Effect of cleaving interchain disulfide bridges on the radius of gyration and maximum length of anti-poly(D-alanyl) antibodies before and after reaction with tetraalanine hapten

(small-angle x-ray scattering/conformational changes/antibody dimensions)

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ABSTRACT   The small-angle x-ray scattering of solutions of rabbit IgG antibodies and their derivatives has been investigated. The reduction and alkylation of the native antibodies cause a small increase of the molecular parameters, indicating a limited expansion of the molecule. Binding of native anti-poly(D-alanyl) antibodies with hapten (80% saturation) causes a significant change of the quaternary structure, expressed by a decrease in the maximum diameter of about 2 nm, of the radius of gyration by 5.5%, and of the volume. The same antibodies, in which the single inter-heavy-chain disulfide bridge was opened by reduction and carboxamidomethylation, do not show any significant decrease in the overall molecular parameters upon reaction with hapten, except for a local structural change in a part of the molecule. These data lend further support to the notion that binding of hapten induces a conformational transition in its specific antibodies and suggest that the opening of the interchain disulfide bridges affects that transition. The dimensions of the intact antibodies calculated from measurements of small-angle x-ray scattering at low concentrations agree closely with those obtained from crystallographic studies.

We have previously shown, by measurements of small-angle x-ray scattering, that the radius of gyration and the volume of anti-poly(D-alanyl) antibodies of the IgG class decrease upon reaction with a tetra-D-alanine hapten (1). Qualitatively similar results were obtained when intact antibodies to p-azophenyl β-lactoside were treated with this hapten (2). More recently, Cser et al. (3) have reported a study by neutron small-angle scattering of antidinitrophenyl antibodies and shown that the antibody molecule contracts upon reaction with the hapten, thus corroborating our conclusion that a more compact antibody conformation may be a general feature of liganded antibodies. In contrast with the above studies, no significant changes in the radius of gyration or volume were observed when either the divalent fragment $(Fab')_2$ or the monovalent fragment Fab′, derived from anti-poly(D-alanyl) antibodies, were treated with a tetra-D-alanine hapten, even though in both cases more than 90% of the combining sites were occupied by the hapten (4). The selective cleavage of the inter-heavy-chain disulfide bridge(s) in an IgG molecule has a marked effect on its native conformation (5–8). As a result of this modification, several of the effector functions located in the Fc region of the molecule are severely impaired (9–11). These include the ability to activate complement and the binding to cellular Fc receptors. On the other hand, this interchain cleavage does not affect the site recognizing the antigen, which is located in the Fab region of the molecule.

We report now a study by small-angle x-ray scattering of rabbit anti-poly(D-alanyl) antibodies in which the interchain

disulfide bridges were opened by reduction and carboxamidomethylation. We report here also that the radius of gyration of an intact IgG molecule, remeasured at low concentrations by small-angle x-ray scattering, closely resembles that calculated from the dimensions obtained by crystallographic studies (12, 13).

MATERIALS AND METHODS

Anti-Poly(D-alanyl) Antibodies. Rabbits were immunized by the antigen poly(D-alanyl) diphtheria toxoid, and the specific anti-poly(D-alanyl) antibodies were purified on a Sepharose poly(D-alanyl) rabbit serum albumin affinity column as described (1).

Reduction and Alkylation. The antibody solution, containing about 40 mg of IgG per ml, was adjusted to 0.1 M Tris·HCl (pH 8.2). Disulfide bridges between light and heavy and between heavy and heavy chains were reduced in 10 mM dithiothreitol at room temperature for 1 hr. The resulting sulfhydryl groups were alkylated by incubation with an excess of iodoacetamide for 30 min. The protein was then dialyzed against 10 mM sodium phosphate/0.15 M NaCl, pH 7.4, to remove excess reagents. The reduction was ascertained by sodium dodecyl sulfate/polyacrylamide gel electrophoresis in the absence of reducing agents (14).

Preparation of Samples for Small-Angle X-Ray Scattering Measurements. Two separate preparations of the above-mentioned proteins were used (I and II). Each protein was divided into two portions. One of them was equilibrated by dialysis with 10 mM sodium phosphate/0.15 M NaCl, pH 7.4, and remained free of hapten. The other was equilibrated with the same buffer, but also included 1 mM (D-alanyl)$_3$-D-alanineamide (15). At this hapten concentration both the intact and the reduced and alkylated antibodies are saturated with tetra-D-alanineamide hapten to the extent of 80%.

Small-Angle X-Ray Measurements. The investigations were carried out with a highly stabilized x-ray generator (Philips PW 1140) with a copper target tube (50 kV, 30 mA) and a Kratky camera with slit collimation (16). The immunoglobulin solutions were placed in Mark capillaries at 4°C. The scattered intensities were measured at 80 different scattering angles in the range between 0.002 and 0.13 radians, with an entrance slit of 120 μm and an electronically programmable step scanning device (17). A proportional counter with an x-ray analysis channel control was used for recording. A pulse height discriminator was focused on the CuK$_\alpha$ line. Concentration series (3, 4, 5, 15, 30, and 40 mg of IgG per ml) were measured and extrapolated to zero concentration (18). Each scattering curve was recorded several times; about 10^5 pulses were counted per scattering angle. Evaluation of the scattering data was performed by computer programs of Zipper (19) and Glatter (20).

Data Evaluation. Small-angle x-ray scattering allows macromolecules to be studied in solution and has proved especially suitable for following conformational changes. Method and evaluation procedures used are described in summarizing papers (21–23).

The result of a small-angle x-ray measurement is a scattering curve in the reciprocal space. By a new method, the indirect Fourier transform of Glatter (23), the pair distance distribution function $p(r)$ in the real space can be calculated with sufficient accuracy. This function gives the frequency of the distances r within a macromolecule by combining any volume element with any other volume element and taking into consideration the difference electron density. From the $p(r)$ function, the radius of gyration R and the maximum diameter D_{max} can be calculated; $p(r)$ becomes zero at values of r equal to or greater than the maximum diameter D_{max} of the particle.

The course of the $p(r)$ functions of different concentrated protein solutions has proved to be an additional check of the homodispersity of protein solutions (18), which looked monodisperse in the analytical ultracentrifuge.

RESULTS

Two different preparations of anti-poly(D-alanyl) antibodies were studied in the following four states: (i) native free antibody, (ii) native antibody saturated with hapten, (iii) free reduced and alkylated antibody, and (iv) reduced and alkylated antibody saturated with hapten. For the evaluation, only scattering curves that seemed to originate from homodisperse solutions—especially the curves from the highly diluted solutions—were used. To check whether the reduction and alkylation on the one hand and the binding of the hapten on the other hand cause a change of the conformation of the antibody, a comparison of the pair distance distribution functions $p(r)$ and of the values of the radius of gyration R, the maximum diameter D_{max}, and the volume V are most suitable.

The reproducibility of the $p(r)$ functions for two different preparations and two different measurements on the same preparation within a period of 6 months is shown in Fig. 1 for the free antibody and for the antibody after 80% saturation with hapten. The experimental error in the values of the radius of gyration is given in Table 1 for the four different types of the antibodies. The final results are summarized in Table 2.

Reaction of Native Antibodies with Hapten. The effect of hapten binding can be immediately seen from the comparison of the pair distance distribution functions in Fig. 2. For both samples, the curve of the antibody saturated with hapten is clearly shifted to smaller distances r; that is, the binding of the hapten causes a decrease of the larger distances. This decrease can be caused by a contraction of the antibody or by any other structural change that shortens the large distances without changing the typical IgG shape. A comparison with Fig. 1 indicates that the effect (shift to shorter distances) clearly exceeds the experimental error level.

The same effect can also be followed by the decrease of the radius of gyration R of the antibody molecule by about 5.5% upon 80% saturation with hapten, as shown in Tables 1 and 2. A decrease of the maximum diameter D_{max} by about 2 nm follows immediately from the shift of the $p(r)$ functions to shorter distances (Fig. 2).

Further, the volume V decreases by about 7% (Table 2). It is necessary to point out that the volume determined by small-angle x-ray scattering is always the hydrated volume; that is, a change in the degree of hydration also changes the value of the volume. Moreover, the volume can never be determined with the same accuracy as the radius of gyration. Especially for rather dilute solutions (as used in this case to prevent aggrega-
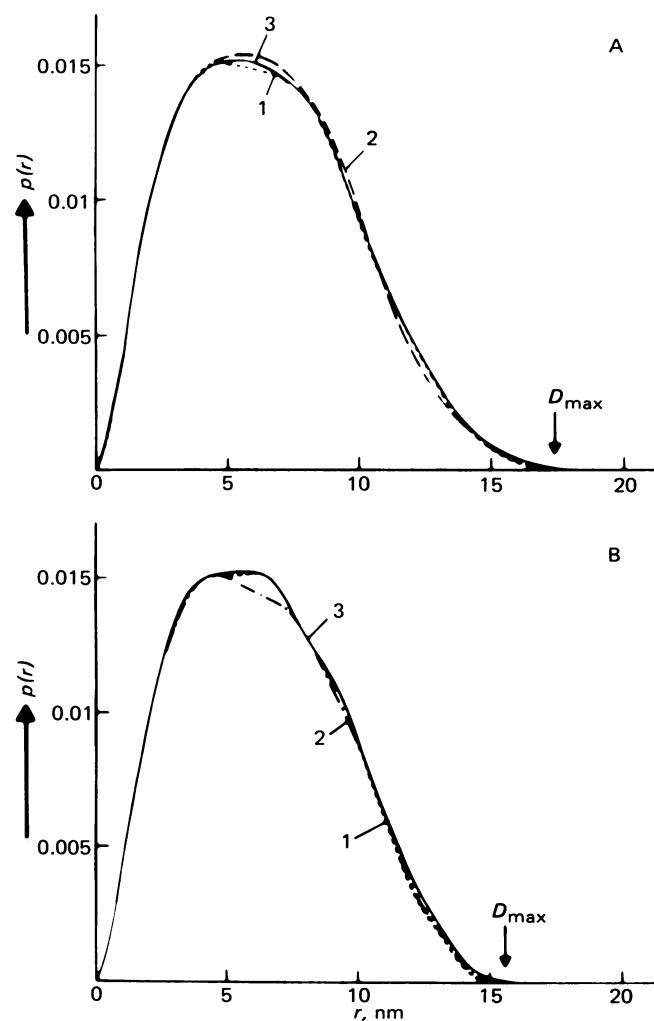

FIG. 1.   (*A*) Distance distribution functions $p(r)$ of native antibodies: antibody 1, first measurement (curve 3, ——); antibody 1, second measurement (curve 1, - - -); antibody 2 (curve 2, – – –). r, Distances between the volume elements dv in the antibody. (*B*) Distance distribution functions $p(r)$ of the same native antibodies after reaction with hapten (80% saturation).

tion), the volume values are afflicted with relatively large errors (Table 2).

Cleavage of Inter-Heavy-Chain Disulfide Bridge. The opening of the interchain disulfide bridges by reduction and carboxamidomethylation results in a small expansion of the

Table 1. Radius of gyration		
Type of antibody	Sample no.	Radius of gyration R, nm
Native	1	5.15
	2	5.17
	3	5.19
Native + hapten (80% saturation)	1	4.88
	2	4.91
	3	4.92
Reduced, alkylated	1	5.25
	2	5.30
Reduced, alkylated + hapten (80% saturation)	1	5.23
	2	5.21

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Table 2. Molecular parameters of antibodies: R = radius of gyration, R_{c1} and R_{c2} = radii of gyration of the cross section, D_{max} = maximum diameter, V = hydrated volume

Type of antibody	R, nm	R_{c1}, nm	R_{c2}, nm	D_{max}, nm	V, nm^3
Native	5.17 ± 0.02	2.63 ± 0.03	1.36 ± 0.02	17.5 ± 1.0	303 ± 15
Native + hapten	4.90 ± 0.02	2.63 ± 0.03	1.39 ± 0.02	15.5 ± 1.0	280 ± 15
Reduced, alkylated	5.27 ± 0.03	2.49 ± 0.03	1.36 ± 0.02	19.0 ± 1.0	268 ± 15
Reduced, alkylated + hapten	5.22 ± 0.03	2.01 ± 0.03	1.39 ± 0.02	18.5 ± 1.0	258 ± 15

antibody molecule. The comparison of the $p(r)$ function of the native with the reduced antibody in Fig. 3A shows a small shift to larger distances. This expansion of the antibody is accompanied with an increase of the radius of gyration by 2% and an increase of the maximum diameter D_{max} by about 1.5 nm (Table 2).

When the reduced and alkylated antibody is saturated with hapten, the typical "contraction" phenomenon that is found with native antibodies is not observed. The small decrease of the radius of gyration R, by only 1%, and the decrease of the maximum diameter D_{max}, by only 0.5 nm, are within the errors of measurement. However, the difference in the shape of the $p(r)$ function (Fig. 3B) indicates that some local change in the quaternary or tertiary structure still occurs, as discussed below.

The volume V of the reduced and alkylated antibodies seems to be lower than that of the native antibodies even though the radius of gyration and the maximum diameter values for the reduced antibody are higher. This observed decrease of the volume is not sufficiently significant; the accuracy of the values of the calculated volume is rather low because, in this case, only diluted solutions were measured. Higher concentrations could not be used because of the presence of aggregates. On the other hand, it is also possible that the increase of the dimensions of the molecule indicated by the increase of the radius of gyration

and the maximum diameter is accompanied by a decrease of the volume. The hydrated volume includes small hollow spaces with a diameter of a few angstroms between the peptide chains, domains, etc. An opening of the disulfide bridges can increase these hollow spaces, which then no longer contribute to the calculated hydrated volume, thus resulting in a decrease of the volume.

Cross Sections. The T- or slightly Y-shaped IgG molecule always shows typical two-stepped cross-section curves (1, 2), as shown in Fig. 4 (curve 1). From the two different slopes of the straight lines (Guinier plot) two different radii of gyration R_{c1} and R_{c2}, summarized in Table 2, can be calculated. In a previous report (24) we were able to show that the smaller cross section (R_{c2}, about 1.4 nm) represents mainly the cross section of the Fab arms, whereas the larger cross section (R_{c1}, about 2.6 nm) is correlated with the middle part of the IgG molecule (hinge region plus Fc fragment). Fig. 4 and Table 2 clearly show that the smaller cross sections (slope of the flat straight lines) are identical; that is, the cross section of the Fab arms is not visibly influenced by the binding of hapten (Fig. 4, curve 2) or reduction (curves 3 and 4).

On the other hand, the wider cross section, related to the middle part (R_{c1}), in the reduced and alkylated antibody is clearly decreased upon hapten binding, an effect that corresponds to the different shape of the $p(r)$ function seen in Fig. 3B for this case.
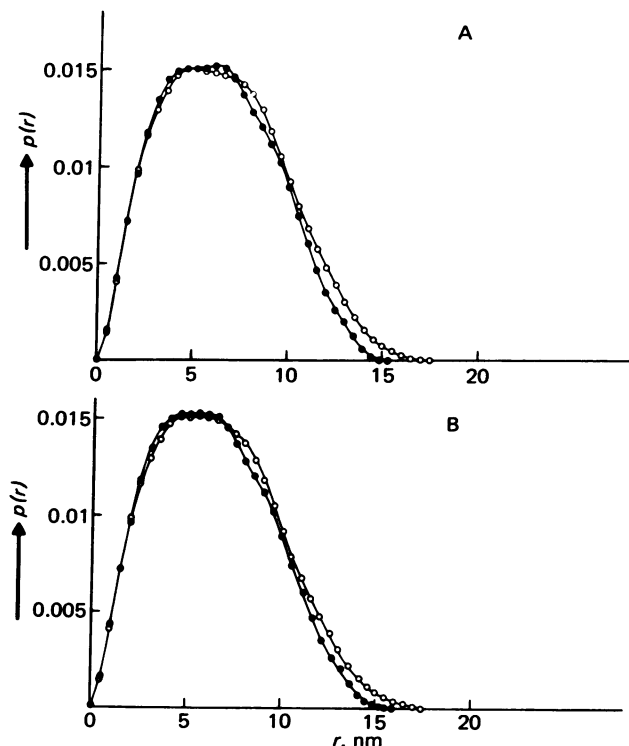

FIG. 2. Comparison of the distance distribution functions $p(r)$ of two (A and B) native antibodies (O) with the functions obtained after saturation with hapten (●).
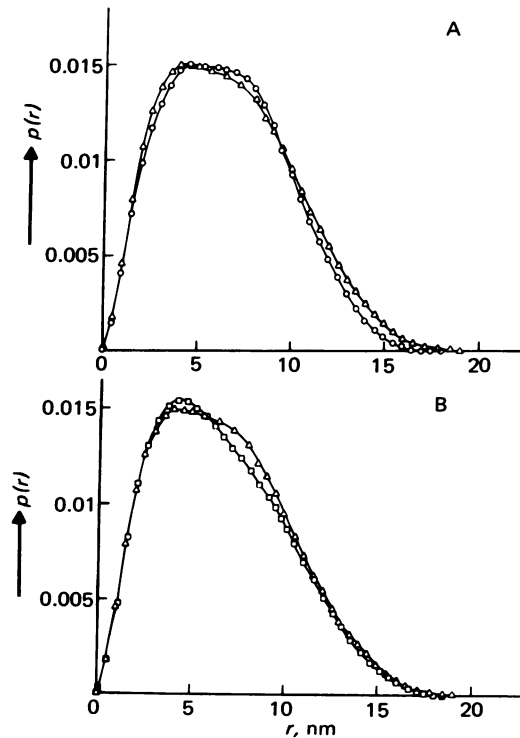

FIG. 3. Comparison of the distance distribution functions $p(r)$ of the native antibody (O) with the reduced and alkylated antibody (△) (A) and of the reduced, alkylated antibody in the free state (△) and after 80% saturation with hapten (□) (B).
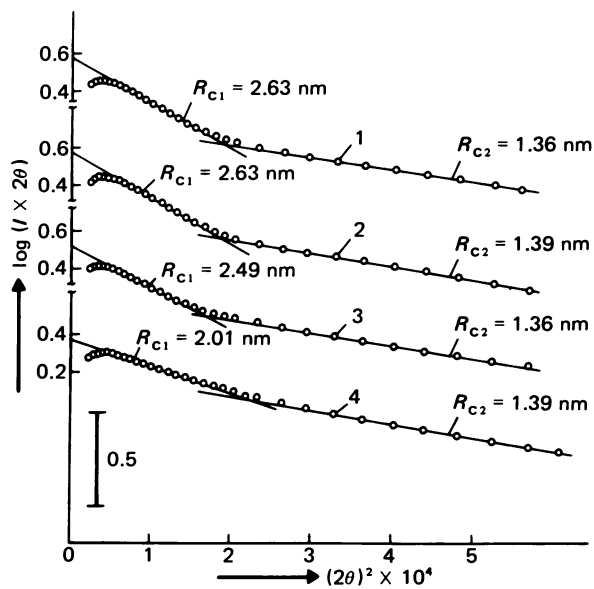
FIG. 4.   Cross-section curves in a Guinier plot of the following antibodies: curve 1, native antibody; curve 2, native antibody after reaction with hapten; curve 3, reduced, alkylated antibody; curve 4, reduced alkylated antibody after reaction with hapten. R_{c1} and R_{c2} are the radii of gyration of the cross section. I, scattered intensity; 2θ, scattering angle.

DISCUSSION

The main conclusion of this study is that the significant changes in the overall molecular parameters (radius of gyration of the whole molecule and maximum diameter) obtained upon measuring the small-angle x-ray scattering of rabbit antibody molecules, with and without its specific hapten, disappear after the only disulfide bridge between the heavy chains is cleaved. Thus, the cleavage affects the native conformation of the antibody molecule.

The scattering of the antibodies (but not of the Fab fragments) investigated earlier by small-angle x-ray measurements (1) always showed a concentration dependence unusual for homodisperse solutions, although the samples looked homodisperse in the analytical ultracentrifuge. Therefore, a remeasurement at very low concentrations with a careful check of homodispersion (18) seemed necessary. The radius of gyration of the native antibody reported here is measured at very low concentrations, and it is very close to that calculated from the dimensions obtained by crystallographic studies (12, 13). The maximum diameter of 17.5 ± 1.0 nm is very similar to the value of 16 nm obtained for the molecule in crystalline state.

The dimensions of intact anti-poly(D-alanyl) antibodies decrease upon saturation with hapten. The effect is best seen in the distance distribution functions, in the decrease of the radius of gyration and of the maximum diameter.

The cleavage of the interchain disulfide bridge by reduction causes a small increase of the distances (Fig. 3A), the radius of gyration, and the maximum diameter. The shift of the $p(r)$ function to larger distances and the increase of the radius of gyration exceed the experimental error level. The decrease of the volume is within the experimental error level, but can also be caused by a change of the hydration.

The reduced and alkylated antibody does not show, upon reaction with hapten, the significant decrease of the distances, radius of gyration, and maximum diameter observed with the native antibodies. Only a very small decrease of the radius of

gyration and maximum diameter, which is within the experimental errors, is observed.

On the other hand, it is of significant interest that the $p(r)$ functions of the reduced and alkylated antibodies show, after saturation with hapten, a clearly different course in the distance region between 5 and 11 nm (Fig. 3B), indicating that some local structural change must have taken place without changing the overall dimensions. The same effect is seen better in cross-section curve 4 of Fig. 4.

To clarify this point, for every elongated particle, a radius of gyration of the cross section can be determined, which represents the root mean square of all the distances of the electrons from the center of gravity at that point (Z-average). Particles with two different types of cross section show two-stepped cross-section curves. For the IgG molecule we know only that the smaller R_{c2} value is related to the Fab, which, when isolated, has similar values. The R_{c1} value must be caused by the wider part of the molecule, related to the hinge region together with the Fc fragment (24). Thus, saturation of the native antibody with hapten results in a clear decrease of the molecular parameters (R and D_{max}), that mainly describe the dimension of the whole molecule. The cross sections (R_{c1} and R_{c2}) are unchanged in this case. The latter does not mean that there cannot be a structural change, but that the distances of the scattering electrons from the center of gravity are unchanged within the experimental error. Saturation of the reduced antibody with hapten, on the other hand, results only in a local change of the structure in the middle part of the molecule (R_{c1}), which does not influence the overall dimensions such as radius of gyration and maximum diameter.

The crucial role played by the interchain disulfide bridge in maintaining the functional activities of the antibody prompted several investigations of its role in modulating the conformational properties of this molecule. The present study contributes to our understanding of the spatial properties of the native in contrast with the reduced and alkylated antibody molecules in solution and its correlation with functional properties and corroborates the results of several previous studies. Thus, the cleavage of this covalent bridge caused an increase in its segmental flexibility, as expressed in the fluorescence anisotropy decay rate of probes located in the antibody combining sites (6). More recently, electron micrographs of reduced and alkylated rabbit anti-dinitrophenyl complexes with its respective divalent hapten were compared with those formed with the intact antibodies (7). A large change in the quarternary structure of the reduced antibodies is induced upon reaction with the divalent hapten causing an effective increase of the antibody Fab arms by 23 Å, thus massively altering the C_H2 domain of the molecule.

Inserting a mercuric ion between the two sulfur atoms of the disulfide bridge allowed the examination of the effect of divalent hapten binding to anti-poly(D-alanyl) antibodies in solution (25). This was achieved by monitoring the exchange rate of chloride ions between the solvent and the coordination sphere of the mercuric probe in the antibody. The marked decrease found in the ^{35}Cl NMR linewidth of the complex is interpreted as a hapten-induced change in the spatial relationship at the hinge. An independent illustration of the role of the disulfide bridges between immunoglobulin domains in modulating their properties has been obtained in studies of light chain dimers. The Bence Jones protein Mcg (26) and the light chain dimer derived from the murine IgA-MOPC 315 (27) revealed pronounced structural changes depending on the state of the disulfide bridging of their COOH terminus. In the latter case the cooperative hapten-binding properties at the site located at the variable end of the dimer is markedly changed upon cleavage of the disulfide bridge.

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