Changes in quaternary structure of IgG upon reduction of the
inter-heavy-chain disulfide bond

(antibodies/complement/protein conformation/electron microscopy)

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ABSTRACT Reduction of the single disulfide bond between
heavy chains in the hinge region of rabbit IgG antibody causes
destabilization of the C4-2 region of the molecule. Our studies
indicate that reduced antibody molecules undergo a large
change in quaternary structure in the C4-2 region upon aggre-
gation with a small bivalent hapten. The conformational change
was observed both in hydrodynamic studies and by electron
microscopy. The sizes of native and reduced antibody complexes
were measured from electron micrographs. These measure-
ments show that reduction of the hinge disulfide allows the C4-2
domains of the antibody to separate under the strain induced
by complex formation. The Fab arms, which are clearly seen
in the electron micrographs of the native complexes, are ex-
tended by a portion of the Fc region to effectively become F(abc
arms in the reduced complexes. The length of the arms is
effectively increased by 23 Å. This results in a massive alteration
in the quaternary structure of the C4-2 region of the molecule,
and this may be the basis of many of the effects of mild redu-
tion on the various effector functions of the antibody molecule.
These findings also support the open structure of the C4-2 region
proposed on the basis of crystallographic analyses, and they
demonstrate how the inter-heavy-chain hinge disulfide restricts
segmental flexibility in the Fe fragment of the IgG molecule.

Immunoglobulins trigger the classical pathway of complement
fixation by activation of the first component of the serum
complement cascade, C1; the immunoglobulins interact directly
with C1 by binding to the structural component of the C1
complex, C1q. Still, the mechanism of activation is not well
understood. While both monomeric and aggregated IgG have
been shown to be capable of binding C1q (1, 2), monomeric
antibodies (with a few notable exceptions) have been found to
be very poor activators of complement (3, 4).

Formation of immune complexes greatly enhances the ability
of antibodies to fix complement. Aggregation of monomeric
IgG by treatment with antigen, heat, or crosslinking reagents
has been found to convert the antibodies to potent activators
of complement (5, 6). Other studies, however, have shown the
requirements for activation to be more complex in that some
critical elements of native antibody structure must be preserved
in the aggregate. Mild reduction of immunoglobulins has been
reported to greatly reduce the complement-fixing ability of the
antibody without affecting antigen binding (7, 8). Investigations
of the effects of mild reduction have demonstrated that the
hinge region disulfide of rabbit IgG is preferentially cleaved
under these conditions (9). The importance of the hinge region
in the mechanism of complement activation has been further
suggested by the localization of the site of C1q binding within
the C4-2 region of the immunoglobulin molecule, at or near the
hinge region (10, 11). However, cleavage of this disulfide has
been reported to have no effect on the tertiary structure of the
antibody or on its ability to bind C1q (12–16).

Crystallographic analyses of human IgG have recently shown
the C4-2 region to be atypical in its lack of contacts between the
individual C4-2 domains (17, 18). Fc fragments of immunog-
oglobulins have been shown to be capable of binding comple-
ment but, surprisingly, their activity seemed to be independent
of the integrity of the hinge disulfide (19, 20). The Fc fragment
of rabbit IgG has also been found to activate complement
while the whole antibody was inactive, suggesting that the Fab
fragments can somehow interact with C4-2 region and might
play a role in the complement activation (19).

These results seem to indicate that the complement fixation
depends upon the quaternary structure of the C4-2 region, and
that the structure of the C4-2 region is modulated in aggregates
by the presence of the Fab arms of the antibody and also by the
hinge disulfide bond between heavy chains.

We have examined the effects of reduction upon small,
well-defined antibody complexes formed between high-affinity
anti-dinitrophenyl (DNP) antibodies and a small bivalent
hapten, N,N'-bis-[2,4-dinitrophenyl]-L-lysine [(DNP)2-L-lysine].
The native and mildly reduced and alkylated antibody com-
plexes were compared with respect to quaternary conformat-
ion both by electron microscopy and by ultracentrifugation.

MATERIALS AND METHODS

Preparation of Rabbit Antibodies. Anti-DNP IgG anti-

bodies were produced in rabbits immunized initially with
dNP-bovine gamma globulin, followed by secondary immu-

izations with DNP-human serum albumin. The antibodies
were purified from the sera by affinity chromatography
on trinitrophenyl-Sepharose. The details of the isolation procedure
will be described elsewhere. The affinity of the antibodies for
dinitrophenol was determined by fluorescence quenching (21)
to be approximately 109 M−1.

Reduction and S-Alkylation of IgG. Antibodies were re-
duced with 7.5 mM 2-mercaptoethanol at pH 7.4 for 45 min
at room temperature. Under these conditions, primarily the
hinge region inter-heavy-chain disulfide bond is reduced (9).
The reduced antibody was then alkylated for 1 hr with 18 mM
iodoacetamide. Excess reducing and alkylating agents were
removed by dialysis at 4°C against 0.01 M sodium phosphate
buffer/0.15 M NaCl, pH 7.4. The extent of reduction of the
hinge disulfide was determined by ultracentrifugation at pH
2, and by sodium dodecyl sulfate/polyacrylamide gel elec-
rophoresis (22).

Ultracentrifugation. Sedimentation velocities were deter-
imined in a Beckman model E analytical ultracentrifuge
equipped with a split-beam photoelectric scanner. A Beckman
model 3801 data coupler was used to interface the centrifuge

Abbreviations: C4-2 region, a portion of the antibody molecule
comprised of the C4-2 domains of the immunoglobulin heavy chains; DNP,
dinitrophenyl.
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with a Digital Equipment Corporation PDP-11/45 computer. The computer was used for on-line data acquisition and off-line data storage and analysis. Sedimentation patterns were obtained from the computer both as absorbance \( (A) \) vs. radius \( (r) \) and derivative \( (dA/dr) \) vs. radius plots. Centrifuge runs were made at 60,000 rpm and from 21°C to 23°C in 0.01 M sodium phosphate buffer/0.15 M NaCl, pH 7.4, and corrected to 20°C for solvent density and viscosity.

**Electron Microscopy.** Samples were mounted and stained by using a procedure we term the "pleated sheet" technique. Antibody preparations at 10 \( \mu g/ml \) in 0.30 M ammonium acetate, pH 6.8, were absorbed to thin carbon films at 0°C. The samples were stained with 1% uranyl formate, and the films were then carefully pleated to trap sample and stain within multiple folds of carbon. Specimens were examined in a JEOL 100B electron microscope at a magnification of 64,000 diameters. Details of this procedure will be described elsewhere.

Length measurements were made from the electron micrographs with an electronic contour-measuring device (25). In order to make the measurements as accurate as possible, the following procedure was used. The images were first projected onto tracing paper placed on the platten of the contour-measuring device. Straight lines were then drawn through the long axis of each of the three hapten-linked Fab pairs of each trimer. The three points of intersection of the three lines determined the length of each Fab pair. The length of each Fab pair was measured automatically by moving the cursor of the contour-measuring device along each side of the tracing, with the intersection points being taken as the ends of each side.

**RESULTS**

**Reduction and Alkylation.** The extent of cleavage of the hinge disulfide was measured by ultracentrifugation at pH 2, conditions under which reduced antibodies split into heavy-light chain half molecules (9). Concentration measurements taken from the ultracentrifuge absorbance vs. radius plot indicate the cleavage of the hinge disulfide to be 85% complete. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of antibody preparation confirmed this result and showed that only small amounts of free heavy and light chains were present due to minimal cleavage of heavy-light chain disulfides.

**Electron Microscopy.** Electron micrographs were taken of the complexes formed at equivalence with the bivalent hapten \( (DNP)_2 \)-lysine and either native or reduced and alkylated antibody. In both cases, monomers, dimers, trimers, and higher polymers were observed. As is predicted from thermodynamic considerations, circular ring structures composed the vast majority of the complexes. A gallery representative of the complexes seen in Fig. 1. Little structural difference can be seen between the native and reduced monomers. However, dimers, trimers, and higher polymers undergo a dramatic increase in size upon reduction of the hinge disulfide. This large change in quaternary structure corresponds to an increase in the effective length of the arms. One hundred ninety-eight measurements were made as described above on the individual hapten-linked Fab pairs of 66 native and 66 reduced trimers. Trimers were chosen for measurement because their triangular shape ensures that the angle of intersection between adjacent Fab arm pairs is relatively constant, and thus introduces little ambiguity in determining the ends of each Fab pair. As is shown in Fig. 2, the reduced trimers clearly exhibit an increase in size. Table 1 summarizes the results obtained in two completely

<table>
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<th>Type of antibody complex</th>
<th>Number of measurements</th>
<th>Length of Fab pairs, Å</th>
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<tr>
<td>Native</td>
<td>78</td>
<td>198 ± 4</td>
</tr>
<tr>
<td>Native</td>
<td>120</td>
<td>193 ± 4</td>
</tr>
<tr>
<td>Reduced</td>
<td>78</td>
<td>241 ± 7</td>
</tr>
<tr>
<td>Reduced</td>
<td>120</td>
<td>241 ± 6</td>
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* Expressed as mean with 95% confidence limits of the sample mean.
independent experiments using different preparations of native and of reduced antibody. Differences between the two sets of data are well within experimental error. The results of both experiments are summarized as a frequency distribution in Fig. 3. The mean increase in the length of an arm is 23 Å, and this difference has high statistical significance (P < 0.001).

In general, the reduced complexes show more flexibility than their native counterparts. A comparison of the monomers shown in Fig. 1 suggests that, after reduction, the Fab arms are free to open to somewhat greater angles relative to each other. Occasionally reduced monomers are seen to open completely, indicating the forces holding the individual C\(\alpha_2\) domains together are relatively weak in the absence of an intact hinge disulfide. Higher complexes show a much greater increase in flexibility. The reduced complexes lose much of the geometrical precision characteristic of the native complexes. This observation is consistent with the introduction of a second hinge at the C\(\alpha_2\)-C\(\beta_3\) region interface.

**Ultracentrifugation.** The results of the electron microscopy were confirmed by ultracentrifugation. The derivative sedimentation patterns of native and reduced antibody monomers are shown in Fig. 4 a and b. The \(s_{20w}\) for the native monomer was found to be 6.54 S and for the reduced and alkylated monomer to be 6.60 S. These values are within experimental error and are not consistent with a large conformational change in the reduced monomer. This result confirms previous observations in this laboratory and also the findings of Olins and Edelman (13) for human IgG. A change in sedimentation behavior was seen, however, when native and reduced antibodies were centrifuged in the presence of one equivalent of (DNP)\(\gamma\)-lysine. The derivative sedimentation patterns are shown in Fig. 4 c and d.

The sedimentation pattern of the native antibody complexes shows partially resolved peaks of monomer, dimer, trimer, tetramer, and higher polymers. The reduced complexes are less well resolved, and the distribution of complexes is shifted to lower sedimentation rates. Because the amount of monomer contained in both preparations is comparable, reduction appears to have caused no destruction of binding sites or large reduction in binding affinity. However, the decrease in sedimentation rates for the reduced complexes may not be due totally to an increase in their frictional ratios. An examination of the distributions of complexes seen in large fields in the electron microscope indicates that while the relative numbers of the various complexes are roughly comparable in both preparations, the distribution of reduced complexes is shifted somewhat towards dimer at the expense of higher complexes. Such a shift can theoretically be attributed to a decrease in the strain associated

**FIG. 2.** Trimeric complexes of native and reduced antibody. (\(\times 320,000\)) Native trimers (a and b) and reduced trimers (c and d).

**FIG. 3.** Frequency plotted as a function of the measured length of hapten-linked Fab pairs in trimeric antibody complexes. (Upper) Measurements from native trimers; (Lower) measurements from reduced trimers.
with closure of the smaller ring structures upon reduction of the hinge disulfide (24). Experimental estimates of the strain associated with ring closure have been found to be significant for tetrameric and smaller size complexes (unpublished data). Thus, both of these factors may be responsible for the apparent decrease in the sedimentation rates of the reduced complexes.

**DISCUSSION**

Reduction of the interheavy-chain hinge disulfide of the IgG molecule interferes with several of the effector functions of the antibody. The complement-fixing ability of the antibody is destroyed (7, 8), incomplete antibodies are converted to direct hemagglutinins (25), and the binding of whole antibody to membrane-bound Fc receptors is abolished by mild reduction (26, 27). However, studies of the structure of monomeric antibodies have provided little understanding of the effects of cleavage of this disulfide bond. Hydrodynamic studies have been unable to detect a large conformational change upon mild reduction (3, 13), and our present study confirms this result. Other physicochemical and immunological studies have yielded no evidence for a change in the tertiary structure of the reduced immunoglobulin (12-15). The binding of monomeric IgG to C1q is unaffected by cleavage of the hinge disulfide (16), suggesting that no change in quaternary structure at the binding site accompanies mild reduction. However, fluorescence depolarization measurements have indicated that the hinge disulfide plays a major role in restricting flexibility within the antibody molecule (28).

The structure of rabbit IgG is interesting in that it exhibits marked flexibility of a segmental nature. Yguerabide et al. (29) have shown that the Fab can move relative to the Fc with an angular range of about 33°. This type of flexibility is due to the separation of the immunoglobulin substructure into discrete domains of interaction. Flexibility in the hinge region allows the relatively compact Fab regions to move relative to one another. Our electron micrographs and those of Valentine and Green (30) show that the angle between Fab arms can vary from nearly 0° to 180°.

The structure of the C3H2 region appears to be unusual in the lack of contacts between individual C3H2 domains. Crystallographic studies of human Fc and IgG have indicated that there are no direct contacts between the C3H2 domains and that they are separated by the carbohydrate chains (17, 18). The tope-exchange studies of Venyaminov et al. (31) have also suggested that the C3H2 region is a relatively open structure and that it undergoes a low-energy destabilization upon reduction of the hinge disulfide. The work of Ellerson et al. (32) further shows there to be little interaction between isolated C3H2 domains in solution. The solvent denaturation studies of Isemann et al. (20) and the fluorescence depolarization measurements of Chan and Cathou (28) have also suggested that increased flexibility in the Fc portion of the molecule occurs as a result of mild reduction. Vuk-Pavlovic and coworkers (33) have recently reported the induction of changes in the nuclear magnetic resonance and circularly polarized fluorescence spectra of rabbit IgG, which had been specifically modified by the insertion of a mercuric ion into the interheavy-chain disulfide bond, upon the binding of a bivalent hapten by the antibodies.

The large change in quaternary structure seen in the electron microscope and in the ultracentrifuge confirms the open flexible structure of the C3H2 region proposed by Huber and coworkers (17) and demonstrates the lack of strong interactions between the C3H2 domains. When reduced antibodies form complexes, the effective length of the Fab increases due to formation of a new hinge near the C3H2-C3H3 switch region. The change in quaternary structure is illustrated in Fig. 5. The 23 Å increase in length corresponds well with the distance between the hinge disulfide and the switch region in the structures proposed on the basis of crystallographic studies, and this distance is less than the entire length of the C3H2 domains, which is about 35 Å. The ultracentrifugation studies suggest that this conformational change does not occur in the monomer and

![Diagram of the Fab arm structure](image-url)

**FIG. 5.** Diagramatic representation of the conformational transition that trimeric antibody complexes undergo upon reduction of the hinge region interheavy-chain disulfide bond. The Fab portions of adjacent antibody monomers are linked by bivalent haptens binding in the antigen combining sites located at the tips of the Fabs. The Fabs are connected by a flexible hinge to the C3H2 and C3H3 domains, which comprise the Fc portion of the molecule. The two C3H2 domains of each antibody are shown as unconnected to illustrate their lack of interaction. Upon cleavage of the interheavy-chain disulfide, the C3H2 domains are free to separate and a second hinge is then formed at the flexible region between the C3H2 and C3H3 domains. The C3H3 domains are tightly associated by noncovalent interactions. The Fab arms effectively become Fab arms, increasing their length and the apparent size of the antibody complex.
apparently confirm a change in conformation for the higher complexes.

That the Fab increases in length by only 23 Å argues that there has been no large change in tertiary structure; there is no evidence for unfolding of the hinge region or switch region peptides. The quaternary structure appears to be stable in the absence of the hinge disulfide, unless the antibody is complexed with other large molecules. It is clear that, when complexes are formed between IgG molecules, the Fab modulates the structure of the Cg2 region.

Transmission of the strain of aggregation through the Fabs to the hinge region appears to cause the molecule to exhibit a segmental type of flexibility within the Fc portion of the molecule. In the absence of an intact disulfide bond, the forces involved are sufficient to overcome the weak Cg2 domain interactions that keep the structure intact.

The massive structural alteration of the Cg2 region that we see upon reduction may be the basis of the loss of complement-fixing ability by the reduced IgG molecule. The conformational change induced by aggregation of the reduced antibody may destroy the complement-binding sites. Investigations of the binding of C1 to aggregated native and reduced antibodies support this alternative (34). On the other hand, alteration of the Cg2 region may make impossible a critical aggregation-induced conformational change in the Fc that is required for complement activation. The aggregation-induced strain that causes the Cg2 domains to separate could provide both the energy and mechanism for such a conformational change in the Fc. With the hinge disulfide intact, such strain could be transmitted segmentally through the Cg1–Cg2 region interface and cause a transformation of the complement-binding site. The resolution of this question will require careful study of the molecular interactions between the antibody and C1q molecules.

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