RESYNTHESIS OF PRECIPITATING ANTIBODY FROM UNIVALENT FRAGMENTS*

A. Nisonoff

Department of Microbiology,
University of Illinois, Urbana, Illinois.

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Porter (1958, 1959) has shown that proteolysis of the bivalent rabbit antibody by papain yields fragments with a sedimentation coefficient of 3.5 S and the capacity to block the homologous precipitin reaction. It has been found (Nisonoff, Wissler, Lipman and Woernley, 1960) that similar results can be obtained by the combined or successive actions of pepsin and a reducing agent. Treatment with pepsin alone causes a reduction in sedimentation coefficient, for the bulk of the antibody protein, from approximately 6.5 S to 5 S and a decrease in molecular weight from 160,000 to 106,000 (Nisonoff, Wissler and Lipman, 1960). This residual molecule is still bivalent. Subsequent treatment with one of several disulfide-splitting reagents yields univalent fragments with $s = 3.5 \pm 0.1$ S and molecular weight 56,000. Since the 3.5 S fragments migrate as a single peak it was concluded that the 5 S molecule is split approximately in half in the process. By amperometric titration of SH groups formed (Nisonoff, Markus and Wissler, 1960), it was found that the splitting into univalent fragments is brought about by rupture of a single, highly labile disulfide bond. There is considerable evidence that the mechanisms of action of papain and pepsin on the antibody are similar, and that the 5 S fragment consists essentially of Porter's fractions I and II, linked through a disulfide bond; (papain, being a sulfhydryl enzyme, is used in conjunction with a disulfide-splitting reagent). Since fractions I and II are nearly identical in amino acid composition and molecular size (Porter, 1959), the antibody appears to consist of two nearly identical active subunits joined by the S-S bond and a third, inactive fragment, which is readily

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removed by papain or pepsin. It is also conceivable that the S-S bond does not actually link the active units, but that the integrity of the bond is necessary for these units to be held together (by other bonds).

The lability of this disulfide bond is shown by the facts that it is reduced by mercaptoethylamine before any of the other 10-12 disulfide bonds in the 5 S molecule (Nisonoff, Markus and Wissler, 1960), and that passage of such molecules through carboxymethylcellulose at pH 5.4 results in a considerable degree of breakdown into univalent, 3.5 S fragments (Nisonoff, Wissler and Lipman, 1960). These fragments were shown to be partially recombined on passage through an IR-120 ion exchange resin or by treatment with a bifunctional organic mercurial.

We have now been able to prepare specifically precipitable antibody in good yield by reoxidation of the univalent, 3.5 S fragments. It has also been found that the critical disulfide bond is available for reduction in the original antibody molecule as well as in the 5 S protein.

Experiments on recombination were done with specifically purified rabbit anti-p-azobenzoate antibody, obtained after immunization of rabbits with a bovine gamma globulin-p-azobenzoate antigen (Nisonoff and Pressman, 1958, 1959). At the optimum antigen concentration 90 µg of the antibody protein, of 200 µg used in the test, was precipitable by an ovalbumin-p-azobenzoate test antigen. The degree of purity was probably higher than indicated since it has been found that the reaction with this test antigen does not go to completion (Nisonoff and Pressman, 1959). The amount of antibody in specific precipitates was estimated by dissolving in 1 ml of 0.02 N NaOH and determining the absorption at 280 mµ. This value was corrected for the contribution by the antigen which in turn was estimated from the absorption at 450 mµ. Precipitating mixtures were allowed to stand for seven days in the refrigerator.

One hundred mg of the purified antibody ($s_{20,w}$ = 6.5 S) was treated with 2 mg of crystallized pepsin in sodium acetate-chloride buffer, pH 4.5, ionic strength 0.14. The volume was 12 ml. After incubating for 16 hrs at 37°, the pH was raised to 8 by addition of 1 N NaOH. A 25% (wt/vol) solution of sodium sulfate was added with stirring to a final concentration of 19% to precipitate

the major protein component. After overnight dialysis against four liters of cold 0.05 M sodium acetate, 61 mg of protein was recovered in a volume of 4.1 ml. A portion of this material was examined in the Spinco Model E Ultracentrifuge at 59,780 rpm (concentration 10 mg/ml, pH 7, 0.1 M sodium acetate). A single symmetrical peak was observed with $s_{20,w} = 4.9$ S. This purified material was tested with a series of concentrations of the ovalbumin-p-azobenzoate antigen; 200 µg of antibody protein was present in each tube. At the optimum antigen concentration 84 µg of antibody precipitated.

The remainder of this 5 S preparation was treated with 0.01 M mercaptoethylamine hydrochloride at pH 5 for one hour at 37° C. A portion of the solution was removed, adjusted to pH 7, ionic strength 0.1, with sodium hydroxide and sodium acetate solutions and examined in the ultracentrifuge with the mercaptoethylamine still present. A single peak was formed with $s_{20,w} = 3.5$ S. The sample used for centrifugation was then dialyzed against cold saline-borate buffer, pH 8. The dialyzed protein formed no visible precipitate when 200 µg portions were mixed with a wide range of concentrations of antigen. One mg completely inhibited the precipitation of 200 µg of the untreated, purified antibody with an optimal concentration of antigen. This was consistent with the previously demonstrated univalence of 3.5 S fragments (Nisonoff, Wissler and Woernley, 1960).

The remainder of the mercaptoethylamine-treated sample was passed through an 8 x 200 mm column of IR-120 cation-exchange resin in the sodium cycle at pH 4.5 and room temperature to remove the positively charged reducing agent. The eluate was then stirred magnetically in a beaker for 3 hours while a stream of oxygen was passed over the surface.[1] A portion of the solution was neutralized to pH 7, the sodium acetate concentration was adjusted to 0.1 M, and the sample was examined in the ultracentrifuge at 59,780 rpm. The concentration of protein was 10 mg/ml. Approximately 70% of the material migrated with $s_{20,w} = 4.8$ S. The velocity of the other, smaller peak could not be accurately estimated, but its position relative to the meniscus after 64 minutes of centrifugation corres-

[1] Preliminary experiments suggested that, as in the dimerization of serum albumin (Edsall *et al.*, 1954), the reaction proceeds more rapidly in slightly acid solution than at neutral pH. If correct, it would indicate that electrostatic repulsion at higher pH overcomes the favorable effect on oxidation of S-S bonds.

ponded closely to that of the 3.5 S material in the previous sample. How much of the recombination occurred on the column is uncertain, but in similar experiments it was found to be 30-50%.

To purify the 5 S protein it was precipitated once with sodium sulfate at a final concentration of 18%. After centrifuging, dissolving the precipitate and dialyzing, 16 mg of protein was recovered at a concentration of 6.2 mg/ml. In the ultracentrifuge, at pH 7, the material migrated almost entirely as a single peak with $s_{20,w} = 5.1$ S. A small amount of a slower moving peak, estimated as less than 5% of the total area, was also present.

This material formed specific precipitates when mixed with appropriate amounts of the ovalbumin-p-azobenzoate antigen. Tests were made at pH 8 with 200 µg portions of antibody protein and varying concentrations of antigen in a total volume of 0.4 ml. With the optimal amount of antigen (0.14 mg), 76 µg of antibody was precipitated. When 6 times as much antigen was used no precipitate formed. Precipitation was completely inhibited by 0.005 M sodium benzoate at pH 8.

The results indicate that polyvalent antibody was resynthesized. The fact that the sedimentation coefficient was approximately the same as that observed prior to reduction with mercaptoethylamine suggests that the reoxidized material was bivalent. The possibility of preparing antibody of mixed specificity is suggested.

The yield of 5 S protein was 27% of that isolated after the initial peptic digestion. If this is corrected for the use of intermediate materials in tests, the yield was about 35-40 %.

In another series of experiments it was found that 3.5 S fragments of rabbit antiovalbumin gamma globulin, having specific blocking activity, can be produced by treatment first with 0.02 M mercaptoethylamine and then with pepsin (2% of the weight of globulin). This is the reverse of the sequence previously used. It was necessary to fix the SH groups formed, by treatment with 0.04 M iodoacetamide, prior to dialysis for removal of the reducing agent. When this was not done the subsequent reaction with pepsin resulted mainly in the forma-

tion of 5 S rather than 3.5 S protein, suggesting that the SH groups were partially reoxidized during dialysis. Treatments with mercaptoethylamine or pepsin were carried out at pH 4.5, 37° C.; dialysis at pH 8 and ~5° C.

The concentration of mercaptoethylamine needed was higher than that required after treatment with pepsin (about 0.008 M). In earlier attempts to reverse the order of treatment, using cysteine (Nisonoff, Wissler, Lipman and Woernley, 1960), the amount of reducing agent used was based on that required after peptic digestion and was inadequate, even with subsequent addition of iodoacetamide. The critical disulfide bond appears to be available for reduction in the original antibody molecule and readily reoxidized upon removal of the reducing agent.

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