Disulfide bond structures of IgG molecules
Structural variations, chemical modifications and possible impacts to stability and biological function

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The disulfide bond structures established decades ago for immunoglobulins have been challenged by findings from extensive characterization of recombinant and human monoclonal IgG antibodies. Non-classical disulfide bond structure was first identified in IgG_4 and later in IgG_2 antibodies. Although, cysteine residues should be in the disulfide bonded states, free sulfhydryls have been detected in all subclasses of IgG antibodies. In addition, disulfide bonds are susceptible to chemical modifications, which can further generate structural variants such as IgG antibodies with trisulfide bond or thioether linkages. Trisulfide bond formation has also been observed for IgG of all subclasses. Degradation of disulfide bond through β-elimination generates free sulfhydryls disulfide and dehydroalanine. Further reaction between free sulfhydryl and dehydroalanine leads to the formation of a non-reducible cross-linked species. Hydrolysis of the dehydroalanine residue contributes substantially to antibody hinge region fragmentation. The effect of these disulfide bond variations on antibody structure, stability and biological function are discussed in this review.

Introduction

The recombinant monoclonal IgG antibodies comprise a rapidly growing group of protein therapeutics. The disulfide bond structure of IgG is highly conserved through evolution and was once considered a uniform and homogeneous structural feature. However, detailed characterization of a large number of IgG molecules has revealed several new structural features in both recombinant and natural human IgG antibodies. These new findings and their effects on IgG structure, stability and biological function are reviewed here.

Classical Disulfide Bond Structures

Disulfide bond structures of the four subclasses of IgG were established in the 1960s.[1-8] These disulfide bond structures are referred to as the classical disulfide bond structures because they

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are widely accepted. As shown in **Figure 1**, there are many similarities and some differences with regard to the disulfide bond structures in the four subclasses of IgG antibodies, IgG_1, IgG_2, IgG_3 and IgG_4. Each IgG contains a total of 12 intra-chain disulfide bonds; each disulfide bond is associated with an individual IgG domain. The two heavy chains are connected in the hinge region by a variable number of disulfide bonds: 2 for IgG_1 and IgG_4, 4 for IgG_2 and 11 for IgG_3. The light chain of the IgG_1 is connected to the heavy chain by a disulfide bond between the last cysteine residue of the light chain and the fifth cysteine residue of the heavy chain. However, for IgG_2, IgG_3 and IgG_4, the light chain is linked to the heavy chain by a disulfide bond between the last cysteine residue of the light chain and the third cysteine residue of the heavy chain.

The level of solvent exposure is different between intra-chain and inter-chain disulfide bonds. Cysteine residues that form inter-chain disulfide bonds are located in the hinge region with the exception of the third cysteine residue of the heavy chain in IgG_2, IgG_3 and IgG_4, which is located between the interface of VH and CH1 domains.[9] Therefore, inter-chain disulfide bonds are highly solvent exposed.[9-12] On the other hand, intra-chain disulfide bonds are buried between the two layers of anti-parallel β-sheet structures within each domain and are not solvent exposed.[9-12] The solvent exposure difference has important implications because exposed cysteine residues are considered more reactive than non-exposed cysteine residues.

Non-Classical Linkage

Disulfide bond structures other than the classical structures shown in **Figure 1** have been observed mainly for IgG_2 and IgG_4, but not for IgG_1 and IgG_3. Only a trace amount of a disulfide bond variant with the two inter heavy chain disulfide bonds in the intra-chain form for IgG_1 has been observed.[13] IgG_3 has repeated amino acid sequence in the hinge region and a total of 11 disulfide bonds in close proximity, which does not allow much flexibility for formation of disulfide bond variants.

Non-classical disulfide bond structures of IgG_2 were first identified in recombinant monoclonal antibodies (mAbs) and then confirmed in human IgG_2 molecules.[14-16] In these publications, the classical disulfide bond structure was referred to as IgG_2A,
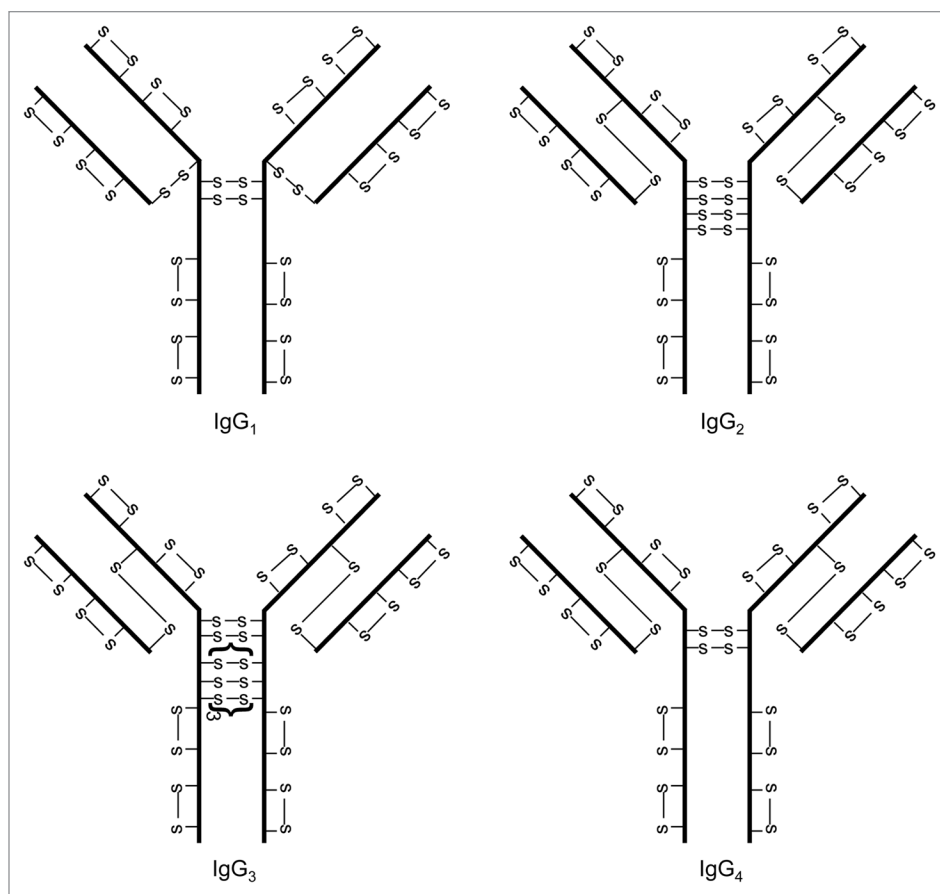
Figure 1. Classical IgG disulfide bond structures.

while the two major non-classical structures were referred to as IgG_2B and IgG_2-A/B, the latter being considered a structural intermediate between IgG_2A and IgG_2B (**Fig. 2**). Distribution of different disulfide bond isoforms is dependent on the type of light chain, IgG_2A is the major form in molecules with λ light chain; IgG_2B is the major form in molecules with κ light chain.[15] A conversion from the IgG_2A form to IgG_2B was observed during cell culture, in vitro incubation with serum and in patient serum.[17] Molecular dynamic simulation study revealed that the sulfur atoms of inter-chain disulfide bonds are highly mobile and can be in close proximity.[18] Therefore, it is not a surprise to observe the coexistence of multiple disulfide bond isoforms for IgG_2 antibodies. In addition to isoforms from different intra-molecule disulfide bond linkages, disulfide bond linked IgG_2 dimer was also found in recombinant IgG_2 from cell culture and in human serum.[19]

By far, IgG_4 is the best known subclass of IgG molecule having non-classical disulfide bond structures (**Fig. 3**). Several interesting observations led to the ultimate finding of the non-classical disulfide bond structures. First, significant amounts of IgG_4 were observed as half-molecules when analyzed by non-reducing sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE),[20-24] but not by size-exclusion chromatography (SEC) run under native conditions,[23] indicating that the two half-molecules are associated by non-covalent interactions rather

than by covalent linkage. Second, polyclonal IgG_4 is unable to cross-link antigen and behaves like a monovalent antibody,[25] while monoclonal IgG_4 can cross-link antigens.[26] Third, IgG_4 as a covalent-linked monomer demonstrates bispecificity in plasma.[26] These observations were explained by the fact that the two inter heavy chain disulfide bonds of IgG_4 are in equilibrium with intra-chain disulfide bond forms.[13,23,24] IgG_1 and IgG_4 differ by one amino acid in the middle hinge region, i.e., there are two proline residues in IgG_1 and a serine and a proline residue in IgG_4. Stable inter heavy chain disulfide bonds of IgG_4 were obtained by replacing the serine residue with a proline residue.[13,23,24] Because of the instability of the inter heavy chain disulfide bonds, bispecific antibody can be formed in vitro in the presence of reducing reagents and in vivo by injection of equal amounts of two recombinant IgG_4 antibodies specific for two different antigens into immunodeficient mice.[27]

Free Sulfhydryls

Presumably, all cysteine residues in IgG are in the disulfide bonded state. However, free sulfhydryls has been routinely detected in IgG molecules, including IgG from serum and recombinant mAbs (**Table 1**).[28-35] It is worthwhile to discuss two important observations in **Table 1**. First, higher level of free sulfhydryls was detected under denaturing conditions compared with native con-
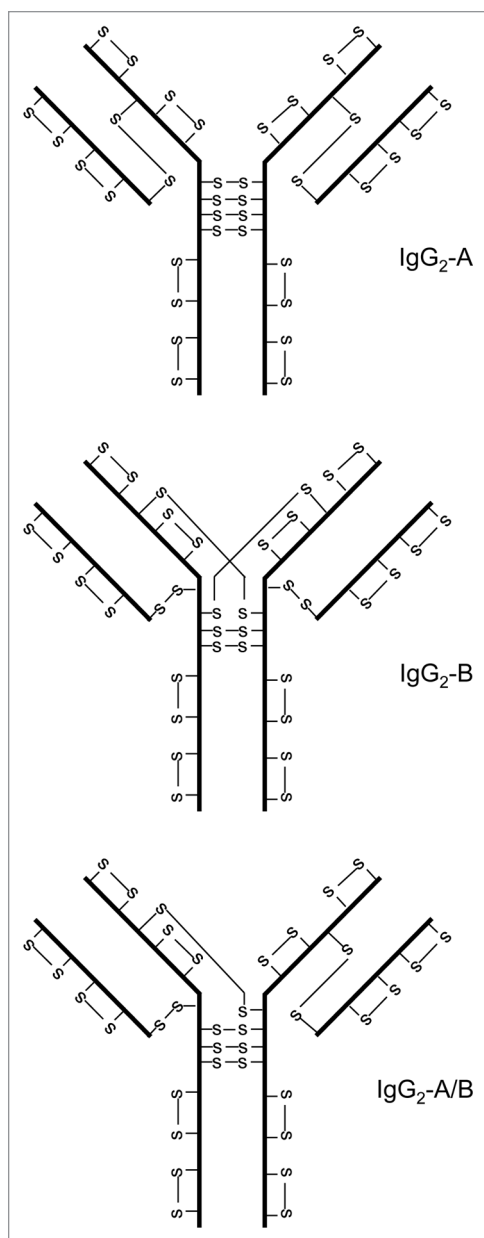
Figure 2. IgG$_2$ disulfide bond isoforms.

ditions. This indicates that free sulfhydryl is associated with cysteine residues involved in both inter and intra chain disulfide bonds. Second, there is a large variation in the levels of free sulfhydryl under denaturing conditions among different studies. This large variation is likely due to a combination of multiple factors, including different IgG types (human or recombinant), different IgG subclasses and experimental variations, e.g., denaturing reagents and denaturing times, which may vary for different IgGs to be fully denatured. Degradation of disulfide bonds that produces free sulfhydryls may occur, as will be discussed later.

While IgG molecules may have different levels of free sulfhydryl, studies suggested a similar distribution of free sulfhydryl among the domain structures, at least for recombinant IgG$_1$.[36,37]

The variable domain has a higher level of free sulfhydryls than that in the constant domain in the light chain. The CH3 domain has the highest level of free sulfhydryls followed by CH1, CH2 and the variable domain in the heavy chain. The lowest level of free sulfhydryls is associated with inter-chain disulfide bonds, suggesting that low level of free sulfhydryls is most likely due to incomplete formation of disulfide bonds. Because inter-chain disulfide bonds with higher solvent exposure level are more prone to degradation than intra-chain disulfide bonds, higher level of free sulfhydryls associated with inter-chain disulfide bonds is expected if free sulfhydryl is generated due to disulfide bond degradation. Distribution other than described above may indicate special cases where particular disulfide bonds are not efficiently formed.

Two special cases have been reported in the literature so far. In both cases, the intra-chain disulfide bond in the heavy chain variable domain is not completely formed at such a level that antibodies with this incomplete disulfide bond were detected by hydrophobic interaction or weak cation exchange chromatography.[38,39] Complete formation of this particular disulfide bond can be achieved by the addition of copper sulfate to cell culture,[40] suggesting that cell culture conditions can affect disulfide bond formation. Antibodies after in vitro incubation in serum or recovered from rat serum after administration showed significant reduction in incomplete disulfide bond formation.[39]

β-Elimination

Under basic conditions, disulfide bonds can decompose through the β-elimination mechanism with the formation of dehydroalanine and persulfide, which can further revert back to a cysteine residue.[41-43] Degradation of the inter light chain and heavy chain disulfide bond of IgG through the β-elimination mechanism followed by cross-linking of the resulting cysteine and dehydroalanine has led to the formation of a non-reducible thioether linkage,[44] which was found at ~0.4% for a recombinant monoclonal IgG$_1$ stored at 4°C and up to 13.6% for a heat-stressed sample. Subsequent hydrolysis of the dehydroalanine is another important mechanism in addition to peptide bond hydrolysis that leads to antibody fragmentation in the hinge region.[45]

Trisulfide Bond Formation

Trisulfide bonding formation is a rare post-translational modification of proteins. The presence of trisulfide bonding was first reported for a recombinant monoclonal IgG$_2$, where one or two of the four inter heavy chain disulfide bonds may exist as a trisulfide bond.[46] Trisulfide bonds were later detected in all subclasses of recombinant IgG antibodies, as well as in human IgG from patients with myeloma.[47] In all cases, higher levels of trisulfide bonds were observed between the cysteine residues that normally form the inter light chain and heavy chain disulfide bonds.[47] Trisulfide bonds in recombinant mAbs are believed to be formed during fermentation as a result of the reaction of an intact disulfide bond with dissolved hydrogen sulfide (H$_2$S).[46,47] This conclusion is supported by the observation that incubation of IgG with
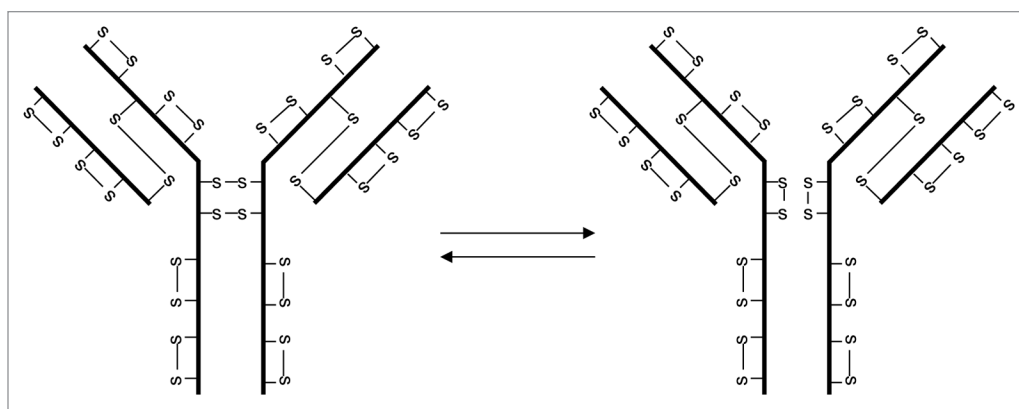
Figure 3. IgG$_4$ disulfide bond isoforms.

Table 1. Level of free sulfhydryl in IgG

Type of IgG	Mole of free SH/Mole of IgG		Reference
	Native	**Denatured**	
Human IgG$_2$	0.24	ND*	28, 29
Human IgG$_1$	0.1–1.1	0.6–4.0	30
Human IgG$_1$, IgG$_4$ and recombinant IgG$_4$	ND*	0.9–2.2	31
Recombinant IgG$_1$, IgG$_2$ and IgG$_4$	0.02–0.08	0.08–0.09	32
Recombinant IgG$_1$	ND*	0.64	33
Recombinant IgG$_2$	0.06	0.59	34
Recombinant IgG$_2$	0.158	0.379	35

ND*, not determined.

H$_2$S resulted in higher levels of trisulfide bonding.[47] Substantial conversion of trisulfide bonds to disulfide bonds was observed when a recombinant IgG$_2$ antibody was incubated with various reducing reagents at pH 7.5.[46] In another study, it was found that trisulfide bonds were stable in buffers at pH 6.5 and in rat serum in vitro. However, complete conversion of the trisulfide bond to a disulfide bond was achieved when a recombinant IgG$_1$ was recovered from rat serum 24 h after intraperitoneal injection.[47] It is hypothesized that trisulfide bond is formed through several reaction intermediates produced by the initial nucleophilic attack of disulfide bond by HS- under an appropriate redox condition.[48]

Structure, Stability and Functions

Information about the structure, stability and functional impacts of non-classical linkage and trisulfide bond is limited. The non-classical structure of the IgG$_2$B is more compact than that of IgG$_2$A, as determined by SEC and analytical ultracentrifugation.[15] IgG$_2$A was shown to have either similar or higher binding affinity and biological activity than IgG$_2$B.[15,16] Studies determined that the presence of trisulfide bonds does not appear to affect the thermal stability or antigen binding.[46,47] Limited information on IgG$_4$ isoforms mainly comes

from mutagenesis studies. Two mutants, one replacing the first middle hinge cysteine with a serine and the other replacing the serine in the middle hinge with a proline, resulted in more stable IgG$_4$ molecules without affecting antigen binding activity.[24] In a separate study, replacing the middle hinge serine with proline resulted in a more stable inter-chain disulfide bond and increased half-life, again without affecting antigen binding activity.[23]

The effect of free sulfhydryl on the structure, stability and biological functions of IgG has been studied using individual domains, as well as intact IgG molecules. Individual domains of C$_L$ domain,[12] CH3 domain[49,50] and single-chain variable fragment[51] without the complete intra-chain disulfide bond showed lower stability, but no substantial structural changes. It is expected that the lack of intra-chain disulfide bond in other domains will have similar destabilizing effect because all IgG domain share similar folding.[52] Incomplete formation of the disulfide bond in the heavy chain variable domain of a recombinant monoclonal antibody resulted in a significant decrease in potency.[38,39] A natural antibody derived from the ABPC48 mouse plasmacytoma, in which the second cysteine residue in the heavy chain variable domain was replaced by a tyrosine residue, is capable of binding antigen,[53] suggesting further that a complete disulfide bond is not a prerequisite for antigen binding. Higher amounts of free sulfhydryl resulted in lower thermal stability of both recombinant and human IgG antibodies.[31] In addition, the higher aggregation propensity of IgG$_2$ compared with IgG$_1$ is also attributed to higher level of free sulfhydryl of IgG$_2$.[35]

Partial reduction has been one of the commonly used methods to study the effect of inter-chain disulfide bond on the structure, stability and biological functions of IgG. Although a global conformational change was not observed,[12,52,54-59] partial reduction increased the flexibility of the hinge region, probably as a result of reduction of inter-chain disulfide bonds, resulting in further separation of the two CH2 domains.[52,55,56,60] An apparent increase in the hydrodynamic sizes of human IgG$_1$, IgG$_2$ and IgG$_4$, but decreased size for IgG$_3$ were also observed upon partial reduction and alkylation, which is again attributed to the structural change in the hinge region and CH2 domain.[61] Highly dependent on the experimental conditions,[62] partial reduction either has no

impact[59,63] or reduces complement activation efficiency.[64-66] The effect of partial reduction on binding to Fc receptors and, consequently, antibody-dependent cell-mediated cytotoxicity (ADCC) is also not consistent, e.g., no effect[67,68] and significantly reduced activity[59,69,70] were observed for different antibodies. One of the critical issues is the degree of reduction of the intra-chain disulfide bonds in different studies. It has been reported that only inter-chain disulfide bonds of human IgG_1 are susceptible to reduction under native conditions.[71] However, reduction of intra-chain disulfide bonds of rabbit IgG under native conditions may be possible.[62,64]

Although levels of free sulfhydryls appear to be low, their presence poses some challenges for recombinant monoclonal antibody formulation. It was found that the majority of the IgG_1 dimer is formed due to formation of intermolecular disulfide bonds,[72] which could result from free sulfhydryls. Dimerization through disulfide bond formation is the major aggregation pathway for a recombinant monoclonal IgG_2 antibody at pH 6.0 after heat stress.[73] A substantial amount of covalently linked aggregates formed via disulfide bonds of an IgG_2 was also found in the aggregates caused by agitation.[34] It is possible that antibodies with incomplete disulfide bonds are more susceptible to unfolding under various stress conditions and, therefore, have a higher propensity for covalent aggregation through disulfide bond formation. IgG antibodies with higher levels of free sulfhydryls also have a greater tendency to expose hydrophobic regions, which can drive the formation of non-covalent aggregates through inter-molecule hydrophobic interactions.

specifically to disulfide bond structures is a potential major contributor to heterogeneity. It has been clearly demonstrated that intra-chain disulfide bonds are in general more stable after antibody assembly. Therefore, the low levels of free sulfhydryl associated with these intra-chain disulfide bonds are probably due to incomplete formation of disulfide bonds. On the other hand, inter-chain disulfide bonds are exposed and less stable, which explains why increased heterogeneity is associated with these bonds. It is thus reasonable to hypothesize that non-classical disulfide bond structures, trisulfide bonding and thioether linkages formation may occur after antibody assembly.

Close attention should be paid to these new disulfide bond-related structures during the development of recombinant mAbs because changes in structures and stability have been observed. Theoretically, administration of non-native disulfide bonded structures to humans has the potential to trigger immune response. Lowering stability can also ultimately lead to non-native structures because of the higher propensity to unfold and form aggregates. More experiments are thus warranted to improve understanding of the effects of disulfide bond related structural variants on the stability, structure and biological functions of IgG molecules.

Conclusion

Heterogeneity is a common feature of recombinant mAbs as a result of post-translational modifications and variation related

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