Comprehensive elucidation of the structural and functional roles of engineered disulfide bonds in antibody Fc fragment

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The Fc-based therapeutic proteins include therapeutic monoclonal antibodies and Fc-fusion proteins. As their common part, antibody Fc fragment is important for maintenance of the molecular structure and function (1). Because instability and aggregation tendency constrain the development of these therapeutic proteins for clinical use, engineering of Fc fragment for improvement of the physicochemical properties including stability and aggregation resistance could be a useful platform for overcoming the obstacles. Many methods, including introduction of covalent bond (e.g. disulfide bond) and modification of noncovalent bond, have been used for optimization of Fc fragment to achieve the expected clinical outcomes (1).

The Fc fragment in an Ig is a dimeric form composed of two copies of CH2 domains and two copies of CH3 domains. In each domain, there is a native disulfide bond that is important for the structural stability (2). It has been shown that the native disulfide bond between Cys367 and Cys325 (all the Fc residues here are numbered according to EU numbering (2)) can support the folding of single CH3 domain, as well as the dimerization process between two CH3 domains, and prevent aggregation formation (3–5). Although the role of native disulfide between Cys261 and Cys321 in the CH2 domain has not been well characterized, we could not observe the soluble expression of isolated CH2 in Escherichia coli after mutations of two native cysteines to other residues (data not shown). Hence, the native disulfide bond should also play an important role in supporting the correct folding of CH2 domain. Because of the important roles of native disulfide bond, it could be highly desired that introduction of additional disulfide bond could stabilize the Fc molecule to make it better toward clinical use.

In a previous study, we engineered an additional disulfide bond by replacement of Phe242 and Lys334 by two cysteines in isolated wildtype CH2 domain (wtCH2) from the IgG1 Fc fragment to identify a mutant termed m01 (6). The melting temperature ($T_m$) of m01 is $\sim 18^\circ$C higher than that of wtCH2, while the urea concentration of 50% unfolding rises from 4.2 to 6.8 M. No obvious conformational change occurs. Following this study, we and other group also reported that introduction of an additional intradomain disulfide bond by mutation of certain amino acids (e.g. replacement of Pro343 and Ala431 by two cysteines, respectively) in CH3 domain leads to $\sim 8^\circ$C or $\sim 35^\circ$C increases of the $T_m$ in the context of an intact Fc fragment or an isolated monomeric CH3 domain, respectively (7, 8). In addition, introduction of interdomain disulfide by replacement of a C-terminal Lys447 to cysteine and mutation of other nearby residues in CH3 domain can also increase the stability and aggregation resistance of the cyclized Fc fragment (9, 10). In a recent study, more residues in
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CH2 domain were selected for induction of additional disulfide bonds, which results in various effects on stability and circulation in vivo (11).

Although current results show that engineering of disulfide bond in Fc is promising for modification of therapeutic monoclonal antibodies and Fc-fusion proteins, it is still not very clear how these introduced disulfide bonds in different domains of Fc fragment contribute to optimization on structural and functional properties of Fc. For example, because unpaired cysteines could be paired correctly to form intradomain disulfide bond, and the dimerization of each monomeric Fc fragment was not affected as desired.

Therefore, the potential change of aggregation propensity of Fc fragment with extra disulfide bonds should be also well evaluated.

To address these issues, we constructed three Fc mutants with additional disulfide bonds in the CH2 domain, the CH3 domain, and both the CH2 and CH3 domains and expressed them in mammalian cells. After purification, a series of experiments were performed for evaluation of the influence of engineered disulfide bonds on structural and functional properties. We found that introduction of disulfide bonds in different domains could make different contributions on improvement of physicochemical properties including stability and aggregation resistance. One Fc mutant with two extra disulfide bonds in the CH2 and CH3 domains, respectively (FcCH2-s-s-, FcCH3-s-s-), has the best physicochemical properties without obvious alteration on Fc-mediated functions. Our results give straightforward evidence that Fc-based therapeutics could be improved through engineering of disulfide bonds in the Fc fragment.

Results

Design, expression, and purification of human IgG1 Fc fragment and its mutants

Three mutants including FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s- based on human IgG1 Fc fragment were designed. In wildtype Fc (wtFc), each CH2 or CH3 domain has one native disulfide bond (Protein Data Bank code 1HZH (12)) (Fig. 1A). A disulfide bond was introduced in CH2 domain through replacement of Leu242 and Lys334 by two cysteines, respectively (6), to construct FcCH2-s-s-, while residues Pro343 and Ala431 in CH3 domain were mutated by two cysteines, respectively (7) to get FcCH3-s-s-. (Fig. 1A). With combination, FcCH2-s-s- was built by simultaneous substitution of Leu242, Lys334, Pro343, and Ala431 by four cysteines (Fig. 1A). The sequence alignment of wtFc and its three mutants was also performed (Fig. 1B).

**FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s- exist as dimeric form**

wtFc and its three mutants could be solubly expressed at comparable level in mammalian cells and purified on protein G resin. Because their theoretic molecular mass calculated by amino acid residues was ~24 kDa, and all of them should be glycosylated at Asn297, the size of corresponding bands was correct as shown in SDS-PAGE (Fig. 2A). To determine the molecular mass of wtFc and its mutants in solution, all four proteins were analyzed with size-exclusion chromatography (SEC) (Fig. 2B). They showed identical elution profiles that only had one unique peak with molecular mass of ~60 kDa calculated by molecular mass standards (Fig. 2B), which indicated that all four proteins existed in a dimeric form. Therefore, the cysteines could be paired correctly to form intradomain disulfide bond, and the dimerization of each monomeric Fc fragment was not affected as desired.

**FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s- are significantly more stable than wtFc**

The secondary structure of these four proteins were measured by CD. The spectra showed that their structure is very similar, consisting of primarily β-strands at 25 °C. Hence, introduction of disulfide bond did not change the overall structure (Fig. 3A). Then the thermal stability of wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH2-s-s- was evaluated (Fig. 3B). The unfolding curves of Fc and its mutants included unfolding of the CH2 and CH3 domains. In the case of wtFc and FcCH3-s-s-, two separate courses, representing unfolding processes of CH2 and CH3 domains, could be obviously observed. The Tm values of CH2 domains in wtFc and FcCH3-s-s- were 73.3 °C, while the Tm of CH3 domain in wtFc was 84.3 °C. Notably, the Tm of CH3 domain of FcCH3-s-s- dramatically increased because of the engineered disulfide bond, which could not be accurately calculated because the unfolding was not completed at 94 °C. In contrast to that of wtCH2 and FcCH3-s-s-, the unfolding course was uniform in the cases of FcCH2-s-s- and FcCH3-s-s- as observed because of the increase of thermoresistance in CH2 domain after introduction of an additional disulfide bond. Therefore, the total Tm of FcCH2-s-s- could be obtained, which was 85.2 °C. Notably, the total Tm of FcCH3-s-s- could be very high (e.g. >90 °C). Because separate introduction of disulfide bonds in CH2 and CH3 domains obviously increased the Tm of each engineered domains, simultaneous introduction of two disulfides in two domains resulted in extreme thermostability of FcCH2-s-s-.

The stability against chemically induced unfolding of wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH2-s-s- was also compared in the presence of urea (Fig. 4). The 50% unfolding of wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH2-s-s- occurred at urea concentrations of 7.3, 8.7, 7.8, and 9.0 M, respectively. Therefore, introduction of disulfide bond in CH2 domain could increase the stability against chemical denaturant more obviously than that in CH3 domain. In general, the trend of improvement of ability against unfolding under thermal pressure and in the presence of denaturant after engineering disulfide bond was very similar, although the contributions from different disulfide bonds may vary.

Additional disulfide bond can increase aggregation resistance

Turbidity assay was performed for evaluation of aggregation propensity under high temperature and acidic conditions. The rates of increase of the absorbance at 320 nm in wtFc and FcCH3-s-s- were faster than that in FcCH2-s-s- and FcCH3-s-s- after incubation at 60 °C, which indicated more aggregates formed in wtFc and FcCH3-s-s- under high temperature pressure (Fig. 5A). The result was confirmed by size-exclusion chromatography at the end of incubation, which showed that no obviously/or very 3
minor soluble big oligomers formed in the case of FcCH2-s-s- and FcCH3-s-s- (Fig. 5B). In contrast, a remarkable second peak eluted earlier in wtFc and FcCH3-s-s- was observed indicating formation of aggregates. Under acidic conditions, FcCH2-s-s- and FcCH3-s-s- were more aggregation-resistant than FcCH2-s-s- and wtFc (Fig. 6). Although the introduced disulfide bond in CH2 domain did not significantly change the aggregation propensity of FcCH2-s-s- at low pH compared with wtFc, it could cooperate with the additional disulfide bond in CH3 domain to further enhance the aggregation resistance (FcCH2-s-s- versus FcCH3-s-s-). Hence, engineering of disulfide bonds in Fc can improve its aggregation resistance. The different positions of introduced disulfide bonds could have different contributions for increase of aggregation resistance under different conditions.

**Comparison of binding of wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s- to Fc receptor**

Although the positions of mutated cysteines were not involved in Fcγ receptor (FcγR) binding (13), it might affect the interaction by conformational change. Therefore, U937, a high FcγRII-expression cell line, was used here for measurement of binding of Fc and its mutants to FcγRII, which could be a representative for estimation of binding to Fc receptors (14). The obvious fluorescence intensity shift was observed when wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s- were added into U937 cells, indicating their binding to FcγRI (Fig. 7). All four proteins exhibited very similar binding behaviors, which meant that additional disulfide bond did not affect the interaction with effector molecules and subsequent Fc-mediated effector functions.
Binding of wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s-CH2-s-s- to neonatal Fc receptor (FcRn) at pH 6.0

Because binding of antibody Fc fragment to FcRn in a pH-dependent manner is one of the key factors for extension of the serum half-life of antibody in vivo (15), the interaction of these four proteins with soluble human FcRn (shFcRn) was analyzed by biolayer interferometry technology (BLI) for evaluation of their serum half-lives after introduction of disulfide bond (Fig. 8). As measured, all the Fc and its mutants bound to FcRn at pH 6.0 with relatively low affinity in range of submicromole to micromole (Table 1). The response curve completely dropped to baseline level when the probe was immersed into pH 7.4 buffer, which indicated FcRn-bound proteins were completely released (data not shown). Although the measured affinities of these four proteins to FcRn had some difference, possible because of different structural stability at acidic conditions, the overall binding pattern of them to FcRn was still quite similar and the introduced disulfide bond might not reduce the serum half-life in vivo.

Discussion

Optimization of Fc fragment is desired to achieve better clinical potentials, which includes modification of physiological functions and improvement of structural properties. To modify Fc functions (e.g. enhancement of FcRn binding and modulation of effector functions), several studies focused on engineering CH2 domain. However, these modifications might decrease the stability of the whole Ig molecule (11, 16, 17), which limits the further application for clinical use.

We and another group previously disclosed that CH2 domain in Fc fragment is relatively unstable against heating and chemical reagent-induced unfolding compared with CH3 domain (6, 8, 18). Interestingly, it has also been shown that Fc unfolding is first triggered by the protonation of acidic residues on CH2 domain under acidic conditions (19). Therefore, CH2 domain is an ideal target for improvement of the stability and aggregation resistance of Fc. We previously identified a CH2 mutant (m01s) that has increased stability, aggregation resis-
tance, and pH-dependent binding to FcRn by introducing an additional disulfide bond and removal of several unstructured residues at the N terminus (6, 20, 21). A recent work found that introduction of a disulfide bond could compensate the reduction of stability caused by aglycosylated IgG (11). In addition to introduction of a disulfide bond, site-directed mutagenesis at several hot spots in the CH2 domain could also increase the stability and aggregation resistance in the context of an intact IgG by modification of noncovalent interactions (22). In addition, replacement of an enhanced aromatic sequon into the N-glycosylated loop DE has been performed that could enhance its thermal stability and resistance of aggregation induced by low pH in the context of a full-length IgG1 (23). The strong interactions between two CH3 domains are important for Fc dimerization. Therefore, the physicochemical properties of CH3 domain also have significant effects on the structural stability of Fc. In a previous study, the stability of bovine IgG1 CH3 domain also have significant effects on the structural stability and aggregation tendency of CH2. Therefore, stabilization of CH2 domain is the major factor to determine the aggregation and unfolding tendency of CH2. Therefore, stabilization of CH2 domain can simultaneously engineering disulfide bonds in CH2 and CH3 domains.

According to our results, introduction of disulfide bond in CH2 domain (FcCH2-s-s-) results in ~10 °C increase in $T_m$ of CH2 domain in the context of Fc fragment, as well as 1.4 M increase in the concentration of urea at 50% unfolding of Fc fragment. In contrast, additional disulfide bond in CH3 domain (FcCH3-s-s+) also leads to an ~10 °C increase in $T_m$ of CH3 domain but only a 0.5 M increase in the urea concentration at middle point of unfolding. The Fc mutant with two additional disulfide bonds (FcCH3-s-s+) has extremely high $T_m$ (>90 °C) and very strong resistance to urea-induced unfolding (concentration at middle point of unfolding is 9.0 M). Based on thermo-induced unfolding experiment, the separate introduction of disulfide bonds into CH2 or CH3 domains could only increase the stability of the corresponding domain because the folding of them is independent. Interestingly, it is observed that the $T_m$ value of CH2 in FcCH2-s-s- is higher than that in FcCH3-s-s+ (Fig. 3), which may be caused by synergic effect derived from simultaneously engineering disulfide bonds in CH2 and CH3 domains.

In the evaluation of aggregation formation under 60 °C incubation, introduction of disulfide bond in CH2 domain can prevent aggregation much better than that in CH3 domain. Because the thermostability of CH2 is much lower than that of CH3, the thermo-induced aggregations mainly depend on aggregation and unfolding tendency of CH2. Therefore, stabilization of CH2 domain is the major factor to determine the aggregation resistance of a whole Fc molecule under heating conditions.

In acidic conditions, it has been shown that CH3 plays critical role in driving intact antibody aggregation among antibody
In accordance with this result, we also found that additional disulfide in CH3 domain (FcCH3-s-s-) could make Fc more aggregation-resistant than that in the CH2 domain (FcCH2-s-s-) under pH 2.0. Therefore, stabilization of CH3 could increase the aggregation resistance of the Fc fragment to low pH more significantly than that of CH2. Notably, although it seems that only engineered disulfide bond in CH2 domain has no obvious contribution to improvement of aggregation resistance to low pH, the synergic effect is still very obvious (Fig. 6). Hence, the effect on stabilization of wtFc could be maximized when CH2 and CH3 domains are engineered with additional disulfide bonds together.

Because introduction of disulfide bond may cause the loss of Fc-mediated functions, we also compared the function of these Fc mutants. All the Fc mutants with additional disulfide bonds still maintain their Fc receptor-binding abilities as wtFc. It has also been reported that the introduced disulfide bond did not obviously change the binding to FcRn (7, 11). Although we observed some slight difference of the affinity to FcRn after engineering of disulfide bond, the general binding was still in a pH-dependent manner. Therefore, the recycling of the protein might not significantly change (e.g. reduce).

In summary, we demonstrated the contribution of introduced disulfide bonds in different Fc domains to the stability, aggregation resistance, and function. The most stable and aggregation-resistant mutant FcCH3-s-s-CH2-s-s- newly identified here could be used for modification of Fc-based therapeutics toward better clinical outcomes.

**Experimental procedures**

**Design, cloning, expression, and purification of wtFc and its mutants**

Human IgG1 Fc fragment (wtFc) was cloned into mammalian expression vector pSecTag2A (Invitrogen) for expression in FreeStyle 293-F (293F) cells. Its three mutants including FcCH2-s-s- (L242C/K334C), FcCH3-s-s- (P343C/A431C), and FcCH3-s-s-CH2-s-s- (L242C/K334C/P343C/A431C) were designed as reported previously (6, 7), constructed by PCR-based site-directed mutagenesis and also cloned into pSecTag2A. The clones were verified by direct sequencing. 293F cells (Invitrogen) were transiently transfected with the expression plasmids by using PEI (Polysciences) and grown in FreeStyleTM 293 expression medium according to the manufacturer’s protocol (Gibco). All proteins were purified by using protein G column (GE Healthcare). The purity was monitored by SDS-PAGE, and the concentration was determined by NanoPhotometer N60 (Implen) with extinction coefficient 1.45 (https://web.expasy.org/protparam/). All the purified fractions were dialyzed against PBS (pH 7.4) (137 mM NaCl, 2.683 mM KCl, 8.1 mM Na2HPO4, 1.76 mM KH2PO4) and stored at −80 °C.

**Thermal stability measurement by CD**

The purified wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s-CH2-s-s- proteins (0.5 mg in 0.5 ml) were loaded onto a Superdex 75 10/300 GL column (GE Healthcare) running on an ÄKTA pure system (GE Healthcare). The purity was monitored by SDS-PAGE, and the concentration was determined by NanoPhotometer N60 (Implen) with extinction coefficient 1.45 (https://web.expasy.org/protparam/). All the purified fractions were dialyzed against PBS (pH 7.4) (137 mM NaCl, 2.683 mM KCl, 8.1 mM Na2HPO4, 1.76 mM KH2PO4) and stored at −80 °C.
proteins were dissolved in PBS (pH 7.4) at the final concentration of 0.4 mg/ml, and the CD spectra were recorded on an Applied Photophysics Chirascan-SF.3 spectrophotometer (Applied Photophysics Ltd.). Wavelength spectra were recorded at 25 °C using a 0.1-cm-path length cuvette for native structure measurements. Thermal stability was measured by recording the CD signal at 216 nm in the temperature range from 25 to 94 °C with heating rate of 0.5 °C/min. The experiments were repeated twice, and the $T_m$ values were presented as means ± S.D.

**Spectrofluorometry**

The purified wtFc, $F_c^{CH_2-s-s}$, $F_c^{CH_3-s-s}$, and $F_c^{CH_2-s-s-CH_2-s-s-}$ were added into PBS (pH 7.4) with urea from 0 to 10 M. The final concentration of each protein was 100 μg/ml. After incubation at 25 °C overnight, the intrinsic fluorescence intensity was recorded on EnVision™ (PerkinElmer) to compare their stability against chemical denaturant. The measurement was performed with excitation wavelength at 280 nm and emission spectra at 340 nm at 25 °C as previously reported (6). The background fluorescence intensity of the solution (buffer + denaturant) was deducted from the sample fluorescence intensity. The experiments were repeated twice, and the values of urea concentration at 50% unfolding of each proteins were presented as means ± S.D.

**Turbidity assay**

The purified wtFc, $F_c^{CH_2-s-s}$, $F_c^{CH_3-s-s}$, and $F_c^{CH_2-s-s-CH_3-s-s-}$ with concentration of 2 mg/ml were prepared by filtering through 0.22-μm filter and centrifuging at 13,000 g for 10 min to make a final 900 μl of working solution. After incubation at 60 °C for 552 h (23 days), the protein samples were centrifuged at 4 °C, 13,000 × g for 15 min, and then the supernatant was injected onto a Superdex 75 10/300 GL column running on ÄKTA pure system to assess oligomer formation. To evaluate their behaviors in acid-induced aggregation, the final concentration of each protein was also adjusted to 2 mg/ml in PBS (pH 2.0 adjusted by HCl). The samples were

### Table 1

| Protein       | $K_{on}$ ($1/\text{ms}$) | $K_{off}$ ($1/\text{s}$) | $K_d$ (nM) | kDa |
|---------------|--------------------------|--------------------------|------------|-----|
| wtFc         | 2.94 × 10^5              | 2.99 × 10^{-1}           | 1020       | 1   |
| $F_c^{CH_2-s-s}$ | 4.12 × 10^5              | 1.09 × 10^{-1}           | 265        | 1   |
| $F_c^{CH_3-s-s}$ | 1.14 × 10^5              | 1.26 × 10^{-1}           | 1100       | 1   |
| $F_c^{CH_2-s-s-CH_3-s-s-}$ | 2.34 × 10^5              | 7.05 × 10^{-2}           | 301        | 1   |

Figure 8. Determination of binding of wtFc, $F_c^{CH_2-s-s}$, $F_c^{CH_3-s-s}$, and $F_c^{CH_2-s-s-CH_3-s-s-}$ to shFcRn at pH 6.0 by BLI. In general, all four proteins bind to shFcRn with high similarity. The experiments were repeated twice, and the results from one representative experiment are presented.
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incubated for up to 34 days at 37 °C without agitation. Turbidity was measured by recording the 320-nm absorbance at different time points.

Flow cytometry

To measure the interactions of wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s- with U937 cells, which can highly express FcγRI (14), each of these four proteins with different concentrations in PBS containing 1% BSA (w/v) were incubated with U937 cells at 4 °C for 1 h. Unbound proteins were washed away with PBS. The goat anti-human IgG Fc cross-adsorbed antibody, DyLight 650 (Invitrogen), used as secondary antibody, was incubated with cells at 4 °C for 1 h. The cells were washed and resuspended in PBS (pH 7.4) for measurement of the fluorescence intensity on CytoFLEX (Beckman Coulter). HeLa cells were used as negative control.

Design, expression, and purification of shFcRn

The genes encoding the α-chain and β2-microglobulin of shFcRn were cloned into the dual promoter vector pVITRO2-neo-mcs (Invivogen) to construct the recombinant expression plasmid pVITRO2-shFcRn. This plasmid was transfected into 293F cells for expression as described above. After 5 days of expression, shFcRn heterodimer was purified from supernatant using a human IgG conjugated Sepharose column (provided by Professor Bing Yan). The shFcRn was bound in PBS at pH 6.0, followed by elution in PBS at pH 7.4. The concentration was determined by NanoPhotometer N60 with extinction coefficient 1.78.

Binding of wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s- to shFcRn

Real-time binding assay between protein FcRn and wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s- was carried out on an Octet QK system (Fortebio) at 25 °C using BLI. Specifically, the shFcRn was labeled with EZ-Link® sulfo-NHS-LC-biotin (Pierce) as described in its instructions. After labeling, the shFcRn was desalted to remove nonreacted biotin with Amicon Ultra-0.5 centrifugal filter unit, 3 kDa (Merck). Then the biotin-labeled shFcRn was loaded into streptavidin biosensors, and wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s- dissolved in PBS containing 0.02% Tween 20 (pH 6.0) were added at a series of concentrations: 5000, 1667, 556, and 185 nM. The affinities of wtFc, FcCH2-s-s-, FcCH3-s-s-, and FcCH3-s-s- to FcRn were calculated according to 1:1 binding model through the Octet QK software (ForteBio) and the equilibrium dissociation constant (K_d) value was equal to the kinetic dissociation rate constant divided by the kinetic association rate constant. After the dissociation step, the regeneration step was performed by changing biosensors into pH 7.4 buffer to make all bound proteins completely dissociate from the biosensors and the baseline return to initial state.

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References

1. Yang, C., Gao, X., and Gong, R. (2017) Engineering of Fc Fragments with optimized physicochemical properties implying improvement of clinical potentials for Fc-based therapeutics. Front. Immunol. 8, 1860 Medline
2. Edelman, G. M., Cunningham, B. A., Gall, W. E., Gottlieb, P. D., Rutishauser, U., and Waxdal, M. J. (1969) The covalent structure of an entire γG immunoglobulin molecule. Proc. Natl. Acad. Sci. U.S.A. 63, 78–85 CrossRef Medline
3. Thies, M. J., Talamo, F., Mayer, M., Bell, S., Ruopolo, M., Marino, G., and Buchner, J. (2002) Folding and oxidation of the antibody domain CH3 of J. Biol. Chem. 281, 1267–1277 CrossRef Medline
4. McAuley, A., Jacob, J., Kolvenbach, C. G., Westland, K., Lee, H. J., Brych, S. R., Rehder, D., Kleemann, G. R., Brems, D. N., and Matsumura, M. (2008) Contributions of a disulfide bond to the structure, stability, and dimerization of human IgG1 antibody CH3 domain. Protein Sci. 17, 95–106 Medline
5. Sakurai, K., Nakahata, R., Lee, Y. H., Kardos, J., Ikegami, T., and Goto, Y. (2015) Effects of a reduced disulfide bond on aggregation properties of the human IgG1 CH3 domain. Biochim. Biophys. Acta 1854, 1526–1535 CrossRef Medline
6. Gong, R., Vu, B. K., Feng, Y., Prieto, D. A., Dyba, M. A., Walsh, J. D., Prabakaran, P., Veenstra, T. D., Tarasov, S. G., Ishima, R., and Dimitrov, D. S. (2009) Engineered human antibody constant domains with increased stability. J. Biol. Chem. 284, 14203–14210 CrossRef Medline
7. Wozniak-Knopp, G., Stadlmann, J., and Rüker, F. (2012) Stabilisation of the Fc fragment of human IgG1 by engineered intradomain disulfide bonds. PLoS One 7, e30083 CrossRef Medline
8. Ying, T., Chen, W., Feng, Y., Wang, Y., Geng, R., and Dimitrov, D. S. (2013) Engineered soluble monomeric IgG1 CH3 domain: generation, mechanisms of function, and implications for design of biological therapeutics. J. Biol. Chem. 288, 25154–25164 CrossRef Medline
9. Wozniak-Knopp, G., and Rüker, F. (2012) A C-terminal interdomain disulfide bond significantly stabilizes the Fc fragment of IgG1. Arch. Biochem. Biophys. 526, 181–187 CrossRef Medline
10. Yageta, S., Shibuya, R., Imamura, H., and Honda, S. (2017) Conformational and coloidal stabilities of human immunoglobulin G Fc and its cyclized variant: independent and compensatory participation of domains in aggregation of multidomain proteins. Mol. Pharm. 14, 699–711 CrossRef Medline
11. Jacobsen, F. W., Stevenson, R., Li, C., Salimi-Moosavi, H., Liu, L., Wen, J., Rao, Q., Daris, K., Buck, L., Miller, S., Ho, S. Y., Wang, W., Chen, Q., Walker, K., Wypych, J., et al. (2017) Engineering an IgG scaffold lacking effector function with optimized developability. J. Biol. Chem. 292, 1865–1875 CrossRef Medline
12. Saphire, E. O., Parren, P. W., Pantophlet, R., Zwick, M. B., Morris, G. M., Ruddle, P. M., Dwek, R. A., Stanfield, R. L., Burton, D. R., and Wilson, I. A. (2001) Crystal structure of a neutralizing human IgG against HIV-1: a template for vaccine design. Science 293, 1155–1159 CrossRef Medline
13. Shields, R. L., Namenuk, A. K., Hong, K., Meng, Y. G., Rae, J. R., Briggs, J., Xie, D., Lai, J., Stadlen, A., Li, B., Fox, J. A., and Presta, L. G. (2001) High resolution mapping of the binding site on human IgG1 for FcγRI, FcγRII, FcγRIIa, and FcRn and design of IgG1 variants with improved binding to the FcγR. J. Biol. Chem. 276, 6591–6604 CrossRef Medline

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14. Ying, T., Feng, Y., Wang, Y., Chen, W., and Dimitrov, D. S. (2014) Mono-
meric IgG1 Fc molecules displaying unique Fc receptor interactions that
are exploitable to treat inflammation-mediated diseases. mAbs 6,
1201–1210 CrossRef Medline
15. Roopenian, D. C., and Akilesh, S. (2007) FcRn: the neonatal Fc receptor
comes of age. Nat. Rev. Immunol. 7, 715–725 CrossRef Medline
16. Dall’Acqua, W. F., Kiener, P. A., and Wu, H. (2006) Properties of human
IgG1s engineered for enhanced binding to the neonatal Fc receptor
(FcRn). J. Biol. Chem. 281, 23514–23524 CrossRef Medline
17. Majumdar, R., Esfandiary, R., Bishop, S. M., Samra, H. S., Middaugh, C. R.,
Volkin, D. B., and Weis, D. D. (2015) Correlations between changes in con-
formational dynamics and physical stability in a mutant IgG1 mAb engi-
neered for extended serum half-life. mAbs 7, 84–95 CrossRef Medline
18. Feige, M. J., Walter, S., and Buchner, J. (2004) Folding mechanism of the
CH2 antibody domain. J. Mol. Biol. 344, 107–118 CrossRef Medline
19. Latypov, R. F., Hogan, S., Lau, H., Gadgil, H., and Liu, D. (2012) Elucidation
of acid-induced unfolding and aggregation of human immunoglobulin
IgG1 and IgG2 Fc. J. Biol. Chem. 287, 1381–1396 CrossRef Medline
20. Gong, R., Wang, Y., Feng, Y., Zhao, Q., and Dimitrov, D. S. (2011) Short-
ened engineered human antibody CH2 domains: increased stability and
binding to the human neonatal Fc receptor. J. Biol. Chem. 286,
27288–27293 CrossRef Medline
21. Gong, R., Wang, Y., Ying, T., Feng, Y., Straker, E., Prabakaran, P., and
Dimitrov, D. S. (2013) N-terminal truncation of an isolated human IgG1
CH2 domain significantly increases its stability and aggregation resis-
tance. Mol. Pharm. 10, 2642–2652 CrossRef Medline
22. Chennamsetty, N., Voynov, V., Kayser, V., Helk, B., and Trout, B. L. (2009)
Design of therapeutic proteins with enhanced stability. Proc. Natl. Acad.
Sci. U.S.A. 106, 11937–11942 CrossRef Medline
23. Chen, W., Kong, L., Connelly, S., Dendle, J. M., Liu, Y., Wilson, I. A.,
Powers, E. T., and Kelly, J. W. (2016) Stabilizing the CH2 domain of an
antibody by engineering in an enhanced aromatic sequon. ACS Chem.
Biol. 11, 1852–1861 CrossRef Medline
24. Demarest, S. J., Rogers, J., and Hansen, G. (2004) Optimization of the
antibody CH3 domain by residue frequency analysis of IgG sequences. J.
Mol. Biol. 335, 41–48 CrossRef Medline
25. Yageta, S., Lauer, T. M., Trout, B. L., and Honda, S. (2015) Conformational
and colloidal stabilities of isolated constant domains of human immuno-
globulin G and their impact on antibody aggregation under acidic condi-
tions. Mol. Pharm. 12, 1443–1455 CrossRef Medline