DETECTION OF ANTIBODY MONOMERS, DIMERS AND POLYMERS UPON INTERACTION OF A HOMOLOGOUS SERIES OF DIVALENT HAPTENS WITH ITS SPECIFIC ANTIBODY

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SUMMARY

The interaction of anti-2,4-dinitrophenyl (DNP)-antibody with a homologous series of divalent haptens of the general formula DNP-NH-$(CH_2)_n$-NH-DNP produced an array of monomeric, dimeric and polymeric species; the types of species formed depended upon the particular hapten used. The hapten series ranged from n = 2 to n = 11. The short divalent haptens, i.e., n = 2 to n = 5, upon interaction with an equimolar quantity of antibody, produced only monomers and dimers. The amount of dimer produced increased as n increased from 2 to 6. The intermediate-sized divalent haptens, i.e., n = 6 to n = 9, showed polymers as well as monomers and dimers. However, the amount of monomer again increased as n equalled, or became greater than, 7. The long divalent haptens, i.e., n = 10 and n = 11, again showed almost all monomer. The results are interpreted in terms of the heterogeneity in depth and location of the antibody binding sites, and possible competing reactions.

The location and depth of the two binding sites on γ-G antibody molecules remains an interesting and important problem.[1-6] The analysis is complicated by the heterogeneous nature of antibody molecules directed towards even a single haptenic group.[7]

In order to gain more insight as to the depths of the various binding sites and the distance(s) between the two sites, we have synthesized a homologous series of divalent haptens and studied their interactions with antibody in the ultracentrifuge.

Our data reveals that a hapten as short as DNP-NH-$(CH_2)_3$-NH-DNP can link two antibody molecules to form a dimer species. This is interpreted as showing that the antigen-binding sites on some of the antibody molecules must be shallow and close to the protein surface. Our very long haptens, i.e., DNP-NH-$(CH_2)_{10}$-NH-DNP and DNP-NH-$(CH_2)_{11}$-NH-DNP, form monomers almost

exclusively, i.e., little or no dimer or polymer. This result is to be expected if we have formed a cross-link of the antigen binding sites on the same molecule by these long haptens; however, alternate explanations will be discussed later.

MATERIALS AND METHODS

Isolation of the DNP antibody from rabbits has been previously described.[6] Titration of the antibody with DNP-glycine showed that 90% of the antigen-binding sites on the antibody were available to hapten binding. The Bis-DNP compounds were prepared as follows: 60 millimoles of 2,4-dinitrofluorobenzene were dissolved in 120 mls of ethanol, and this solution was added (with stirring) to the diamine solution (30 millimoles of diamine in 50 mls of 1N $NaHCO_3$). The mixture was stirred for two hours and the yellow precipitate was isolated. The precipitate was washed alternately with water and ethanol and then heat dried. Elemental analyses on the divalent haptens showed the carbon, hydrogen and nitrogen contents to be within 0.3% of the theoretical values. The melting points also agreed quite well with that of another laboratory (N. Michael Green, private communication). The antibody concentration used was 3.14 mgs/ml (based on an $\varepsilon = 1.36$ ml/mg) which corresponded to a 2.09×10^{-5} M solution assuming a molecular weight of 150,000 for the antibody. All hapten solutions had a concentration of 1.87×10^{-3} M. A hapten/antibody ratio of 1.0 was obtained by adding 9.0 microliters of the hapten solution, dissolved in dimethylformamide, to 0.80 mls of the antibody solution. The solutions were then mixed and the ultracentrifuge patterns recorded within 30 minutes after mixing. The sedimentation-velocity runs were performed in a Beckman-Spinco Model E analytical ultracentrifuge at 20.0° using schlieren optics. The antibody monomer had the characteristic $S^o_{w,20}$ of 6.7 while the dimer value was 9.8.

TABLE I

PERCENT OF ANTIBODY MONOMER, DIMER AND POLYMER AT A HAPTEN/ANTIBODY RATIO OF 1.0

Hapten	% Monomer	% Dimer	% Polymer
No Hapten	100	0	0
Bis-DNP 1,2-diaminoethane	100	0	0
Bis-DNP 1,3-diaminopropane	74	26	0
Bis-DNP 1,4-diaminobutane	55	45	0
Bis-DNP 1,5-diaminopentane	33	67	0
Bis-DNP 1,6-diaminohexane	15	75	10
Bis-DNP 1,7-diaminoheptane	29	46	25
Bis-DNP 1,8-diaminooctane	37	40	23
Bis-DNP 1,9-diaminononane	52	29	19
Bis-DNP 1,10-diaminodecane	89	11	0
Bis-DNP 1,11-diaminoundecane	95	5	0

The range of the percentages is ± 5%. The concentration of the monomer was determined by comparing the area under the schlieren peak with that of a reference immunoglobulin whose concentration was known. In the antibody solutions where only monomer and dimer were present, the dimer concentration could be determined by subtracting the monomer concentration from the total antibody concentration. In the solutions which contained dimer and polymer in addition to the monomer, the dimer concentration was determined by comparison of the area under the dimer peak with that of a reference antibody solution which formed only monomer and dimer (i.e., DNP-antibody plus Bis-DNP-lysine) and whose monomer and dimer concentrations were known. The polymer concentration could then be determined by subtracting the monomer and dimer concentration from the total antibody concentration.

RESULTS AND DISCUSSION

Purified DNP-antibody was interacted with a homologous series of divalent haptens of the general formula DNP-NH-$(CH_2)_n$-NH-DNP, where n ranged from 2 to 11. The schlieren patterns, obtained from the 10 haptens, are shown in Figures 1A and 1B. The control solution, i.e., no hapten added, showed the presence of only monomer and exhibited an $S^{o}_{w,20}$ of 6.7. The shortest divalent hapten, i.e., n = 2, also showed only monomer, indicating that this divalent

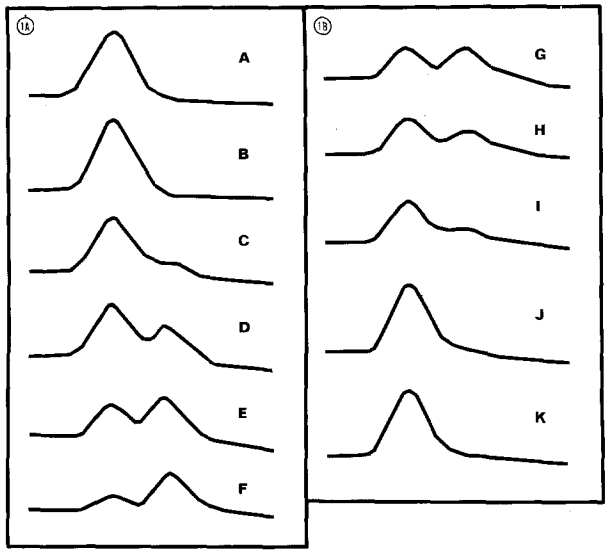

Figure 1A: Microcomparitor tracings of schlieren plates. The peak on the left represents the antibody monomer while the peak on the right represents the dimer. Recordings were made 24 minutes after the maximum rotor speed of 60,000 rpm was reached. All solutions, except the control (A), had a hapten/antibody ratio of 1.0. Haptens used were: (B) Bis-DNP 1,2-diaminoethane, (C) Bis-DNP 1,3-diaminopropane, (D) Bis-DNP 1,4-diaminobutane, (E) Bis-DNP 1,5-diaminopentane, (F) Bis-DNP 1,6-diaminohexane.

Figure 1B: Microcomparitor tracings of schlieren plates. Conditions used are the same as described in Figure 1A. Haptens used were: (G) Bis-DNP 1,7-diaminoheptane, (H) Bis-DNP 1,8-diaminooctane, (I) Bis-DNP 1,9 diaminononane, (J) Bis-DNP 1,10-diaminodecane, (K) Bis-DNP 1,11-diaminoundecane.

hapten was not long enough to bridge two binding sites on two antibody molecules, although it, of course, had to bind to one site.

Beginning with the n = 3 hapten, the monomer concentration decreased with a concurrent increase in dimer concentration. The n = 3 compound showed a significant amount of dimer, i.e., 26%. The formation of dimer with the n = 3 compound is an interesting observation since it shows that the antigen-binding sites on some of the antibodies are close to the surface of the protein. It must be remembered that antibody molecules, even those elicited towards one haptenic group, are heterogeneous.[7] It is likely that in our antibody preparation, which contains antibodies against only one haptenic group, there are many different kinds of molecules, some with shallow binding sites and others with deeper binding sites, but all specific for the DNP group.

As longer haptens, i.e., n = 4, 5 and 6 are used, the amount of dimer increased, reaching the maximum amount with the n = 6 hapten. It is with the n = 6 compound that polymer formation is first seen. With haptens longer than n = 6, one sees a progressive decrease in the amount of dimer (and polymer) as the divalent hapten becomes longer. This is accompanied by an increase in monomer concentration (see Table I). For example, with the n = 11 hapten, fully 95% of the antibody existed as monomer and only 5% as dimer.

Only the intermediate length haptens, i.e., n = 6 to 9, showed polymer (trimer or higher) formation with the amount of polymer varying between 10 and 25% (see Table I).

CONCLUSIONS

The results reported in this communication are consistent with previous observations concerning the heterogeneity of antibody molecules directed towards a single haptenic group.[7] Our view of the antigen-binding site in the antibody molecule is that the depth of the site is not absolute for a particular antigenic determinant. The depth of the site varies from very shallow (and, hence, close to the protein surface) to quite deep sites for other molecules. Thus, if one takes a short divalent hapten, i.e., n = 2, 3 or 4, only those antibody molecules with shallow sites will be sterically able to form dimers. The antibody molecules with deep binding sites would "swallow up" the short divalent hapten, thereby precluding a bridge to a second antibody. Eventually, however, one would predict that it should be possible to form 100% dimer (or polymer) with a long enough divalent hapten. It should also be possible to eventually produce a long enough hapten to link or "handcuff" the two sites on the same antibody molecule.

It was with these aims in mind that our experiments began. The shortest divalent hapten to show the presence of antibody dimer was the n = 3 compound. It must be assumed that the antibody is directed towards, at least, the entire benzene ring and the nitrogen to which it is attached. This allows a maximum distance of $\cong$ 6 Å (4 x 1.5 Å for the two carbon-carbon single bonds and the two carbon-nitrogen single bonds) for the distance between the two binding

sites. If the binding site is directed towards a greater portion of the hapten than the ring and amino nitrogen, then this distance is correspondingly reduced. This allows us the prediction that the two antigen-binding sites are separated by 4 ± 2 Å. This means that the Fab portions of the antibody molecules in the dimer are practically touching each other, a fact that was also concluded by Valentine and Green in their electron microscope studies of antibody-hapten complexes.[3]

As predicted the amount of dimer increased as n increased, up until $n \approx 6$; and, then from 7 to 11, one sees a reduction in the amount of dimer (and polymer) with a concurrent increase in the amount of monomer. The situation of eventually reaching 100% dimer is not seen (see Table I and Figures 1A and 1B).

We offer three possible explanations for this observation. With the longer haptens, we may be starting to form the cross-linked monomer. Preliminary computer programs have indicated that the order of stability is: cross-linked monomer > dimer > opened (or non-cross-linked) monomer. As n increased from $n = 7$ to $n = 11$, more of the antibody might be able sterically to internally cross-link, which would result in the observation of a shift from dimer to the more stable cross-linked monomer.

A second possible interpretation is that as the hapten length becomes longer, it also becomes more hydrophobic. This would allow non-specific binding to apolar regions on the antibody. This would have the net effect of reducing the hapten concentration in the solution so that only a fraction of it would be available to act at the antigen-binding sites. If the hapten is not able to react at the binding sites, dimer formation is precluded.

A third possibillity is the folding up of the hapten molecule on itself. The DNP group is largely hydrophobic in nature. At a critical methylene length, i.e., $n = 7$ or greater, the favorable condition could be an interaction of the DNP group and the methylenes and the molecule could be present in a folded-up condition. The high dielectric constant of the essentially aqueous solution would promote such folding up. The net effect of this

situation is the same as in the second possibility, less or no hapten is available for dimer (or polymer) formation. Experiments are presently underway to determine which of these three possibilities (or other alternatives) is the correct one.

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