Supporting information for:

Mechanism of Immunoglobulin G4 Fab-arm Exchange

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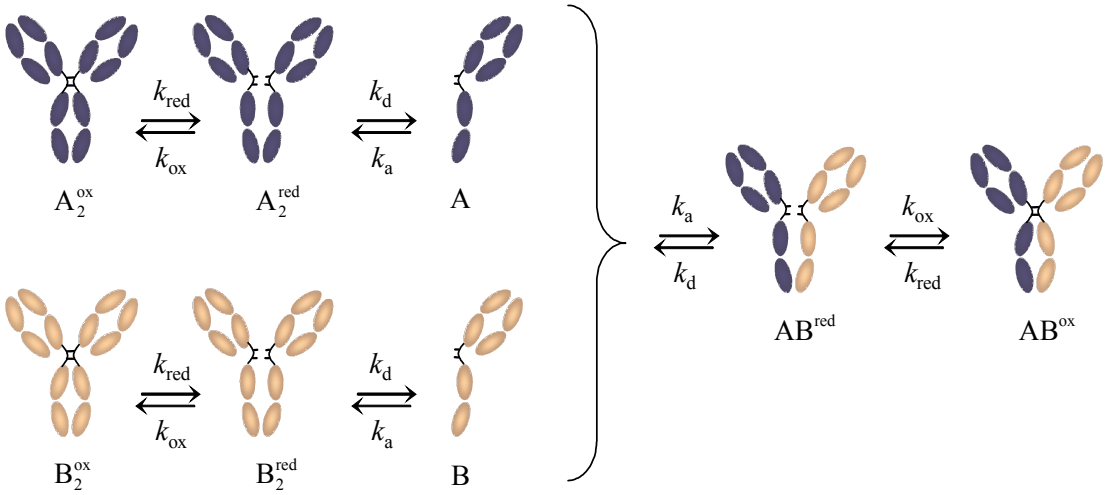
Derivation of kinetic model

In Scheme S1, differential equations are given for the general case where two kinds of IgG4, i.e., A and B, are to be found either as covalent dimers (i.e., A_2^{ox} ; B_2^{ox}), or as non-covalent dimers (i.e., A_2^{red} ; B_2^{red}). The non-covalent dimers may be in a reduced form or be intra-chain isomers. The equilibrium between both kinds of dimers is described in a simplified way as the quotient of two first-order rate constants, k_{red} and k_{ox}. This simplified description is justified on the basis that it suffices to reproduce the observed kinetics, and the fact that it is often found that such a description, leaving out intermediate reduction products, satisfactorily describes the overall reduction process (1). Moreover, in our case, k_{red}, and, indirectly, k_{ox}, are available from experiment. A_2^{red} and B_2^{red} are assumed to be able to dissociate with a single rate constant k_d, and A and B are assumed to be able to associate to either form homodimers or heterodimers (AB^{red}), the latter again in equilibrium with the covalent form (AB^{ox}). The dissociation and association rate constants are defined such that for the homodimerization ($A_2 \leftrightarrow 2\ A$) the dissociation constant is given by Equation 9 (Scheme S2). This process thus equals that described by the third and fourth terms of Equation 3. The rate of formation of A via dissociation of A_2^{red} (fourth term in Equation 3) is twice the rate of disappearance of A_2^{red} via dissociation, because for each molecule of A_2^{red} that dissociates two molecules of A are formed. Hence, in Equation 5, a factor of two appears in the first term. Similarly, the rate of disappearance of A via formation of A_2^{red} is twice the rate of formation of A_2^{red} (second term in Equation 5). Disappearance of A via formation of the heterodimer AB^{red}, described by the third term in Equation 5, also proceeds with a rate of $2k_a$, since both encounters between A with B and B with A can result in dimer formation (i.e., the total amount of encounters between any A and any B is larger than between A and A). Finally, the rate of formation of A via dissociation of a heterodimer AB^{red} is equal to the rate of disappearance of AB^{red}, since for each molecule of AB^{red} only one molecule of A is formed. Therefore, the first term in Equation 7 equals the third term in Equation 5. Equations involving B are derived analogously.



$$\frac{d[A_2^{ox}]}{dt} = -k_{red}[A_2^{ox}] + k_{ox}[A_2^{red}] \quad (1)$$

$$\frac{d[B_2^{ox}]}{dt} = -k_{red}[B_2^{ox}] + k_{ox}[B_2^{red}] \quad (2)$$

$$\frac{d[A_2^{red}]}{dt} = k_{red}[A_2^{ox}] - k_{ox}[A_2^{red}] - k_d[A_2^{red}] + k_a[A]^2 \quad (3)$$

$$\frac{d[B_2^{red}]}{dt} = k_{red}[B_2^{ox}] - k_{ox}[B_2^{red}] - k_d[B_2^{red}] + k_a[B]^2 \quad (4)$$

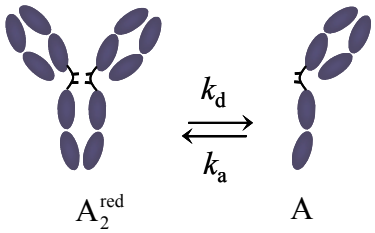
$$\frac{d[A]}{dt} = 2k_d[A_2^{red}] - 2k_a[A]^2 - 2k_a[A][B] + k_d[AB^{red}] \quad (5)$$

$$\frac{d[B]}{dt} = 2k_d[B_2^{red}] - 2k_a[B]^2 - 2k_a[A][B] + k_d[AB^{red}] \quad (6)$$

$$\frac{d[AB^{red}]}{dt} = 2k_a[A][B] - k_d[AB^{red}] - k_{ox}[AB^{red}] + k_{red}[AB^{ox}] \quad (7)$$

$$\frac{d[AB^{ox}]}{dt} = k_{ox}[AB^{red}] - k_{red}[AB^{ox}] \quad (8)$$

Scheme S1.



$$K_d = \frac{k_d}{k_a} \qquad (9)$$

Scheme S2.

$$\frac{d[A_2]}{dt} = -k_d[A_2] + k_a[A]^2 \qquad (10)$$

$$\frac{d[B_2]}{dt} = -k_d[B_2] + k_a[B]^2 \qquad (11)$$

$$\frac{d[A]}{dt} = 2k_d[A_2] - 2k_a[A]^2 - 2k_a[A][B] + k_d[AB] \qquad (12)$$

$$\frac{d[B]}{dt} = 2k_d[B_2] - 2k_a[B]^2 - 2k_a[A][B] + k_d[AB] \qquad (13)$$

$$\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB] \qquad (14)$$

Scheme S3.

Numerical integration

Three cases were considered: A) hingeless IgG4 Fc fragments, B) IgG4/DTT, and C) IgG4 or IgG4 Fc/GSH.

A. In case no covalent dimer can be formed, such as for the hingeless Fc fragments, species A_2^{ox}, B_2^{ox}, and AB^{ox} do not exist and Equations 1, 2, and 8 no longer apply. In Equations 3 and 4, the first two terms cancel, as do the last two terms in Equation 7. This leads to the simplified series of equations as shown in Scheme S3. This scheme was used to calculate the kinetics profile for the exchange reaction of the hingeless IgG4 Fc fragments. Integrations were carried out using a

fourth-order Runge-Kutta algoritm using a step-size of 0.6 ms. The dissociation constant was taken to be $k_d = 1.2\times10^{-3}$ s^{-1}, and the association constant to be $k_a = 0.6\times10^{6}$ s^{-1} M^{-1} (based on the dissociation constant, Table S1). Initial equilibrium concentrations of A_2 and A were calculated (A_2 being in equilibrium with A) using a concentration of ($A_2 + 0.5$ A) equal to 13 nM, and for B, equimolar amounts were used. The resulting kinetics of formation of AB are shown in Figure 6A. The observed kinetics, i.e., the FRET signal at 620 nm, is expected to correlate with the appearance of AB.

B. In case of the exchange reaction of IgG4 reduced with 0.3 mM DTT, an average rate constant for the reduction was estimated using SDS-PAGE and FRET (Figure S4, Table S2) to be $k_{red} = 1.5\times10^{-3}$ s^{-1}. The reduction was found to be essentially irreversible and complete. Therefore, we arbitrarily assigned a value of $k_{ox} = 1.5\times10^{-5}$ s^{-1} to the reverse process. As explained above (Derivation of Kinetic Model), we assumed IgG4 to exist either in oxidized or reduced form (i.e., with or without disulfide bonds between the heavy chains). Furthermore, we assume that no intra-chain isomers are formed and that all dissociated half-molecules A or B exist with a fully reduced hinge. Therefore, the dissociation rate constant was chosen to be $k_d = 2.0\times10^{-3}$ s^{-1}, equal to the observed rate of exchange for reduced IgG4. Using the dissociation constant as reported in Table S1, the association constant was taken to equal $k_a = 0.5\times10^{6}$ s^{-1} M^{-1}. First, a simulation was performed for 12000 seconds using initial concentrations of A_2^{ox} and B_2^{ox} of 13 nM. This run appears as the kinetics profile 'without pre-incubation' in Figure 6B. From this simulation, equilibrium values for A_2^{ox}, B_2^{ox}, A_2^{red}, B_2^{red}, A, B, AB^{red}, and AB^{ox} were obtained. A second simulation was performed, mimicking the situation where A and B are incubated with DTT separately and then mixed. For this simulation, initial concentrations of A_2^{ox}, B_2^{ox}, A_2^{red}, and B_2^{red} were taken to be twice the equilibrium value, and concentrations of AB^{red} and AB^{ox} to be zero. The observed kinetics is expected to correlate with $AB^{red} + AB^{ox} \approx AB^{red}$. Similar calculations were carried out in case of 0.1 mM DTT, taking an average rate constant of reduction of $k_{red} = 0.55\times10^{-3}$ s^{-1} and again a hundred-fold lower oxidation rate constant, and the results are also shown in Figure 6B.

C. In case of the exchange reactions of either IgG4 or IgG4 Fc in the presence of 1 mM of GSH, we proceeded essentially as for the case of DTT. Reduction was found to be far from complete Figure S4D). Instead, we estimated both the rate of formation as well as the equilibrium

concentration of isomers without disulfide bonds between the heavy chains by incubation of fluorescently labeled IgG4 (natalizumab) or IgG4 Fc with 1 mM of GSH, drawing samples at different time points that were quenched with iodoacetamide and analyzed for the percentage of material that participates in the exchange reaction using the FRET assay (Figure S4, Table 2, Table S2). Isomers without disulfide bonds between the heavy chains will include the intra-chain isomer, but in theory can also be reduced IgG4 or any intermediate with one or more molecules of GSH associated with the hinge cysteines. Thus, one can no longer speak of rate of reduction or oxidation, and instead we refer to these processes as rate of formation of non-covalent isomers (k_{nc} instead of k_{red}) and of covalent isomers (k_c instead of k_{ox}). For IgG4 Fc, both reduced and intra-chain isomers were found to dissociate at equal rates, and therefore, this situation may still be described by Equations 1–8 (i.e., whether A and B represent mainly intra-chain isomers or reduced isomers or a mixture seems not to be relevant since all isomers dissociate and recombine at similar rates). Reduced IgG4 was found to dissociate almost twice as fast as the intra-chain isomer. Thus, for the latter, a more complex situation may exist. However, given the limited reduction power of GSH, most of the non-covalent isomers most likely represent the intra-chain isomer, corresponding to A and B in Scheme S1. Thus, two simulations were carried out: for IgG4, using values of k_{nc} (= k_{red}) = 0.57×10^{-3} s^{-1}, k_c (= k_{ox}) = 5.1×10^{-3} s^{-1}, k_d = 1.1×10^{-3} s^{-1}, and k_a = 0.5×10^{6} s^{-1} M^{-1} (Figure S5); and for IgG4 Fc, k_{nc} (= k_{red}) = 2.9×10^{-3} s^{-1}, k_c (= k_{ox}) = 7.6×10^{-3} s^{-1}, k_d = 1.2×10^{-3} s^{-1} and k_a = 0.5×10^{6} s^{-1} M^{-1}. These simulations correspond fairly closely to experiment. A simulation for IgG4 using a value of k_d = 2×10^{-3} s^{-1} resembled the experiment less, suggesting that any non-covalent isomer dissociates with a rate close to that observed for the intra-chain isomer rather than the reduced isomer. Observed kinetics is expected to correlate with $AB^{nc} + AB^{c}$.

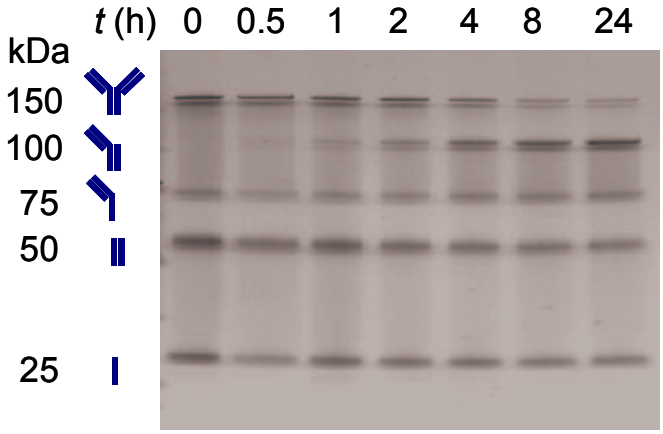


Figure S1. Non-reducing SDS-PAGE shows formation of the hybrid 100 kD product, implying that interchain disulfide bonds re-form. Bands were made visible with silver staining.

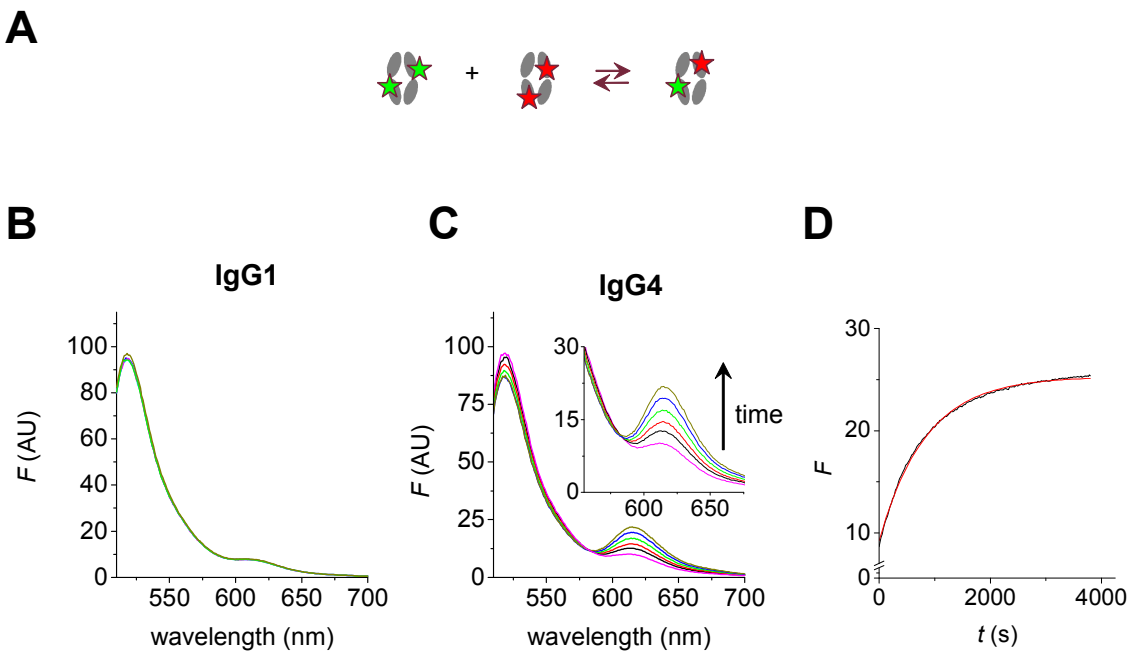


Figure S2. A) Exchange process for hingeless IgG4 Fc fragments fluorescently labeled with either Dy488 or Dy594. B) For hingeless IgG1 Fc fragments (labeled with Dy488/Dy594), no change in fluorescence spectrum is observed. C) Mixing both types of fluorescently labeled hingeless IgG4 Fc fragments on the other hand results in time-dependent increase of the FRET signal (excitation at 488 nm). D) This increase monitored at 620 nm resulting in first-order kinetics.

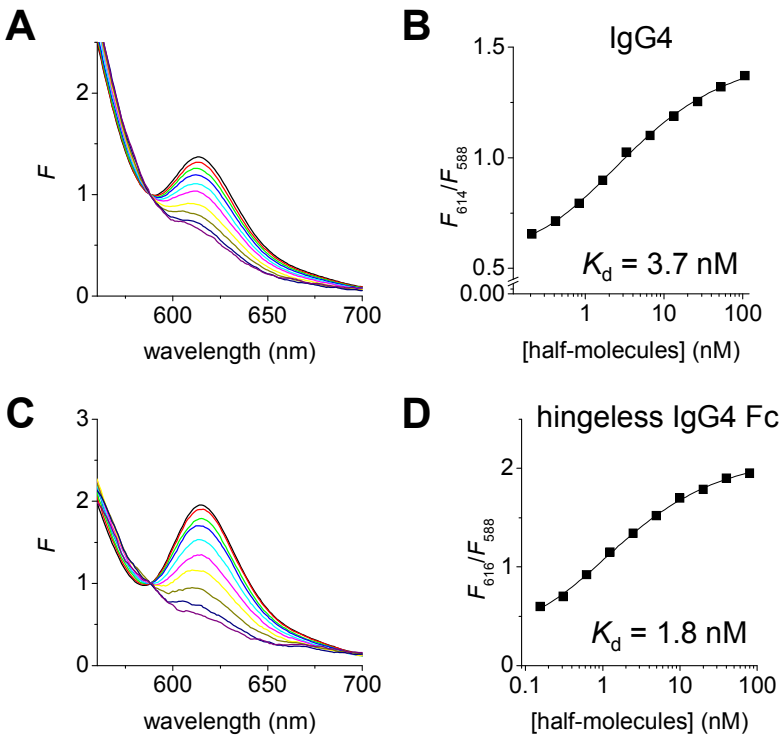


Figure S3. Determination of dissociation constants of IgG4 or hingeless IgG4 Fc fragments into half-molecules. Fluorescence spectra were recorded for two-fold dilutions of equimolar amounts of Dy488 or Dy594-labeled IgG4 (A,B) or hingeless IgG4 Fc (C,D) after equilibrating for 2 hours at 37 ^{o}C. Spectra were normalized on the isosbestic point (588 nm as determined in kinetics experiments) (A,C). Normalized FRET maxima were plotted against concentration of half-molecules and an dissociation constant calculated by fitting a homo-dimerization model to the data.

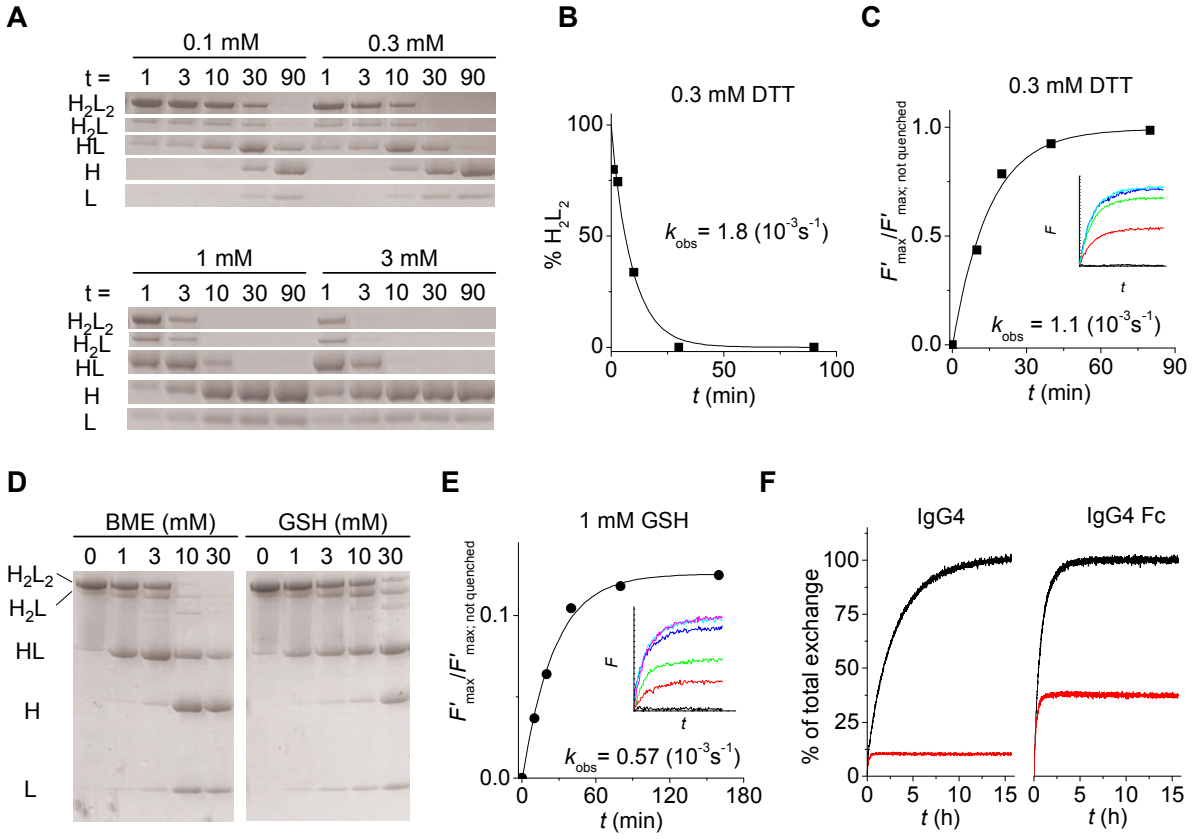


Figure S4. Kinetics of reduction of IgG4. A) Tysabri was reduced with different concentrations of DTT at 37 °C, samples were drawn at 1, 3, 10, 30, and 90 minutes, quenched with iodoacetamide, and analyzed with non-reducing SDS-PAGE/Coomassie staining. Rate of reduction decreases with decreasing concentration of DTT, but even at 0.1 mM of DTT complete reduction of the hinge disulfide bonds is observed after 90 minutes. B) Intensities of bands representing H_2L_2 (obtained using ImageJ) were plotted against time for the reduction of IgG4 at 0.3 mM of DTT and a pseudo-first order rate constant was calculated (Table S2). C) Samples of Tysabri were incubated with 0.3 mM DTT for different times and quenched with iodoacetamide. After mixing, the exchange reaction was monitored using FRET (inset) and end-point values were plotted against time, and a pseudo-first-order rate constant was calculated. $F'_{max} = F_{max} - F_0$. D) Tysabri was incubated with different concentrations of BME or GSH for 24 hours at 37 °C, quenched with iodoacetamide, and analyzed using non-reduced SDS-PAGE. E) Samples of Tysabri were incubated with 1 mM GSH for different times and quenched with iodoacetamide. After mixing, the exchange reaction was monitored using FRET (inset) and end-point values were plotted against time, and a pseudo-first-order rate constant was calculated. F) Fractions of non-covalent isoforms of IgG4 (natalizumab) and IgG4 Fc were determined after incubation with 1 mM of GSH (37 °C; 120 minutes). Samples were either quenched with iodoacetamide (red curves) or directly used for analysis using FRET (black curves). Concentrations of IgG4 and IgG4 Fc were 90 and 40 nM, respectively. The percentage of total exchange was calculated as $100 \times (F_t - F_0)/(F_{max,GSH} - F_{0,GSH})$, with F_0 fluorescence at t=0, F_t the fluorescence at time t, and $F_{max,GSH}$ the end-point value reached by monitoring the reaction in the presence of 1 mM GSH.

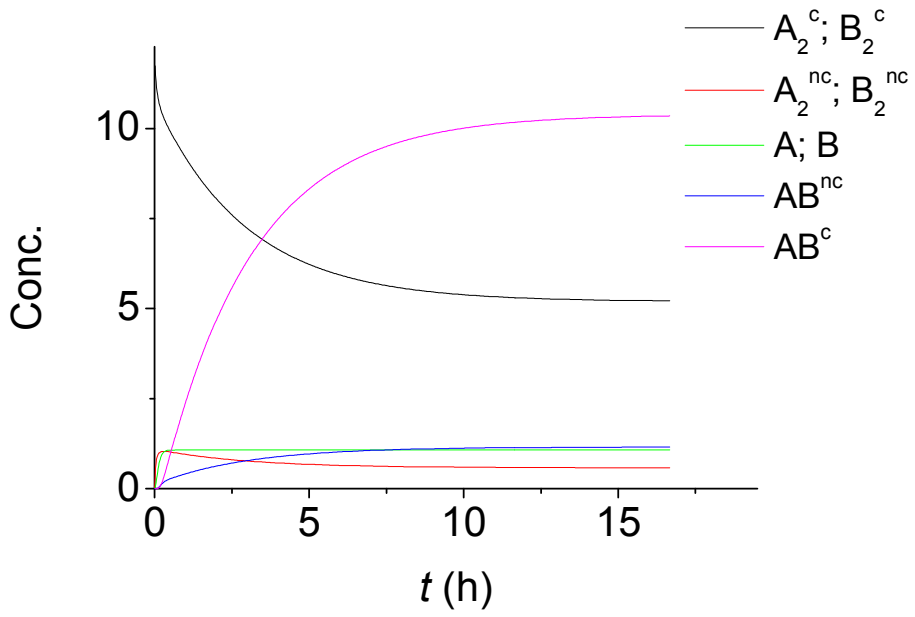


Figure S5. Results from a simulation of the exchange process of IgG4 (natalizumab) with 1 mM GSH.

Table S1. Dissociation constants for IgG4 or fragments thereof dissociating into half-molecules at 37 °C.

	K_d (nM)
IgG4	3.8 (0.4) [a]
IgG4 Fc	2.7 (0.3)
Hingeless IgG4 Fc	1.9 (0.3)

a) Standard error between parentheses

Table S2. Observed rate constants (10^{-3} s^{-1}) for the dissappearance of IgG4 isomers with inter-heavy chain disulfide bonds (in presence of DTT), or formation of non-covalently bound isomers (in presence of DTT or GSH) at 37 °C.

	DTT		**GSH**
	0.1 mM	**0.3 mM**	**1 mM**
IgG4 natalizumab	0.7 [a]/0.4 [b]	1.8 [a]/1.1 [b]	0.57 [b]
IgG4 Fc	n.d.	n.d.	2.9 [b]

a) Value obtained using SDS-PAGE as explained in legend to Figure S4 A and B.

b) Value obtained using FRET as explained in legend to Figure S4 B and D.

Reference List

1. Gilbert, H. F. (1995) *Methods Enzymol.* **251,** 8-28