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Favourable interaction between heavy and light chains arrests the undesirable oligomerization of heavy chains in the refolding of denatured and reduced immunoglobulin G

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Abstract. Recently we developed a slow dialysis method that effectively refolds denatured and reduced immunoglobulin G (IgG) [Maeda, Ueda and Imoto (1996) Prot. Engng **9:** 95–100]. This method allows both individual and simultaneous refolding of denatured and reduced H and L chains. Analysis by SDS-polyacrylamide gel electrophoresis revealed that some oligomers were formed through disulfide bonds when H chains were refolded individually. It was also shown that the extent of IgG obtained by rejoining the mixture of refolded H and L chains which had been refolded individually was similar to that obtained by refolding denatured and reduced whole IgG. The results indicated that a favourable interaction between H and L chains prevented formation of H-chain oligomers to yield intact IgG. The present results suggest a mechanism whereby individually folded chains might associate to form IgG molecules in vivo.

Key words. Aggregation; H chain; immunoglobulin G; L chain; protein folding; slow dialysis.

The mechanism of protein folding is a theme of continuing interest in modern biology. The refolding processes of monomeric proteins such as BPTI [1], single antibody domain [2] and hirudin [3] from reduced form in vitro have been extensively explored. On the other hand, our understanding of refolding of oligomeric proteins from their reduced form in vitro has progressed very little. Fab is an oligomeric protein whose refolding process has been investigated in detail [4–9]. Lilie et al. have carried out the refolding of reduced Fab [from κ/IgG1 (mouse)] at a concentration of 5 μM using a rapid dilution method under conditions that yielded ~60% refolding [7], whereas denatured and reduced [Ig[Gκ/IgG1 (mouse)] was hardly renatured under similar condition (about 5%) [10]. Although there may be subtle differences in conditions between these experiments, the dramatic difference in the refolding yield between Fab and whole IgG may depend largely on the structure of the protein: that is, Fab does not have a Fc region. Because antibodies are beginning to be used in biomedical treatment, and since the Fc region is involved in functions such as activation of the complement system and binding to receptors on cells, whole IgG may have an advantage to use for biomedical treatment. Recently we demonstrated via a slow dialysis that denatured and reduced whole IgG can be renatured intact in high yield (70–80%) in vitro [10]. This method makes it possible to analyse the refolding mechanism of denatured and reduced whole IgG. In this paper, we examine the role of H and L chains on refolding of monoclonal IgG by comparing the refolding of denatured and reduced whole IgG with that of its respective H and L chains.

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Materials and methods

Materials. Oxidized glutathione, cystamine dihydrochloride and 2-mercaptoethanol were purchased from Nacalai Tesque (Kyoto, Japan). BrCN-activated Sepharose 4B and Sephacryl S-200 were purchased from Pharmacia. A column of TSK-GEL G3000SW (7.8 × 600 mm) was purchased from Tosoh Co. Ltd. (Tokyo). P-Goat Anti-Mouse IgG + A + M (H + L) was purchased from Zymed (USA). *p*-Nitrophenylphosphate, disodium salt (PNPP), was obtained from Wako Pure Chemical (Osaka, Japan). Guanidium hydrochloride (Gdn-HCl) was purchased from Kanto Chemical (Tokyo). All other chemicals used were of the highest quality commercially available.

Preparation of the monoclonal antibody (mAb). Hybridoma cells were developed by fusing murine myeloma (P3U1) and splenocytes obtained from mice immunized with hen lysozyme according to the method of Kohler and Milstein [11]. Positive clones were selected by enzyme-linked immunosorbent assay (ELISA) using the purified hen lysozyme as a probe. One positive clone was propagated, and the antibody was produced as ascites in mice. This antibody, designated mAb LKS103 (κ/IgG1), was purified by affinity chromatography were lysozyme is linked to BrCN-activated Sepharose 4B as described previously [12].

Refolding of denatured and reduced whole IgG, H and L chains and renaturation of the mixutre of refolded H and L chains. Three milligrams of H chain and 1.8 mg of L chain were dissolved in 1.5 ml of 8 M urea solution, and 500 µl of each solution was mixed. In addition, the respective H and L chain solutions were diluted twofold with 8 M urea solution. After 1 ml each of the H and L chain solutions and the mixture solution were degassed, 5 µl of 2-mercaptoethanol was added to each of these solutions. Each solution was incubated at 40 °C for 1 h under a nitrogen atmosphere (reduced solution). To each reduced solution, 16.2 mg of oxidized glutathione dissolved in 200 µl of 8 M urea solution was added (redox solution). Each protein was then refolded by the slow dialysis method using the systematic renaturation device described previously [13]. The redox solution was dialysed against 100 ml of the redox solution containing 8 M urea with stirring. The urea concentration of the dialysing bottle was diluted continuously with 400 ml of the redox solution without urea at a flow rate of 0.1 ml/min using a high-pressure pump (Hitachi 655 Liquid Chromatography) for HPLC at 4 °C. For renaturation of refolded chains, 500 µl of each refolded H chain and L chain solution without urea as mixed in the presence of 2-mercaptoethanol and oxidized glutathione and allowed to stand at 4 °C for 1 h.

Determination of the refolding yield. Determination of the refolding yield was performed by the ELISA system as previously described [10]. The refolding yield of refolded H and L chains and refolded IgG were calculated based on the standard curve obtained from the binding affinity of native lysozyme against intact IgG at various concentrations.

Fluorescence spectroscopic study of the refolded or denatured IgG. Each refolded solution was diluted 10- or 20-fold with the renaturation buffer with or without 8 M urea. Each solution was then centrifuged to remove the precipitate. Tryptophyl fluorescence of the supernatant was measured with a Hitachi F-2000 fluorescence spectrophotometer. Tryptophyl fluorescence was measured with excitation at 280 nm, and emission spectra were recorded in the 300–400 nm range.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 10% acrylamide in slab gels and staining with Coomassie Brilliant Blue R-250 as previously described [14].

Gel chromatography. Analysis of the refolded species on gel chromatography connected to HPLC was carried out using a column of TSK-GEL G3000SW (7.8 × 600 mm), equilibrated with 10% acetic acid at a flow rate of 1.0 ml/min. The refolded species were directly applied to the column, and the protein elutions were detected by monitoring at 280 nm.

Results

Separation of H and L chains. We used an anti-hen lysozyme monoclonal antibody as IgG. After reduction of disulfide bonds in whole IgG, H and L chains can be separated from each other by depressing noncovalent interactions between them using Sephacryl S-200 equilibrated with 10% acetic acid containing 6 M Gdn-HCl according to the reported procedure [15]. IgG (7 mg) was dissolved in 1 ml of 8 M urea solution [0.584 M Tris-HCl buffer at pH 8.6 containing 8.125 M urea, 5.37 mM *N*,*N*,*N*′,*N*′-ethylenediamine tetraacetic acid (EDTA)] and reduced with 20 µl of 2-mercaptoethanol at 40 °C for 1 h under nitrogen atmosphere. After reduction, 1 g of cystamine dihydrochloride was added to the reduction solution as a solid and allowed to react for 2 h at 40 °C in order to block the SH groups in the denatured and reduced IgG and prevent the reformation of cross-links between the H and L chains. The solution was applied to a column of Sephacryl S-200 (1.5 × 200 cm). It was eluted with 10% acetic acid containing 6 M Gdn-HCl (fig. 1). The H and L chain fractions were collected and dialysed against 10% acetic acid. The dialysates were then lyophilized. Using this procedure, we obtained purified H and L chains, respectively.

Refolding of H and L chains. Free thiols can easily be reproduced from modified cysteines which have been blocked with cystamine dihydrochloride. Thus, after reduction of the purified H and L chains, the denatured

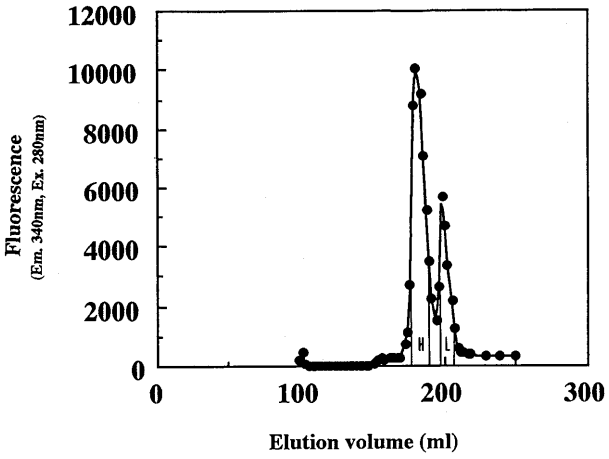

Figure 1. Gel chromatography of reduced and aklylated IgG on a column of Sephacryl S-200 (1.5 × 200 cm). The column was eluted with 10% acetic acid containing 6 M Gdn-HCl.

and reduced H and L chains at a concentration of 10 μM were renatured separately by the SH–SS interchange reaction catalysed by 2-mercaptoethanol and oxidized glutathione at pH 8.0 using slow dialysis. Tryptophyl fluorescence spectra of both refolded H and L chains had a maximum wavelength at 340 nm (fig. 2, A and B). The tryptophyl fluorescence spectra of the denatured H and L chains had a maximum at 352 nm (fig. 2, A and B). The difference in the maximum wavelengths indicated that tryptophan residues in the refolded H and L chains were shielded from the solvent. Thus the refolded H and L chains seemed to have some sort of structure. However, both refolded H and L chains had almost no binding activity to antigen (lysozyme) using enzyme-linked immunosolvent assay (ELISA) (table 1). From analysis by SDS-PAGE of the refolded H and L chains under reducing and nonreducing conditions, we found that refolded L chains formed monomers (fig. 3; lanes 3 and 6), whereas refolded H chains formed various amounts of oligomer (fig. 3; lanes 2 and 5).

When denatured and reduced H and L chains were mixed in the presence of 2-mercaptoethanol, oxidized glutathione and 8 M urea, the refolding yield of this mixture was 83% at a concentration of 10 μM by means of slow dialysis (table 1). In contrast, when refolded H chains and L chains were mixed in the presence of 2-mercaptoethanol and oxidized glutathione in the absence of denaturant, the refolding yield was 62% at a concentration of 5 μM (table 1). To confirm whether the species bound to lysozyme in ELISA was IgG, each refolded mixture was directly applied to the column of TSK-GEL G3000SW, gel chromatography, connected

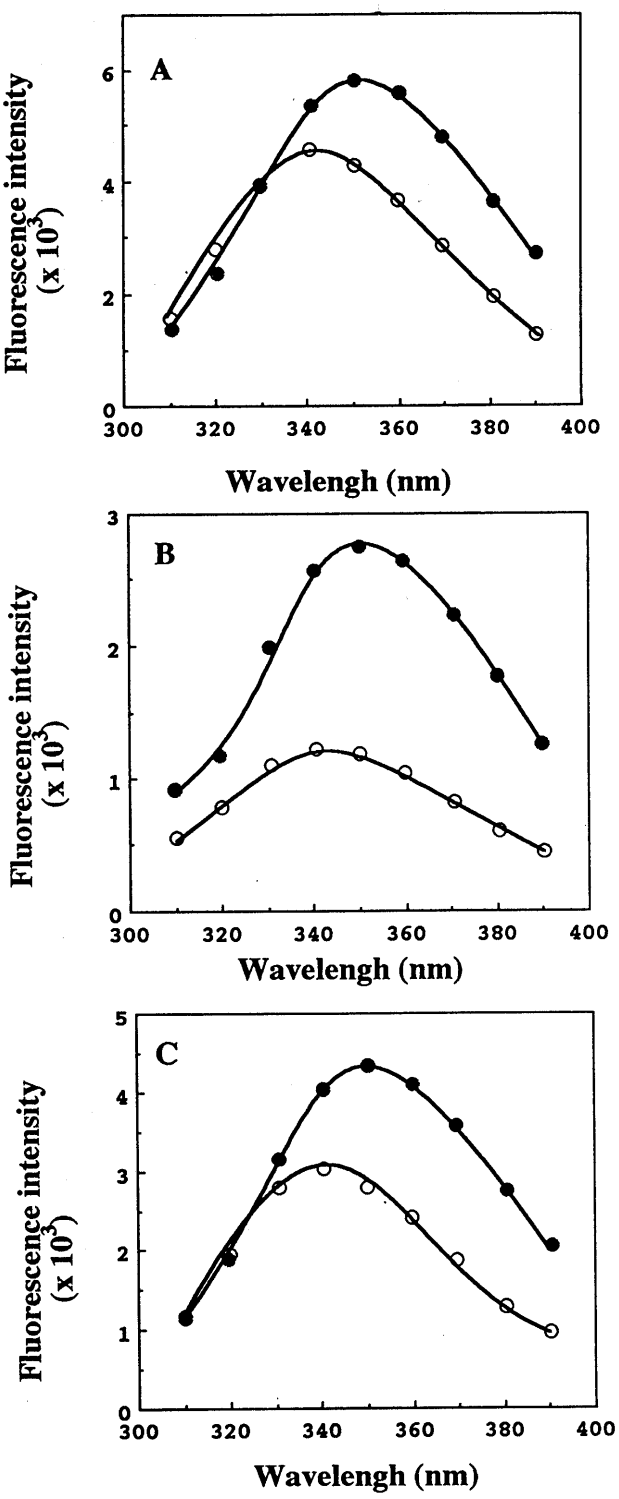

Figure 2. Fluorescence emission spectra of denatured (●) and refolded H chain (○). (*A*), L chain (*B*) and IgG (*C*). Spectra were measured at 25 °C with an excitation wavelength of 280 nm.

to HPLC (fig. 4). Figure 4B shows the pattern of refolded species obtained from the mixture of individu-

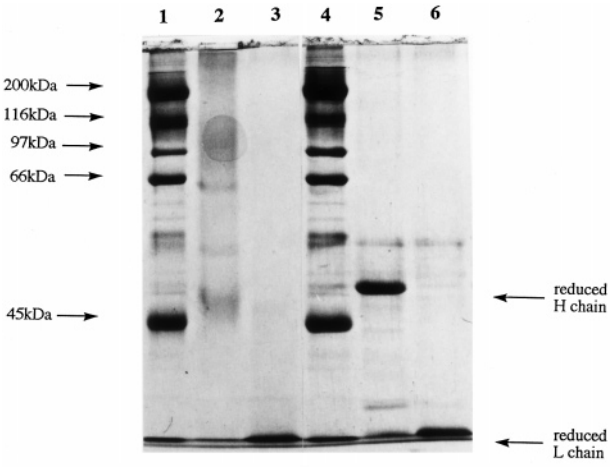

Figure 3. SDS-PAGE of refolded H chain and L chain. Lanes 1 and 4: molecular weight size marker; 2: refolded H chain on non-reduced gel; 3: refolded L chain on nonreduced gel; 5: refolded H chain on reduced gel; 6: refolded L chain on reduced gel.

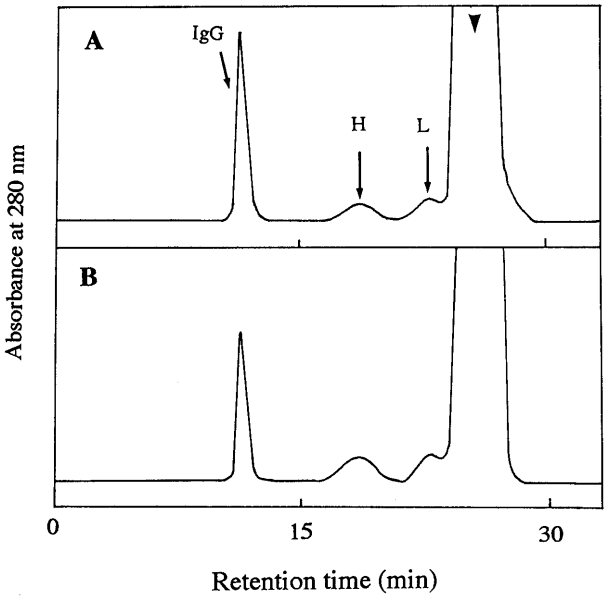

Figure 4. Gel chromatography HPLC of the refolded species of denatured and reduced H and L chains on TSK-GEL G3000SW (7.8 × 600 mm) (*A*) from the simultaneous refolding of denatured and reduced H and L chains; (*B*) from the mixture of the individual refolding of denatured and reduced H and L chains. The column was eluted with 10% acetic acid at a flow rate of 1.0 ml/min. Arrowhead indicates the peaks resulted from low molecular weight.

ally refolded H and L chains, on gel chromatography under nonreducing conditions. For comparison, the pattern of simultaneously refolded H and L chains is shown in figure 4A. Clearly, the elution time of each major fraction was identical to that of authentic IgG, whereas the size of each peak depended on the concentration of H and L chains. We previously demonstrated that the refolded species obtained by simultaneous refolding of H and L chains was active, intact IgG, by affinity chromatography, SDS-PAGE and fluorescence spectroscopy [10]. Our present results are consistent with our previous ones. The species bound to lysozyme in ELISA, obtained in two different refolding procedures, were confirmed to result from intact IgG. Figure 5 shows these refolding yields at the respective concentrations obtained plotted over the refolding yields of denatured and renatured IgG at various concentrations obtained in the previous report [10]. The result indicated that the refolding yields obtained in this study were reasonably consistent with the yield expected from their protein concentrations. That is, the extent of correctly refolded IgG obtained by mixing the refolded heavy and light chains, which had been refolded individually, was similar to that obtained by refolding denatured and reduced whole IgG.

Table 1. Refolding yield of reduced and denatured H and L chains and mixture of H and L chains by means of slow dialysis at pH 8 and 4 °C.

Condition[a]	Refolding yield[b] (%)
H chain only	0.08
L chain only	0.26
H and L chains[c]	83
H and L chains[d]	62

[a]Renaturation was carried out at the concentration of 10 µl (H chain), 10 µM (L chain), 10 µM (mixture of denatured and reduced H and L chains) and 5 µM (mixture of refolded H and L chains).
[b]The value was calculated based on the standard curve obtained by the binding affinity of native lysozyme against intact IgG at various concentrations.
[c]Refolding of denatured and reduced H and L chains in the presence of 2-mercaptoethanol, oxidized glutathione and 8 M urea.
[d]Refolding by the mixture of the refolded H and L chains in the presence of 2-mercaptoethanol and oxidized glutathione, without urea.

Discussion

The refolding yield of denatured and reduced Fab was reported to be quite high even under condition using rapid dilution [7] but that of denatured and reduced IgG was considerably lower under similar conditions [10]. As these monoclonal antibodies are derived from mouse IgG (κ/IgG1), their Fc regions may be nearly identical. Thus, the Fc region of the H chain would be

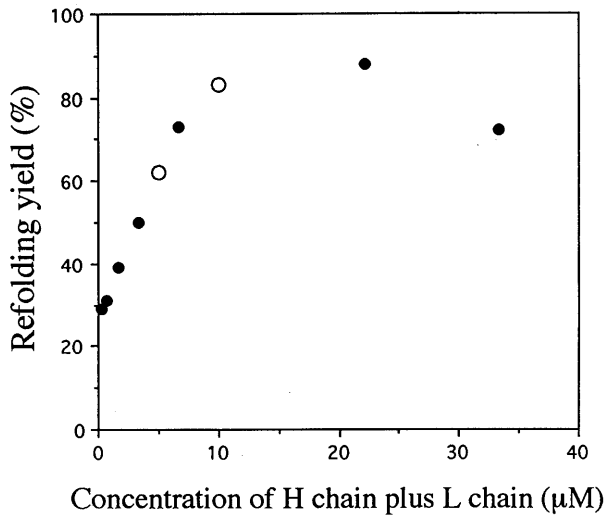

Figure 5. Concentration dependencies of refolding yield of denatured and reduced IgG. Filled circles were cited in a previous report [10]. The present data are shown by open circles.

attributable to the difference in the refolding yield between Fab and whole IgG. A previous paper [10] describes refolding of denatured and reduced whole IgG in high yield using a slow dialysis method. The present work describes the effect of the Fc region on refolding of denatured and reduced whole IgG via slow dialysis. As shown in figure 3, various oligomers formed through disulfide bonds in the refolding of reduced H chains, and H chains had a tendency to form oligomers, whereas refolded L chains gave monomeric species. However, the partial formation of oligomers of refolded L chains was observed by gel chromatography as described above (data not shown) indicated that noncovalent interaction between refolded L chains had occurred. On the other hand, tryptophyl fluorescence spectra of both refolded species showed a maximum wavelength at 340 nm (fig. 2). This result suggests that tryptophan residues in both refodled H and L chains were shielded from the solvent, and hence that the compact structure of each subunit would indicate formation of disulfide bonds in the intradomain of each subunit. In case of the IgG1 employed here, there are three intermolecular disulfide bonds between H and L chains. Thus interactions between H chains, especially between Fc regions, would take place at high concentrations of H chains, and formation of various oligomers via disulfide bond with various molecular weights might occur. However, Fab has only one intermolecular disulfide bond, and is much less likely to form nonspecific intermolecular disulfide bonds. Thus, the reported difference in the refolding yield between Fab and whole IgG may be the result of their structural differences.

As previously reported, refolding of IgG was concentration-dependent because the association of four subunits was required (fig. 5) [10]. Considering the refolding yield on the protein concentration, it was found that the extent of the intact IgG obtained by refolding from denatured and reduced whole IgG was similar to that obtained by the mixture of refolded H and L chains, which had been refolded individually. These results suggest that the favourable interaction of a monomeric species of refolded L chains with refolded H chains to form intact IgG may depress the formation of H chain oligomers and dissociate noncovalent oligomers of refolded L chains to monomeric species. That is, in the refolding of denatured and reduced whole IgG, favourable interactions between H chains and L chains would arrest undesirable interactions between H chains. It is also known that molecular chaperones depress aggregation of proteins. Because slow dialysis had little effect on aggregation of proteins, we were able to estimate the folding mechanism of IgG from its nascent form in vivo. The evidence obtained experimentally here – that the interaction between refolded H chains and L chains can form intact IgG – suggests that, once H and L chains are synthesized, interaction between denatured and reduced H and L chains on ribosome is not necessary to form intact IgG. This idea may be reasonable, insofar as molecular chapersones immediately bind to nascent protein owing to depression of aggregation. Indeed, the genes that encode H and L chains are located on different chromosomes. If interaction between denatured and reduced H and L chains were required to form intact IgG, these genes would have to be located on the same chromosome for effective production of IgG.

Aided by the techniques of genetic engineering, by a spate of efforts various types of antibody derivatives for medical use, such as single chain F_V [16], bispecific single chain F_V [17] and minibody [18] were prepared. Moreover, when we consider that since V_H [19] and L chains [20] both appear to have functional activity, H and L chains may be candidates for simple functional antibody derivatives. L chains appear to be suitable for use alone, as their oligomers are only partly formed, but H chains are not, since they promote aggregation. However, if disulfide bonds were eliminated from H chains, H chains might also be suitable for use alone.

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