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Linkage and Assembly of Polymeric IgA Immunoglobulins†

Rose Marie Chapuis‡ and Marian Elliott Koshland*

ABSTRACT: The intersubunit linkage of polymeric IgA immunoglobulins was determined from studies of the products of reductive and cyanogen bromide cleavage. Under conditions of limited dithioerythritol reduction tetramer IgA molecules were cleaved to yield two monomers and a J chain containing dimer. The stability of the dimer and the conservation of the J chain disulfides indicated that the J chain joins two monomer subunits. Evidence confirming the J chain dimer clasp was obtained from the depolymerization of tetramer and dimer IgA by cyanogen bromide treatment. The cleavage studies also showed that (a) the S–S bonds directly joining the other subunits are located at the same penultimate α chain half-cystines that constitute the site of J chain attachment and (b) during limited reduction the monomer–monomer bonds undergo interchange to release subunits without a concomitant generation of α chain thiols. These linkage data provide strong support for the assembly of IgA and IgM polymers by sequential disulfide exchanges beginning with the formation of a J chain containing dimer.

Many of the structural features of the polymeric immunoglobulins have been determined. The polymers are known to be composed of IgG-like monomers and a single J chain linked by disulfide bonds to form closed, planar molecules (Metzger, 1970; Cebra and Small, 1967; Halpern and Koshland, 1970, 1973). The size of the polymers is a function of the particular heavy chain present in the monomeric subunits. IgM, which contains a μ-type heavy chain, is almost invariably a pentamer, while IgA, which contains an α-type heavy chain, is polydisperse, dimers and tetramers being the most common forms.

The linkage between the monomers and J chain and the mechanism of their polymerization are less well understood. Analyses of the products of limited IgM reduction have indicated that the J chain is located as a disulfide clasp between two of the monomers while the remaining subunits are joined by direct S–S bonds. On the basis of these results, the assembly of IgM was postulated to proceed by a series of sequential disulfide exchanges beginning with the formation of a J-containing dimer (Chapuis and Koshland, 1974). However, proof of the postulated mechanism requires more precise information concerning the intersubunit bonds. For example, the available data do not exclude a monomer linkage for J chain. It is possible that the J chain is joined to a single subunit rather than to two subunits, by insertion in an intrasubunit μ–μ bond. Moreover, the available data do not establish the exact location of the monomer–monomer bonds. A half-cystine in the C_H3 domain of the μ chain is alkylated after limited IgM reduction (Beale and Feinstein, 1969; Frangione et al., 1971; Putnam et al., 1973), but it is not clear whether the residue is directly involved in intersubunit bonding or whether it is modified as a result of disulfide interchange during reductive cleavage.

Studies of a human IgA_1 myeloma protein were undertaken to resolve these linkage questions. The polydisperse IgA had the advantage over pentamer IgM that native dimers as well as tetramers could be isolated and used in analyzing the intersubunit structure. In addition, the α_1 chains contain a limited number of methionine residues located at strategic positions for distinguishing inter- and intrasubunit bonds. The present paper describes the combined application of reductive and cyanogen bromide cleavage to determine the mechanism of IgA polymerization.

Materials and Methods

Preparation of Tetramer IgA. A human myeloma IgA (Hol) was isolated from serum by repeated precipitation with 18% Na_2SO_4 in the presence of 10^{-3} *M* EDTA and 10^{-2} *M* iodoacetamide. After dialysis to remove Na_2SO_4 the preparation was filtered through a column of Sepharose 6B equilibrated with Tris–saline buffer (0.02 *M* Tris-HCl (pH 8.0)–0.15 *M* NaCl–10^{-3} *M* EDTA–0.01% NaN_3). The major protein peak in the eluate consisted exclusively of IgA as judged by immunoelectrophoresis against a polyvalent anti-human serum antibody. The ascending portion

†From the Department of Bacteriology and Immunology, University of California, Berkeley, California 94720. *Received November 13, 1974.* Supported by Research Grant AI 07079 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

‡Present address: Institut de Biochimie, Université de Lausanne, 1005 Lausanne, Switzerland.

Table I: Polypeptide Composition of Dimer and Tetramer Myeloma IgA.

Chain	Total nmoles Recovered: Total ^{14}C cpm	Total nmoles Recovered: [^{14}C]-CMCys[a]	Total nmoles Recovered: Chain[b]	Chains per Polymer
Dimer				
α–light	42,686	3.59	0.171	4.0
J	3,033	0.255	0.0425	0.995
Tetramer				
α	89,801	7.63	0.477	8.0
light	27,393	2.31	0.463	7.8
J	4,611	0.387	0.0645	1.09

[a] CMCys, carboxymethylcysteine. [b] Calculated from values of 16, 5, and 6 for the half-cystine contents of α, light, and J chains, respectively.

of the peak was shown by ultracentrifugation to contain 13.8% trimer, 71.1% tetramer, and 14.8% larger aggregates (Figure 1) and was used as an enriched tetramer fraction without further purification. The descending portion which contained the smaller size polymers was used as a source of dimers.

Preparation of Dimer and Monomer IgA. Native dimers, as well as dimers and monomers generated by reductive cleavage of IgA, were separated from the other polymer components either by gel electrophoresis or sucrose gradient centrifugation. The electrophoresis was performed on sodium dodecyl sulfate 3.5% polyacrylamide gels according to the method of Fairbanks et al. (1971). The gels were then frozen and sliced, and the sections containing dimer and monomer were removed. The pooled slices of each protein were ground in a tissue homogenizer with 4 volumes of 9 *M* urea–0.2 *M* Tris-HCl (pH 8.0)–2 m*M* EDTA. After 2 hr at 4° the eluate was removed by centrifugation and the elution was repeated. The protein in the combined eluates was concentrated by ultrafiltration, dialyzed against 6.7 *M* guanidine-HCl–0.05 *M* Tris-HCl (pH 8.0)–2 m*M* EDTA, followed by dialysis against 1 *M* acetic acid, and then lyophilized.

Density gradient centrifugation was carried out using linear 5–25% sucrose gradients in 0.015 *M* Tris-HCl (pH 8.0)–0.125 *M* NaCl–0.2 m*M* iodoacetamide–0.75 m*M* EDTA–0.015% NaN_3; 3 mg of protein was layered on each gradient and the tubes were centrifuged at 39,000 rpm in a SW 40 titanium rotor for 17.5 hr. Dimer and monomer peaks were pooled from the eluates of six gradients, concentrated, dialyzed, and repurified by the same procedure.

Reductive Cleavage of IgA Polymers. For stoichiometric measurements the IgA polymers (3 mg/ml) were completely reduced with 50 m*M* dithioerythritol in 6.7 *M* guanidine-HCl–0.2 *M* Tris-HCl (pH 8.0)–2 m*M* EDTA for 2 hr at room temperature and alkylated at 0° by adding a 2.2-fold molar excess of [^{14}C]iodoacetamide (1.2×10^7 cpm/μmol). For measurements of intersubunit linkage the IgA polymers were first partially reduced and alkylated with [^{14}C]iodoacetic acid (1.19×10^7 cpm/μmol) and then completely reduced and alkylated with [^{3}H]iodoacetic acid (8.49×10^5 cpm/μmol). The details of this differential radioalkylation method have been described elsewhere (Chapuis and Koshland, 1974).

Chain Isolation and Determination of Radioactivity. The J chain was separated by electrophoresis of the reduced and alkylated samples on alkaline urea 4.15% polyacrylamide gels (Reisfeld and Small, 1966). Because the Hol α and light chains could not be resolved on the basis of charge, these chains were separated by electrophoresis of the reduced and alkylated samples on sodium dodecyl sulfate 10% polyacrylamide gels. The gels were frozen, sliced, and prepared for radioactivity measurements as described previously (Wilde and Koshland, 1973). The label incorporated in the α and J chains was obtained directly from counting of the appropriate gels. The label incorporated into light chain was determined by counting the light–J fraction isolated on sodium dodecyl sulfate gels and correcting for the contribution of the J chain (see Figure 3).

Cyanogen Bromide Cleavage of IgA. Samples of polymer and monomer IgA were dialyzed against acetic acid, taken to dryness, and then cleaved in 75% formic acid with a 20-fold w/w excess of cyanogen bromide. After 24-hr incubation in the dark at room temperature the reagents were removed by lyophilization. The size of the cleavage products was assayed by dissolution of the dried material in 10 *M* urea and 2% sodium dodecyl sulfate, boiling for 1 min, and electrophoresis on sodium dodecyl sulfate 3.5% polyacrylamide gels.

Ultracentrifuge Analyses. Sedimentation analysis was performed on a Beckman Spinco Model E analytical ultracentrifuge equipped with schlieren optics. Double sector cells with quartz windows were used in a AnD rotor. Relative amounts of protein were determined from planimetric measurements of the areas under the peaks. No correction was applied for radial dilution.

Results

Because the polydisperse IgA chosen for these studies was a product of neoplastic cells, preliminary experiments were carried out to establish that the polymers were correctly assembled. Dimers and tetramers were isolated and their polypeptide compositions were analyzed by completely reducing the disulfide bonds and alkylating the thiols with [^{14}C]iodoacetamide. The chains were then separated by gel electrophoresis and the yield of each was determined from measurements of the ^{14}C content and the known number of half-cystine residues per chain. The results obtained are shown in Table I. The observed compositions were in good agreement with the stoichiometry of α, light, and J chains in normal IgA dimers and tetramers (Halpern and Koshland, 1973). In the case of the tetramer, the slightly high recoveries of J chain could be explained by the amount of trimer contaminating the preparation (Figure 1).

The assembly of disulfide bridges was analyzed by alkylating the tetramers with [^{14}C]iodoacetamide. No significant amounts of label were detected after reaction in aqueous buffer, but 2.85 mol/mol of tetramer were incorporated after reaction in 6.7 *M* guanidine. To determine the distribution of the ^{14}C-alkylated thiols, the tetramer samples were completely reduced and alkylated with [^{3}H]iodoacetic acid and the constituent chains were examined. Essentially equimolar quantities of [^{14}C]carboxymethylcysteine were found in the α and light chains, 0.188 and 0.160 mol/mol, respectively, and only 0.011 mol/mol of J chain. This distribution indicated that disulfide bridges were not present between 1/5 of the α and light chains in the tetramers. At the same time the absence of significant ^{14}C label in the J chain and the absence of additional ^{14}C label in the α chains indicated that the intersubunit bonds had been completely as-

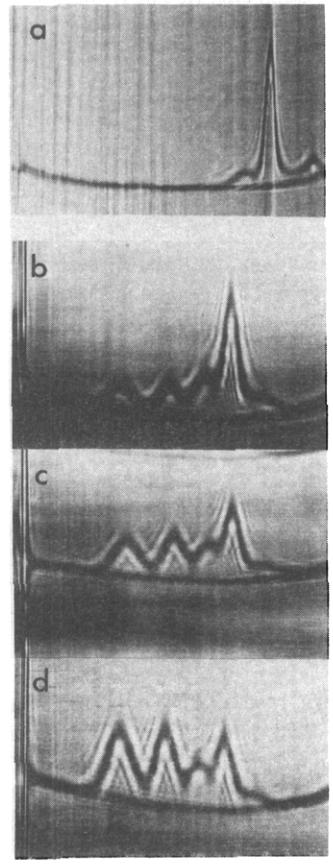

FIGURE 1: Schlieren patterns of tetramer IgA preparation before and after limited reduction. (a) Tetramer, 4 mg/ml in 7 *M* guanidine–0.05 *M* Tris-HCl (pH 8.0)–2 m*M* EDTA; after 64 min at 67,770 rpm; T = 20°. (b, c, d) Tetramer reduced with 0.1, 0.2, and 0.3 m*M* dithioerythritol respectively; 4 mg/ml of protein in Tris–saline buffer, 0.2 *M* iodoacetamide, after 32 min at 59,780 rpm; T = 20°.

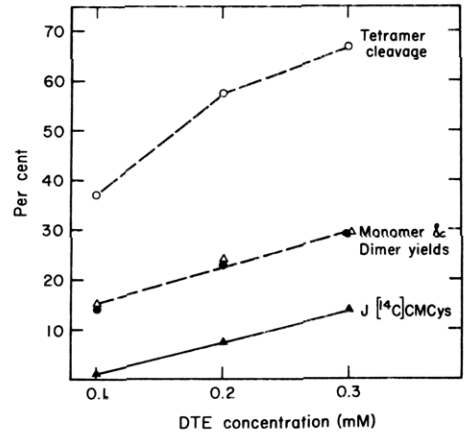

FIGURE 2: Correlation of disulfide bond cleavage with depolymerization. (▲—▲) % of J chains with one [^{14}C]carboxymethylcysteine/chain; (△- -△) % release of monomers; (●—●) % release of dimers; (○—○) % cleavage of tetramer.

sembled. Similar results have been obtained for a human Waldenström's IgM except that a higher percentage of the pentamer light chains were covalently attached (Chapuis and Koshland, 1974).

Once the IgA myeloma tetramers were shown to be comparable in structure to normal polymers, studies of their intersubunit linkage could be undertaken. The first approach was to analyze the linkage of J chain using the methods developed in previous investigations of pentamer IgM (Cha-

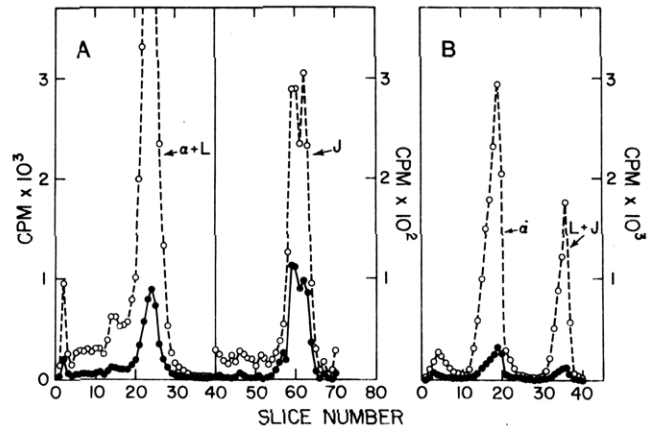

FIGURE 3: Electrophoresis of tetramer IgA after limited reduction and ^{14}C alkylation followed by complete reduction and ^{3}H alkylation. (● —●) ^{14}C cpm; (○- - -○) ^{3}H cpm. (A) Separation of J chain by alkaline urea 4.5% polyacrylamide gel electrophoresis. (B) Separation of α chain from light–J chain fraction by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis.

puis and Koshland, 1974). The tetramers were reduced under conditions which gave selective cleavage of intersubunit bonds. After the SH groups were alkylated with [^{14}C]iodoacetic acid, the subunit products were quantitated by velocity ultracentrifugation. The cleavage achieved is illustrated by the schlieren patterns shown in Figure 1 and the yields of monomers and dimers plotted in Figure 2. To correlate the subunit yields with the number of J chain disulfides broken, the partially reduced preparation was then completely reduced and alkylated with [^{3}H]iodoacetic acid. The J chain was isolated by electrophoresis on alkaline urea gels and the half-cystines alkylated after limited reduction were calculated from the ratio of the ^{14}C content to the total ^{14}C and ^{3}H content. The quantitative separation of the J chain is illustrated by the left-hand gel pattern in Figure 3, and the disulfide cleavage results are given in Figure 2 as the percentage of J chain containing one [^{14}C]carboxymethylcysteine residue per mol.

It is evident from the relationships in Figure 2 that the cleavage of J chain disulfides could not account for the extent of depolymerization and thus the J chain could not be joined to each of the tetramer subunits. After reduction with 0.1 m*M* dithioerythritol minimal amounts of ^{14}C label, equivalent to one reacted half-cystine/70 chains, were incorporated into J chain. Yet 36.6% of the tetramers were cleaved, 14% to monomers and 15% to dimers. Moreover, the same discrepancy between the percentage of J chain labeled and the percentage of subunits released was observed after reduction with higher dithioerythritol concentrations.

More definitive evidence for the location of J chain was obtained from the size distribution of the cleavage products. Equal amounts of dimers and monomers were released even when 70% of the tetramers were cleaved. The resistance of the dimers to further reductive cleavage indicated that the component subunits were linked, not through the regular monomer–monomer bond, but through stronger disulfide bonds to the J chain. This interpretation was supported by analyses of the dimers and monomers generated by limited reduction with 0.2 m*M* dithioerythritol. The subunits isolated by elution from sodium dodecyl sulfate polyacrylamide gels were completely reduced and alkylated with [^{3}H]iodoacetic acid to determine their J content and the extent of J chain disulfide cleavage. Measurements of radioactivity (Table II) showed that the dimer product contained stoi-

Table II: Cleavage of J and α Chain Disulfide Bonds during Limited Reduction of Tetramer IgA.

Sample	% Containing 1 mol of J Chain	Molar Content of [^{14}C]CMCys[a]: J Chain	α Chain: Obsd	α Chain: Cor[b]	α Chain: % of Expected[c]
Tetramer 57.9% cleaved	109	0.082	0.136	0.109	31.2
Dimer product	93.3	0.034	0.211	0.184	36.8
Monomer product	3.7	0.48	0.309	0.282	28.2

[a] CMCys, carboxymethylcysteine. [b] Corrected for the number of alkylated half-cystines contributed by the breakage of α–light chain bonds. [c] Calculated from the yields of monomer and dimer and the assumption of two ^{14}C-labeled residues per monomer and per dimer.

Table III: Number of Alkylated Half-Cystines per α Chain after Limited Reduction of Tetramer IgA.

Dithioerythritol Concn (mM)	Molar Content of [^{14}C]CMCys[a]: Obsd	Cor[b]	% of Expected[c]
0.1	0.078	0.062	28.5
0.2	0.136	0.109	31.2
0.3	0.225	0.177	40.6

[a] CMCys, carboxymethylcysteine. [b] Corrected for the number of alkylated half-cystines contributed by the breakage of α–light chain bonds. [c] Calculated from the yields of monomer and dimer and the assumption of two ^{14}C-labeled residues per monomer and per dimer.

chiometric amounts of essentially intact J chain. On the other hand, the monomer product was found to contain a small percentage of partially alkylated J chain. These results were very similar to those obtained in analyses of IgM cleavage products (Chapuis and Koshland, 1974). In both cases, the data eliminated the possibility that J chain is joined to more than two polymer subunits, but left open the possibility that J chain may be attached within a single subunit.

Although the J chain cleavage data were similar for IgA and IgM, significant differences were observed in the cleavage of the respective heavy chain disulfides. After limited reduction of IgM the number of ^{14}C-alkylated half-cystines/μ chain was found to correspond with the amounts of subunits released. The experimentally determined incorporation of carboxymethylcysteine agreed within 2% with the theoretical values calculated from the yields of reduction products and the assumption of two ^{14}C-labeled residues per monomer, dimer, trimer, and tetramer. After limited reduction of tetramer IgA, however, the number of ^{14}C-alkylated half-cystines/α chain was consistently between one-third and one-half the values expected from the extent of

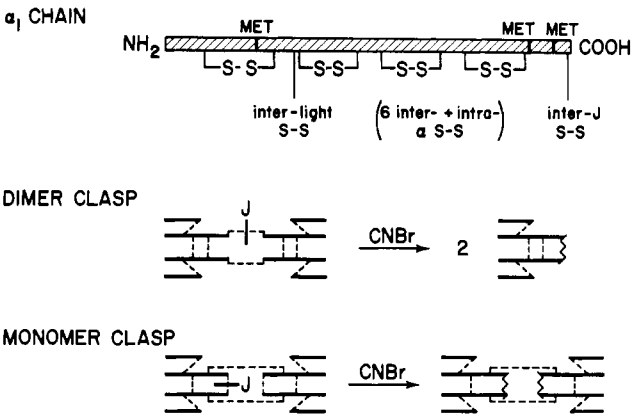

FIGURE 4: Differentiation of alternative IgA linkage models by the products of cyanogen bromide cleavage.

depolymerization (Tables II and III). These results could not be explained on the basis of insufficient alkylating reagent. The incorporation of ^{14}C label into α chains was not detectably increased by the use of a tenfold rather than a threefold molar excess of iodoacetic acid over the dithioerythritol concentration. The results also could not readily be explained by sequestering of thiols (Smith and Ballou, 1974) since 28–40% of the expected number of SH groups was alkylated. The most likely explanation was the initiation of sulfhydryl–disulfide interchange during the reductive cleavage. Such a process would generate subunits without concomitant release of SH groups if stable intrasubunit bonds were formed in the subunits freed by interchange. The differences between the cleavage of IgA and IgM disulfides could then be accounted for by differences in the reductive susceptibility of the respective intrasubunit bonds. In these circumstances, it is evident that alkylation does not provide an accurate measure of the tetramer IgA intersubunit bonds cleaved or their location in the α chains.

To resolve the problem of polymer linkage, an alternative cleavage approach was employed. The choice of cyanogen bromide was based on data obtained in recent structural analyses of human IgA (see diagram at the top of Figure 4). First, studies of the cyanogen bromide cleavage products have shown that J chain is attached to the penultimate half-cystine in the α chain (Mestecky and Schrohenloher, 1974). Treatment of polymer IgA with cyanogen bromide was found to cleave the α chain at a methionine located nine residues from the C-terminus and to release a fragment containing J chain disulfide bonded through the penultimate half-cystine to the C-terminal octapeptide. Second, sequence studies of the α_1 chain have shown that the constant region contains only two methionines, 9 and 39 residues, respectively, from the C-terminus, and the variable region may contain an additional methionine usually located between the intrachain disulfide bridge (Chuang et al., 1973). Cleavage of the light chain was not a factor since the κ chain of the Hol IgA_1 used in these studies lacked methionine altogether.

The position of the methionine residues and the number of intrasubunit α–α bonds (Wolfenstein-Todel et al., 1974) indicated that cyanogen bromide cleavage of IgA_1 polymers would not disrupt the 4-chain structure of the individual subunits. The only effect would be the loss of small peptides from the C-terminal, and possibly also the NH_2-terminal, end of the α chains. The cleavage of the C-terminal peptides would, however, affect the polymer structure differ-

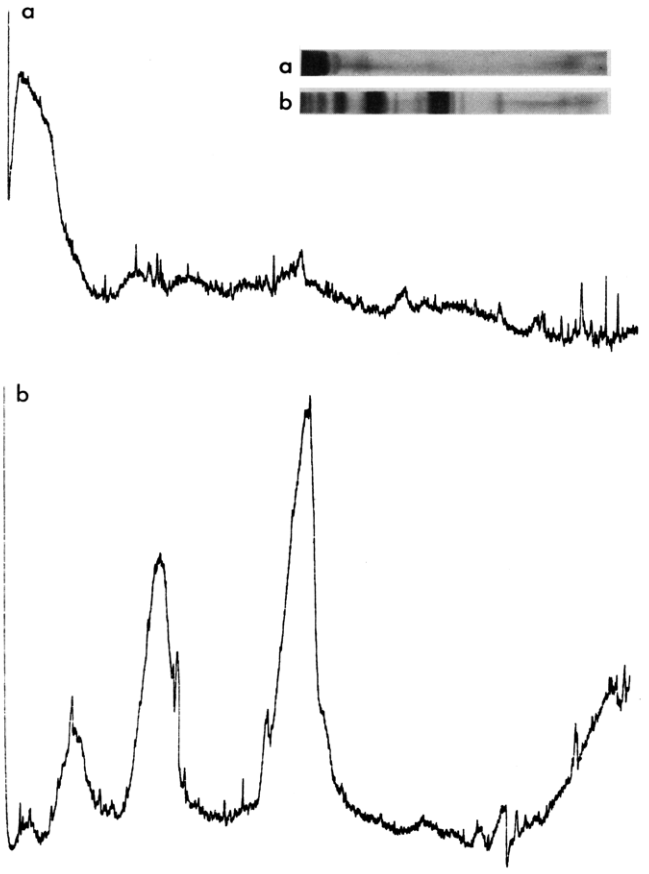

FIGURE 5: Electrophoresis of tetramer IgA before (a) and after (b) cyanogen bromide cleavage; 20 and 50 μg applied, respectively. Protein in sodium dodecyl sulfate 3.5% polyacrylamide gels was determined by absorption measurements at 278 nm, using a Zeiss spectrophotometer equipped with a linear transporter.

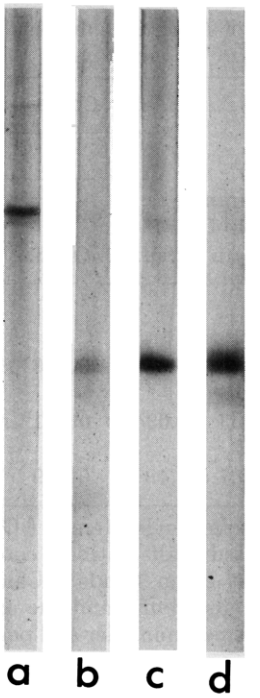

FIGURE 6: Electrophoresis of dimer and monomer IgA before and after cyanogen bromide treatment. Sodium dodecyl sulfate 3.5% polyacrylamide gels; (a) dimer control; (b) cleaved dimer; (c) monomer control; (d) cleaved monomer.

ently depending on the linkage of J chain and the position of the intersubunit disulfide bridges. The dimer clasp model (Figure 4) postulates that J chain is inserted between the penultimate half-cystines of adjacent subunits and to maintain the symmetry of disulfide structure characteristic of immunoglobulins, the polymers are closed by S–S bonds between residues at the same penultimate positions. If this model is correct, the IgA polymers should be converted to monomers by cyanogen bromide cleavage. The alternative monomer clasp model (Figure 4) postulates that J chain is inserted within an intrasubunit bond formed by the penultimate half-cystines. Again to maintain symmetry, each subunit has an intrasubunit α–α bond at this position, and the subunits are joined by disulfide bonds located NH_2-terminal to the position of cyanogen bromide attack, probably in the C_H2 domains. If this model is correct, the polymeric structure should be conserved after cyanogen bromide treatment.

These alternatives were tested using tetramers and dimers isolated from the native Hol IgA_1. Monomers isolated from the reductive cleavage products of Hol IgA provided a subunit control. The preparations were treated with excess cyanogen bromide in denaturing acid solvent and then assayed for size changes by electrophoresis on sodium dodecyl sulfate 3.5% polyacrylamide gels. The gel patterns obtained for each protein before and after cleavage are shown in Figures 5 and 6. As expected from the location of the methionine residues, cyanogen bromide treatment did not significantly increase the electrophoretic mobility of the monomer (Figure 6). A maximum loss of 38 residues from each α chain would decrease the monomer molecular weight by 6%, too small a size change to be distinguished by the assay system used. On the other hand, cyanogen bromide treatment disrupted the polymers' structure in a pattern consistent with the predictions of the dimer clasp model. The tetramers were broken down mostly to dimers and monomers, and the dimers were quantitatively converted to monomers. The differences in the extent of tetramer and dimer cleavage probably reflected differences in the accessibility of the methionine residues. Since the Fc domains are known to form the central core of the polymers (Chesebro et al., 1968; Munn et al., 1971), the tighter packing of the tetramer subunits may have protected some methionines from reaction. Conversely, the denaturing solvents used in the dimer isolation may have opened the dimer Fc structure and made the methionine residues more accessible to attack.

Discussion

The studies of polymer IgA linkage presented in this paper clearly show that the J chain is disulfide bonded between adjacent IgA subunits rather than within one subunit. The evidence was obtained from two different experimental approaches. First, limited reduction of tetramer IgA was found to release equivalent amounts of monomer and dimer; the monomer product contained trace quantities of partially alkylated J chain, whereas the dimer product contained stoichiometric amounts of unmodified J chain. These results indicate that the S–S bonds directly joining the tetramer subunits were cleaved under the reducing conditions used, but two of the four monomers remained linked through intact disulfide bridges to the J chain. In the second approach, cyanogen bromide cleavage of tetramer and dimer IgA was found to convert the polymers to monomeric subunits. These results are consistent with the linkage predicted by a J chain dimer clasp and the positions of the α-

chain methionines. The results cannot be explained by a monomer linkage of J chain unless an asymmetric arrangement of intersubunit bonds is invoked. This type of arrangement is highly unlikely in view of the structural symmetry observed in electron microscope studies of dimer IgA (Munn et al., 1971).

In addition to resolving the J chain linkage, the cyanogen bromide experiments place the monomer–monomer bonds at the penultimate half-cystines of the α chains. Since J chain is also attached to half-cystines in the same position (Mestecky and Schrohenloher, 1974), all the IgA intersubunit bonding must be concentrated at the C-terminus of the Fc regions. Cross-linking in this region is consistent with electron micrographs of dimer IgA that show the Fc structures abutted end to end and the Fab arms extending outward to form a dumbbell-shaped molecule (Munn et al., 1971). The position of cross-linking is also consistent with the information on heavy chain primary structure. The penultimate half-cystine lies within a 19 residue C-terminal peptide that has been implicated in polymer assembly because it is found uniquely in α and μ chains (Chuang et al., 1973).

The question then arises of whether the C-terminal μ-chain peptide is responsible for all the intersubunit cross-linking in pentamer IgM. The present evidence indicates that the J chain position is identical in IgM and IgA; the J chain has been shown to be disulfide bonded to the penultimate half-cystine in the μ chain (Mestecky and Schrohenloher, 1974), and dimers containing unmodified J chain have been isolated after limited IgM reduction (Chapuis and Koshland, 1974). The evidence concerning the position of the IgM monomer–monomer bonds is conflicting. Both the attachment of J chain and the pronounced homology between the C-terminal sequences of μ and α chains suggest intersubunit linkage at the penultimate half-cystines. On the other hand, the half-cystines alkylated after limited reduction of the pentamer have been located at position 414 in the μ chain rather than at the penultimate 575 position (Beale and Feinstein, 1969; Frangione et al., 1971; Putnam et al., 1973).

In previous studies of IgM linkage, a simple mechanism of disulfide interchange was proposed to explain polymer assembly (Chapuis and Koshland, 1974). The J chain was assumed to initiate polymerization by presenting a free thiol to a monomer disulfide bond; an exchange takes place that results in the formation of an S–S bond between J chain and one of the monomer heavy chains and the liberation of an SH group on the opposing heavy chain. In rapid succession a similar reaction takes place between a second J chain thiol and another monomer to produce a J chain containing dimer. The heavy chain thiols can then either be joined to close the dimer or can induce additional exchanges with other monomer disulfides to produce larger polymers.

The present studies of polymer IgA linkage provide further support for this mechanism. For example, the assignment of an initiating function to the J chain was based on the relative resistance of J chain disulfides in IgM reductive cleavage. In polymeric IgA the difference in the lability of the intersubunit bonds is even greater; the J chain S–S bonds were found to remain intact under reducing conditions that cleaved most of the monomer–monomer bonds. The proposed rearrangement of disulfide bonds was deduced from several lines of evidence: the alkylation of half-cystines at position 414 in the μ chain, the attachment of J chain to the penultimate heavy chain half-cystine, and the requirement of disulfide interchange enzyme for correct assembly (Della Corte and Parkhouse, 1973). The reductive cleavage of tetramer IgA provides more direct evidence for disulfide interchange. Measurements of the alkylated half-cystines in the α chains showed that subunits are released without the generation of a corresponding number of α-chain thiols.

Although the IgA linkage data identify the penultimate half-cystines as participants in the assembly interchange reaction, the participation of other α-chain half-cystines has not been resolved. This information cannot be obtained by reduction and alkylation of polymeric IgA, because measurements of alkylated half-cystines indicate that disulfide interchange occurs during reduction forming intrasubunit bonds resistant to selective cleavage. There is evidence from cyanogen bromide cleavage studies of myeloma monomer IgA that the penultimate α-chain half-cystines are not bonded to each other, but are linked with half-cystines located elsewhere in the α chain. This evidence is based on the observation that no C-terminal peptides were released from the α chains until the cyanogen bromide fragments were reduced and alkylated (Prahl et al., 1971). However, since the disulfide structures of secreted and intracellular monomers may not be equivalent, studies of intracellular monomers in IgA polymer synthesizing cells will be required to define the exact pathway of disulfide rearrangement and the half-cystines involved.

The IgA linkage data do not indicate any differences in intersubunit bonding that would account for the heterogeneity of the IgA polymers compared to the homogeneity of the IgM polymer. On the basis of present information the most likely explanation is conformational differences around the intersubunit half-cystines. The neighboring residues are known to contribute to the specificity of the assembly process since hybrid molecules were not observed when IgA and IgM monomers were simultaneously reassembled in the presence of J chain and disulfide interchange enzyme (Della Corte and Parkhouse, 1973). Thus, the conformation of the IgA monomers may permit the formation of stable dimers, trimers, and tetramers while the conformation of the IgM monomers may impose enough steric constraints so that only the pentameric form is stable.

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Shape and Volume of Fragments Fab′ and $(Fab')_2$ of Anti-Poly(D-alanyl) Antibodies in the Presence and Absence of Tetra-D-alanine as Determined by Small-Angle X-Ray Scattering†

Ingrid Pilz, Otto Kratky, Arieh Licht, and Michael Sela*

ABSTRACT: The conformation of two fragments derived from anti-poly(D-alanyl) antibodies, the divalent fragment $(Fab')_2$ and the monovalent fragment Fab′, was studied by small-angle X-ray scattering before and after interaction with the tetra-D-alanine amide hapten. More than 90% of the combining sites were occupied by the hapten. No significant changes were observed in the volume or in the radius of gyration, with either of the fragments. This contrasts with the significant decrease in these two parameters found upon reacting the hapten with intact anti-poly(D-alanyl) antibodies (I. Pilz, O. Kratky, A. Licht, and M. Sela (1973), *Biochemistry 12,* 4998). For Fab′, the radius of the whole particle was found to be 3.48 nm in the absence of the hapten and 3.46 nm in its presence, the radius of gyration of the cross-section was 1.37 nm without hapten and 1.38 nm in its presence, and the volume of the particle was 92 nm^3 in the absence of the hapten and 91 nm^3 in its presence. For $(Fab')_2$ the respective values were 5.06 and 5.05, 1.38 and 1.37, and 182 and 182. These results suggest that a conformational change occurs within the antibody molecule, but not within its Fab fragment, upon reaction with the tetraalanine hapten.

In a recent study (Pilz et al., 1973b) by small-angle X-ray scattering of anti-poly(D-alanyl) antibodies, a significant volume contraction could be observed upon interaction with the tetra-D-alanine hapten. The anti-poly(D-alanyl) antibodies obtained by immunization with poly(D-alanyl) diphtheria toxoid showed a decrease of the volume by 10% and a decrease of the radius of gyration by 7.7% when 90% of the binding sites were occupied by hapten. Qualitatively similar results were obtained when intact antibodies of a different specificity (anti-*p*-azophenyl β-lactoside) were reacted with the homologous hapten (Pilz et al., 1973a).

In view of these results it was of interest to find out whether the interaction of the hapten with fragments derived from an intact antibody, and still possessing active combining sites, would be also accompanied by changes in radius of gyration and in volume. For this purpose, we have prepared from anti-poly(D-alanyl) antibodies by peptic digestion the divalent fragment $(Fab')_2$. From this divalent fragment we prepared by reduction and alkylation the monovalent fragment Fab′. Both Fab′ and $(Fab')_2$ were reacted with tetra-D-alanine amide, and their volumes and radii of gyration were determined by small-angle X-ray scattering, before and after the interaction with the hapten. The results show clearly that the radius of gyration and the volume of each one of the two antibody fragments studied did not change significantly as a result of the interaction with the tetrapeptide.

Materials and Methods

The preparation of the antigen poly(D-alanyl) diphtheria toxoid has been previously described (Licht et al., 1971). It contained 20 D-alanine residues attached per protein molecule, distributed with the average of 2.8 chains, containing 7.1 D-alanine residues/chain. Preparation of the ligands

† From the Institute for Physical Chemistry, University of Graz, Graz, Austria (I.P.), Institut für Röntgenfeinstrukturforschung, Graz, Austria (O.K.), and Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel (A.L. and M.S.). *Received September 27, 1974.* This study was supported in part by the "Österreichischen Fonds zur Förderung der wissenschaftlichen Forschung," by Grant 06-010 from the National Institutes of Health, U.S. Public Health Service, Bethesda, Md., and Grant No. 280 of the United States-Israel Binational Science Foundation.