DETECTION OF TWO SPECIES OF ANTIBODY MOLECULES WITH THE SAME SPECIFICITY

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SUMMARY

Two types of anti-2,4 dinitrophenyl γ-G immunoglobulin molecules are characterized: those which can form dimer upon interaction with the divalent hapten, α-ε-dinitrophenyl lysine, and those which cannot form dimer. The two types of antibody molecules are separable by ultracentrifugation and by Sephadex G-200 chromatography. It is proposed that the two types of molecules result from two different orientations in space of the antigen combining sites.

The three-dimensional conformation of antibody molecules, and the location of the antigen binding sites, have been the subjects of much recent discussion about antibody structure. Electron micrographic studies[1,2] seem to indicate that the active sites of the antibody molecule are located at the tip of the Fab pieces. Cathou and Werner[3] have reported, as the result of circular dichroism studies, that the antibody active site seems to be composed of amino acid residues from distant parts of the molecule, similar to enzyme active sites. However, there is no direct evidence (e.g., x-ray crystallography data) pinpointing the three-dimensional location of the antigen combining site on the antibody molecule. As a result of the experiments reported here, we propose that antibody molecules with the same specificity may have antigen combining sites with different three-dimensional orientations in space.

It has been reported[4,5] that when anti-2,4-dinitrophenyl (DNP) antibody interacts with a divalent hapten, such as α-ε-DNP-lysine, monomer and dimer are formed exclusively. This differs from the results of earlier workers,[6-8] who reported higher order species present upon interaction with α-ε-DNP-

lysine. The discrepancy seems to depend upon whether aggregated species are present in the antibody preparation before the addition of α-ε-DNP-lysine. In this laboratory, antibody preparations, with no detectable aggregation, give monomer and dimer exclusively upon interaction with the divalent hapten, α-ε-DNP-lysine. The monomer and dimer were found to be stable entities, separable by sedimentation velocity and by Sephadex G-200 chromatography.[4,5] It has therefore been hypothesized[5] that two types of antibody molecules were present in the original preparation: those which can form dimer and those which cannot. The purpose of this communication is to describe experiments which confirm the hypothesis of two types of antibody molecules.

MATERIAL AND METHODS

The anti-2,4-dinitrophenyl antibody was prepared from rabbits as previously described.[5,9] The preparation used was shown to have greater than 90% binding capacity for hapten. The divalent hapten used in all experiments was α-ε-DNP-lysine. Separation of monomer and dimer was performed by Sephadex G-200 chromatography. A 2.0 ml sample, containing 40 mg of anti-DNP antibody, in 0.15 M NaCl, plus equimolar α-ε-DNP-lysine, was applied to a Sephadex G-200 column (1.9 x 35 cm) which had been equilibrated with 0.15 M NaCl. (A non-ultraviolet light absorbing, low molecular weight, presumably carbohydrate contaminant, was eluted from the G-200 column in one of the experiments, but was shown not to interfere with the results.)

Hapten was removed from the pooled dimer fractions and from the pooled monomer fractions, resulting from the G-200 chromatography, by Dowex 1 chromatography. The antibody was concentrated, when necessary, by ultrafiltration.

All sedimentation velocity experiments were performed in a Beckman-Spinco Model E ultracentrifuge at 20°, using schlieren optics.

RESULTS AND DISCUSSION

The purpose of the experiments to be described, was to test if there were two species of molecules present in the rabbit anti-2,4-dinitrophenyl

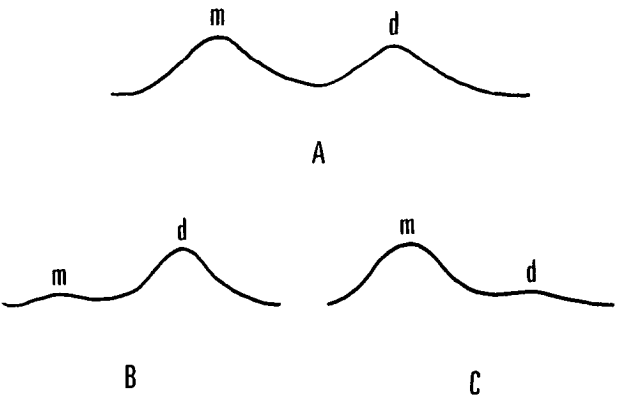

FIGURE 1: (A) Monomer (m)-Dimer (d) mixture after interaction of antibody with equimolar α-ε-DNP-lysine and before G-200 chromatography; (B) Isolated dimer; (c) Isolated monomer.

(DNP) antibody system. First, the anti-DNP antibody was interacted with equimolar α-ε-DNP-lysine. Then, the resulting monomer-dimer mixture was separated by Sephadex G-200 chromatography. Next, the hapten was removed from the dimer and from the monomer by passage through Dowex 1. And finally, equimolar hapten was readded to the original dimer and to the original monomer, to see if the monomer reformed only monomer and if the dimer reformed only dimer.

Figure 1 summarizes the results of the Sephadex G-200 chromatography. Figure 1, and all other figures in this communication, are tracings of the schlieren patterns, magnified ten times, obtained from sedimentation velocity experiments in the ultracentrifuge. Figure 1A shows the monomer-dimer mixture before G-200 chromatography. Figure 1B shows the separated dimer, and Figure 1C shows the separated monomer. It is seen that the isolated monomer has only a very small amount of dimer present, and the isolated dimer has only a very small amount of monomer present.

First, the studies on the isolated dimer will be described. The dimer was converted to about 70% monomer by adding a one hundred-fold excess of 2,4-dinitrophenol, which replaced the α-ε-DNP-lysine. After passage through

Dowex 1, to remove the hapten, the schlieren pattern shown in Figure 2A was obtained, for the unbound antibody. (Figure 2A should be compared with Figure 1B.) The 30% dimer still present in the preparation was found to correlate closely with the amount of residual hapten observed spectrophotometrically. It can be assumed that this fraction of the antibody was very high affinity antibody, so that treatment with 2,4-dinitrophenol did not replace the tenaciously bound α-ε-DNP-lysine, which in turn was not removed by the Dowex 1 column.

Next, equimolar α-ε-DNP-lysine was readded to the antibody and centrifuged. Figure 2B shows that almost complete conversion of the antibody to dimer was obtained upon addition of equimolar hapten.

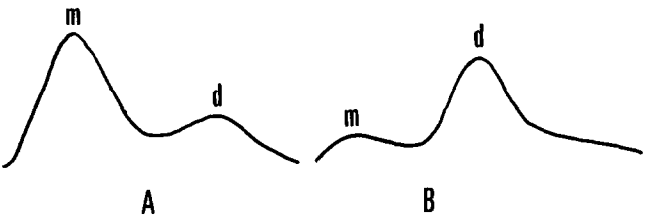

FIGURE 2: (A) Original dimer after removal of most α-ε-DNP-lysine; (B) Original dimer after readdition of equimolar α-ε-DNP-lysine.

It is instructive to compare Figures 1A and 2B at this point. It should be noted that addition, to the original antibody preparation, of less than equimolar or more than equimolar divalent hapten causes an increase in the amount of monomer present[5] and not in the amount of dimer present. The conclusion is that the isolated dimeric species seems to be unique in its ability to form dimer and that at equimolar divalent hapten, essentially all the molecules form dimer.

Figure 3 shows what happens to the antibody from Figure 2A when one-half equimolar α-ε-DNP-lysine (Figure 3A) and when twice equimolar α-ε-DNP-lysine (Figure 3B) were added. It is seen that, as in the case of the original antibody preparation, both more than and less than equimolar

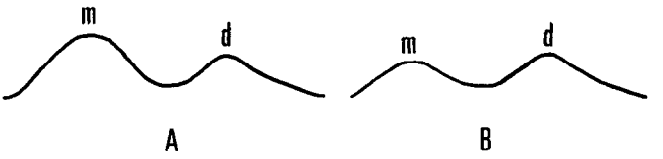

FIGURE 3: (A) Original dimer after readdition of one-half equimolar α-ε-DNP-lysine; (B) Original dimer after readdition of twice equimolar α-ε-DNP-lysine.

divalent hapten causes an increase in the amount of monomer present.

Next, the experiments performed with the isolated monomer from the G-200 column will be described. The monomer was passed through Dowex 1, without prior treatment with 2,4-dinitrophenol. The schlieren pattern of the eluted protein is shown in Figure 4A. The amount of residual dimer is about the same as before passage through Dowex 1 (see Figure 1C). Next, equimolar α-ε-DNP-lysine was added. The resulting schlieren pattern is shown in Figure 4B. It is seen that the monomeric protein remained as monomer, with only the slightest increase in the amount of dimer present.

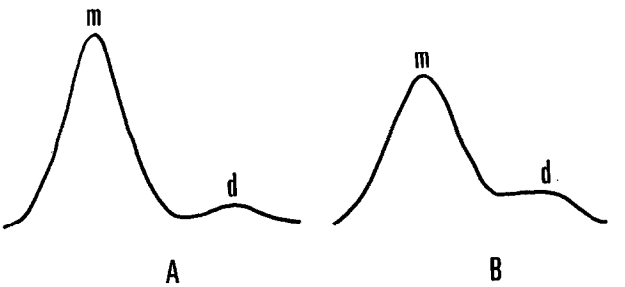

FIGURE 4: (A) Original monomer after removal of most α-ε-DNP-lysine; (B) Original monomer after readdition of equimolar α-ε-DNP-lysine.

When one-half equimolar α-ε-DNP-lysine was added to the protein from Figure 4A, essentially the same schlieren pattern was obtained as when equimolar divalent hapten was added (compare Figures 4B and 5A). Addition of twice equimolar hapten led to the predicted result of conversion of the small amount of dimer to essentially all monomer (see Figure 5B).

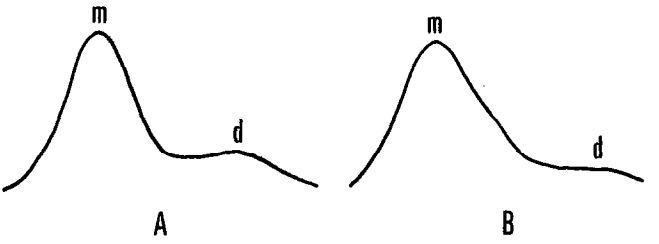

FIGURE 5: (A) Original monomer after readdition of one-half equimolar α-ε-DNP-lysine; (B) Original monomer after readdition of twice equimolar α-ε-DNP-lysine.

CONCLUSIONS

The conclusion is that in a preparation of rabbit anti-DNP antibody, there exist two types of molecules: those which can form dimer and those which cannot form dimer upon interaction with the divalent hapten α-ε-DNP-lysine. The two types of molecules seem to exist in about equal quantities. Since all the antibody molecules do bind hapten, it might be concluded that half the molecules have their active sites located in such a way that dimer formation is possible, while half cannot so form dimer. Another possibility is that the monomeric molecules are able to form an internally cross-linked species, as has been previously suggested.[5] Such a species would be the most stable thermodynamically,[5] so that formation of dimer would be precluded. The species forming dimer, then, would be those whose active sites were located in such a way as not to permit the existence of an internally cross-linked molecule.

REFERENCES

1. Feinstein, A. and Rowe, A. J., Nature 205, 147 (1965).
2. Valentine, R. C. and Green, N. M., J. Mol. Biol. 27, 615 (1967).
3. Cathou, R. E. and Werner, T. C., Biochemistry 9, 3149 (1970).
4. Hsia, J. C. and Piette, L. H., Arch. Biochem. Biophys. 129, 296 (1969).
5. Warner, C. and Schumaker, V., Biochemistry 9, 451 (1970).

6. Velick, S. F., Parker, C. W. and Eisen, H. N., Proc. Natl. Acad. Sci., U.S. 46, 1470 (1960).

7. Epstein, S. I., Doty, P. and Boyd, W. C., J. Amer. Chem. Soc. 78, 3306 (1956).

8. Epstein, S. I. and Singer, S. J., J. Amer. Chem. Soc. 80, 1274 (1958).

9. Eisen, H. N., Gray, W., Little, J. R. and Simms, E. S., Methods Immunol. Immunochem. 1, 351 (1967).