations using metadynamics revealed that a restriction of the $F(ab)_2$ hinge angle was a key driver of the increased conformational restriction.

In conclusion, mAbs with less flexible hinge regions are restricted in the conformational space accessible to them, which enables increased receptor agonism. These findings highlight the importance of hinge variation in modulating antibody activity.

References

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