MECHANISM OF FORMATION OF UNIVALENT FRAGMENTS OF RABBIT ANTIBODY[1]

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Among a number of studies of the proteolysis of antibodies the recent work of Porter (1950, 1958, 1959) is unique in that he found evidence for the presence of univalent fragments. Such fragments, about one third as large as the original molecule, were formed on treatment of the bivalent rabbit antibody molecule with papain. The evidence for univalence was the capacity to inhibit precipitation of the same, untreated antibody with homologous antigen; and, in addition, the fact that he was able to separate two active fractions from the papain digest by chromatography. Of various proteolytic enzymes tested only papain yielded split products that blocked precipitation.

Through measurements of hapten-binding capacity, we have found that the digestion of a rabbit antihapten antibody with papain has little effect on the number of active combining sites, the equilibrium constant for their interaction with hapten, or the degree of heterogeneity, although precipitating activity is lost (Nisonoff and Woernley, 1959a, 1959b). Determination of the maximum hapten-binding capacity demonstrated quantitatively that the fragments are univalent (Nisonoff and Woernley, 1959b, Nisonoff, Wissler and Woernley, 1959).

The present report describes the formation of univalent split products of rabbit antiovalbumin with another enzyme, pepsin, and presents data bearing on the mechanism of the reaction. As indicated below such fragments can be made by including cysteine in the mixture during the peptic digestion. Cysteine has been

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used as an activator in experiments described above on digestion of antibody with papain, since the latter is a sulfhydryl enzyme. However, pepsin is not activated normally by cysteine. It would appear, therefore, that cysteine acts directly on the antibody, probably by reduction of disulfide bonds, and that this action, together with that of an enzyme, is necessary to cause separation of sections of the molecule containing the two active sites. Additional evidence for this hypothesis is the fact that the breakdown into univalent fragments can be carried out in two stages: hydrolysis with pepsin at pH 5, followed by treatment with L-cysteine or another reducing agent, 2-mercaptoethylamine, at pH 8 (at which pH pepsin is inactive).

The data in Table I show that pepsin alone at pH 4 or, to a lesser extent, at pH 5 causes a reduction in sedimentation constant ($s_{20,w}$) of a large fraction of the rabbit antiovalbumin γ-globulin from about 7 to 4.9-5.5. However, most of the residual antibody is evidently still bivalent since it forms about 70% as much specific precipitate as an equal weight of untreated antibody. However, with 0.01 M L-cysteine also present at pH 4 or pH 5, the fragments produced no longer form specific precipitates, but have acquired the capacity to block the homologous precipitin reaction. Also, the $s_{20,w}$ value of the fragments is 3.4-3.5, which agrees within experimental error with the values observed after treatment with papain (3.4-3.8).

Further evidence for the similarity of fragments produced by pepsin-cysteine, or papain-cysteine was provided by the quantitative inhibitory effects of the two types of fragment on the homologous precipitin reaction. The antiovalbumin γ-globulin was treated with papain as described by Nisonoff and Woernley (1959a). The sedimentation constant ($s_{20,w}$) of the resulting fragments was 3.5. This preparation and one obtained by treatment with pepsin (1% of the globulin) and L-cysteine (0.01M) at pH 5 under conditions described in Table I were tested for their inhibitory effect on the precipitin reaction of the untreated antibody. The conditions used were those given in the third footnote of Table I. In each case 0.7 mg of fragments caused a reduction of less than 30% in the amount of precipitate formed, whereas 1.4 mg resulted in more than 75% inhibition. Since

TABLE I

Effect of Cysteine Concentration and pH on the Peptic Digestion of Rabbit Antiovalbumin γ-Globulin*

pH of Reaction	Cysteine Conc. moles/l	% of Original Protein Dialyzable	Amount of Specific Ppt. % of control**	Blocking Activity mg. for 50%*** inhibition	$s_{20,w}$
4	0	19	65	-	5.5 (>90%) +slower material
4	0.01	31	0	Between 0.7-1.4	3.4 (>90%) +slower material
5	0	13	74	-	7.0 (40%) 4.9 (>50%)
5	0.01	17	0	0.7-1.4	3.5 (>95%)
6	0	4	85	-	6.7 (>95%)
6	0.01	5	74	-	6.9 (>90%) +slower material
5 and 8†	0.01	14	0	0.7-1.4	3.4 (80%) 6.4 (20%)
5 and 8‡ (2-mercapto-ethylamine)	0	11	0	0.7-1.4	3.4 (77%) 6.4 (23%)

* Reactions for 18 hrs. at 37° except as indicated. Mixtures contained 85 mg. antiovalbumin γ-globulin, 2.5 mg crystalline pepsin, total volume 3 ml. Buffer contained 0.07 M acetate and 0.05 M chloride (sodium salts). At the end of the reaction mixtures were adjusted to pH 8 and dialyzed *vs.* cold saline-borate buffer, pH 8, μ = 0.16.

** Data for optimum antigen concentration. Reactions in duplicate, in saline-borate buffer, pH 8, μ = 0.16, for five days in refrigerator. Protein concentration of antibody solution (treated or untreated), 7 mg/ml. Each tube contained 0.2 ml each of antibody and antigen. Average value of mean deviation, 5 μg of ppt. Control (with untreated antibody), 298 μg of ppt. Amounts of ppt. estimated from optical density at 280 mμ in 0.02 N NaOH.

*** Weight of treated protein necessary to cause a 50% reduction in amount of ppt. formed by 1.4 mg of untreated antiovalbumin γ-globulin reacting with slightly less then an optimum concentration of antigen. In each case where blocking occurred 1.4 mg caused more than 70% inhibition. Amount of ppt. formed in absence of inhibitor, 266 μg. No effects were observed on precipitation in a heterologous system (bovine-γ-globulin-rabbit antibovine-γ-globulin).

† Antibody was treated with pepsin (18 hrs., 37°, pH 5), adjusted to pH 8, dialyzed vs. saline-borate buffer, treated with 0.01 M cysteine (8 hrs., 37°, pH 8) and redialyzed.

‡ Same as above, except that 2-mercaptoethylamine was substituted for cysteine.

the blocking capacity of the fragments produced by papain is attributable to the fact that they are univalent, it seems evident that the combined action of pepsin and L-cysteine on the antibody also yields univalent fragments, having approximately the same sedimentation constant.

As indicated by the last two experiments in Table I, fragments with $s_{20,w}$ = 3.3 or 3.4, which inhibit precipitation, are also formed by the successive action of pepsin at pH 5 and either cysteine or 2-mercaptoethylamine at pH 8. This indicates that the breakdown into univalent fragments can take place in two stages, the latter probably involving reduction of a disulfide bond. So far we have not been able to produce fragments having these characteristics by the action of cysteine, followed by dialysis and treatment with pepsin, despite efforts to prevent reoxidation by the addition of an excess of iodoacetamide prior to dialysis and treatment with pepsin.

The formation of fragments with blocking activity is not observed upon treatment with pepsin and cysteine at pH 6 (Table I), by the action of cysteine alone at pH 5 or pH 8, or by successive treatment with pepsin and cysteine or pepsin and 2-mercaptoethylamine at pH 8. Nor does either reducing agent at pH 8 have any appreciable effect on the sedimentation constant or specific precipitation. Using mixtures of 3% pepsin (based on the weight of globulin) and cysteine (0.01 M) at pH 5 we observed very little breakdown of the antibody molecule in 48 hours at 0° and only partial hydrolysis in 24 hours at 25°. The minimum quantities of reagents needed for essentially complete hydrolysis into fragments with $s \cong 3.5$ (pH 5, 18 hours, 37°) are approximately 0.003 M cysteine (with 3% pepsin present) and 1% pepsin (with 0.01 M cysteine).

We suggest that the two active fragments, similar in size and blocking activity to the chromatographically-separable fractions labelled I and II by Porter, are held together only by one or more disulfide bonds; and that the function of the pepsin is to split off an inactive fragment, possibly exposing disulfide bonds to the action of a reducing agent. Since a reducing agent has always been used with papain, it seems quite possible that proteolysis with that enzyme also occurs in

two stages; this and other aspects of the mechanism are under investigation.

References

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