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Review

Engineering disulfide bonds within an antibody☆,☆☆

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ABSTRACT

Antibodies have evolved to function in oxidative, extracellular environments. A pair of cysteines in close proximity will oxidatively react to form a disulfide bond that fixes and stabilizes the tertiary structure of a protein. Immunoglobulin G (IgG) includes several disulfide bonds, and the patterns of inter-chain disulfide bonds characterize different IgG sub-classes. Moreover, the Ig-fold domains are characterized by a buried intra-domain disulfide bond, which is important for its structural stability. However, the intra-domain disulfide bond can be replaced without crucial effects on the structure and function, if the domain structure is intrinsically stable or has been stabilized by protein engineering. In previous studies, disulfide bonds were removed by amino-acid substitution indicating that Val and/or Ala (i.e. Ala–Ala, Ala–Val, Val–Ala, and Val–Ala) pairs were preferred for cysteine replacement in the Ig-fold domain. As such, these mutations may be useful for the intracellular use of antibodies. Recently, additional intra-domain disulfide bonds have been shown to stabilize Ig-fold domains and whole IgGs. In heavy chain variable or light chain variable domains, the introduction of additional disulfide bonds into the framework region did not reduce antigen-binding affinity, suggesting that generating disulfide bonds may be a method for stabilizing IgG and antibody fragments, such as the antigen-binding fragment, and single-chain and single-domain antibodies. This article is part of a Special Issue entitled: Recent advances in molecular engineering of antibody.

1. Disulfide bonds in full-length antibodies

Disulfide bonds are formed by the oxidation of 2 thiol groups within Cys residues and in many extracellular proteins. In an oxidative environment, disulfide bonds fix and stabilize the tertiary structures of proteins. Therefore, antibodies contain a number of disulfide bonds. Typically, immunoglobulin G (IgG) has 6 intra-domain disulfide bonds. IgG1 (Fig. 1A), IgG2 (Fig. 1B), IgG3 (Fig. 1C), and IgG4 (Fig. 1D) have 4, 6, 13, and 4 inter-chain disulfide bonds, respectively. Inter-chain disulfide bonds that connect two heavy chains are critically important for the correct combination of heavy chains. A large fraction of plasma IgG4 molecules have two different antigen-binding sites (i.e., they are bispecific) [2]. Transplantation of the heavy-chain hinge region of IgG4 into other IgG subclass molecules resulted in a failure to form disulfide bridges between the two heavy chains [3,4]. The SerH241 at the hinge region, in place of the proline that is dominant in other IgG subtypes, was responsible for this phenomenon [5]. In IgG4, the disulfide bond connecting 2 heavy chains is more susceptible to reduction than those of other IgG sub-classes [6], and the 2 cysteines in the hinge region are intra-molecularly connected (Fig. 1D). Therefore, the presence of disulfide bonds between 2 heavy chains prohibits the exchange of heavy chains from other antibodies and maintains the monospecificity of an antibody.

IgG2 has different isoforms, which contain 2 non-classical disulfide bond linkages (Fig. 1B) [7,8]. Classically, one disulfide linkage connects 2 heavy chains (Cys H232-Cys H232), and another disulfide linkage connects the heavy and light chains (Cys H127- CysL214). In the IgG2 isoforms, CysL214 is connected to CysH232, and CysH127 is connected to CysH233. The 2 IgG2 isoforms are IgG2B and IgG2A/B. IgG2B has 2 sets of non-classical disulfide bonds, and IgG2A/B has 1 set of non-classical disulfide bonds. Electron microscopy and 3D reconstruction of IgG2 revealed that it had an asymmetrical structure. Specifically, one subunit corresponding to antigen-binding fragment (Fab) was in close proximity to the upper portion of the region corresponding to crystallizable fragment (Fc) subunit (second heavy chain constant

Abbreviations: IgG, Immunoglobulin G; VH, Heavy chain variable; CH, Heavy chain constant; VL, Light chain variable; CL, Light chain constant; Fab, Antigen-binding fragment; Fc, Crystallizable fragment; CDR, Complementarity determining region; Fv, Variable fragment; scFv, Single chain antibody fragment; VHH, VH domain of the heavy-chain antibody; T_m, Mid-point temperature of thermal unfolding; β2m, β2-microglobulin; IAC, Intracellular antibody capture; hPSA, Human prostate-specific antigen; VSG, Variant surface glycoproteins; GFP, Green fluorescent protein; hCG, Human chorionic gonadotropin; ΔS_U, Entropic value of the unfolding reaction; ΔH_U, Enthalpic value of the unfolding reaction

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☆☆ In this manuscript, Kabat numbering system is used to indicate the most of amino acid positions [1].

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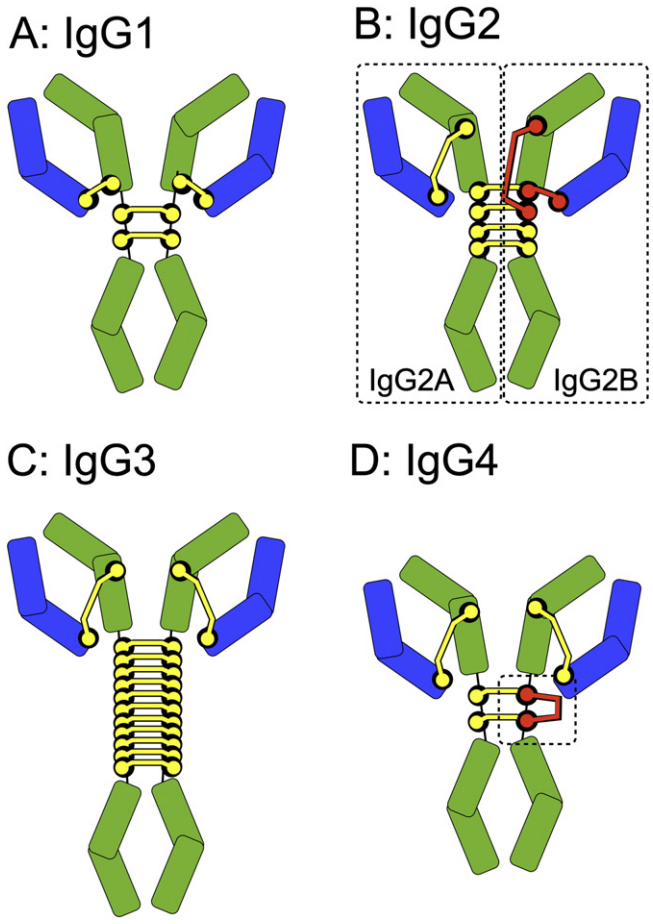

Fig. 1. Patterns of disulfide linkage in IgG1 (A), IgG2 (B), IgG3 (C), and IgG4 (D). IgGs consist of 2 heavy chains (green), 2 light chain (blue), and a canonical inter-chain disulfide bond (yellow line) connecting the chains. In IgG2 and IgG4, non-canonical inter-chain disulfide bonds (red line) are formed.

(CH) domain), and the other Fab subunit was far from Fc [9]. This structure is considered to be that of IgG2A/B isoform.

The disulfide bond between the light and heavy chains is important for the stability of the Fab. In IgG1, the C-terminal cysteines of the CH1 domain (CysH233) and light chain constant (CL) domain (CysL214) form a disulfide bond between the heavy and light chain. In contrast, other IgG subtypes, such as IgG4, have a Cys at the end of the first β strand of CH1. Although the positions of cysteines within the primary sequences differed between IgG1 and other subtypes, their spatial locations were similar [10]. The Fab of IgG1 had a mid-point temperature of thermal unfolding (T_m) that was 11 °C higher than that of IgG4, which has the same variable regions as IgG1 [11]. Introduction of an IgG1-type disulfide bond between the heavy and light chains of the IgG4 Fab significantly increased the T_m, indicating that the IgG1-type disulfide bond between the heavy and light chain increased the thermodynamic stability of the Fab region.

2. Removal of intra-domain disulfide bonds in Ig-fold domains

In antibodies, Ig-fold domains (i.e. heavy chain variable (VH), light chain variable (VL), CH, and CL domains) normally have 1 intra-domain disulfide bond [12] (Fig. 2). This disulfide bond connects the B and F β strands of the Ig-fold domain [13,14]. In 1970, Litman first showed that reduction and alkylation of the intra-chain disulfide bonds of polyclonal antibodies caused a large conformational change [15]. The studies identified the structural role for this disulfide bond in each domain using an isolated fragment of the CL domain (CL fragment). At the time, recombinant proteins were not standard; in fact, the first active recombinant mammalian protein, dihydrofolate reductase, was not obtained from *Escherichia coli* until 1978 [16]. Therefore, the CL domain was prepared by limited proteolysis of Bence Jones proteins. The Bence Jones protein is the light chain of an antibody [17] isolated from the urine of patients with multiple myeloma. Similar to other Ig-fold domains, the CL domain has a characteristic disulfide bond buried within the molecule. Goto and Hamaguchi [18] prepared a human CL fragment from type λ Bence Jones protein by papain digestion. The CL fragment containing a reduced intra-domain disulfide bond exhibited only minor changes in its secondary and tertiary conformations but a significant decrease in its thermodynamic stability. These results indicated that disulfide-bond formation in the CL fragment was essential for its stability but not its structure. On the other hand, modification of the reduced thiol by iodoacetamide or iodoacetic acid induced protein unfolding, suggesting that the introduction of bulky alkyl groups inside the domain destroyed the Ig-fold structure. Ishiwata et al. prepared a series of C-terminal-truncated CL fragments by limited carboxypeptidase P or Y proteolysis, resulting in the deletion of 3 to 18 C-terminal amino acids. Although the deletion of 3 amino acids did not affect the biophysical properties of the CL fragment, deletion of 7 or more amino acids induced partial unfolding of the protein, in which the secondary and tertiary structures as well as the thermodynamic stability were significantly affected [19]. In addition, reduction of the disulfide bond of the truncated CL fragment lacking seven amino acids resulted in the complete unfolding of CL fragment.

The effect of reducing disulfide bonds within variable domains was studied using the variable fragment (Fv) of the monoclonal antibody McPC603 [20]. Reduction of the Fv fragment by β-mercaptoethanol caused irreversible inactivation and aggregation at 37 °C. However, refolding of the reduced fragment did not yield a functional antigen-binding Fv fragment. This indicated that the disulfide bond conferred stability both in the irreversible denaturation process as well as in the refolding reaction. However, there was no structural information available about the reduced VL and VH domains of McPC603. Subsequently, Ohage et al. showed that the stabilized VL domain mutants of McPC603 folded under reducing conditions [21]. In addition, the free energy of unfolding (ΔG_U) of these mutants was measured by analyzing denaturant-induced unfolding curves. The difference in ΔG_U between the oxidized and reduced mutants ranged from 14 to 19 kJ/mol. The stability of the wild-type oxidized VL domain of McPC603 was 13.5 kJ/mol, suggesting that the domain unfolded under reducing conditions. These data suggested that the domain's intrinsic stability determines whether unfolding occurs upon reduction of the disulfide bond.

The VH domain of an antibody derived from the ABPC48 plasmacytoma lacked an intra-domain disulfide bond but continued to interact with its antigen, levan [22,23]. This was the first empirical evidence that the disulfide bond is not essential for antibody function. The second Cys residue in the VH domain, Cys H92, was replaced by Tyr in the VH of ABPC48. Prova et al. restored the missing disulfide bond in the single chain Fv fragment (scFv) of ABPC48 [24]. The mutant scFv of ABPC48 with an intra-domain disulfide bond exhibited significantly higher stability than the wild-type and even higher stability than other scFvs, which explained why ABPC48 remained stable despite the loss of the disulfide bond. The intra-domain disulfide bond is typically buried inside the hydrophobic core of the Ig-fold domain; thus, modification of the CysH22 by a bulky reagent likely disrupted its 3D structure, as observed in the CL fragment. The modified scFv, in which the free thiol was modified by glutathione or 4-(2-aminoethyl)-benzene sulfonyl fluoride, was functional and equally stable as the unmodified protein, suggesting that CysH22 had moved to the surface and was exposed to the solvent.

Frisch et al. produced a recombinant VL domain of Bence Jones protein REI [25]. The mutant VL domain of REI, where CysL23 was substituted with Val, lacked a disulfide bond and displayed decreased