THE EFFECT OF LIMITED NITRATION ON ANTIBODIES TO DINITROPHENYL GROUP *

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1. Introduction

The studies of Singer and his colleagues, using affinity labelling, have demonstrated that tyrosine residues are present in the combining sites of antibodies of a fairly wide range of specificities, including antibodies to the 2,4-dinitrophenyl (DNP) group [1,2]. Iodination studies also inferred tyrosine to be present in the combining site of different antibodies [3,4]. However, in the iodination experiments a substantial decrease in antibody activity was achieved only when a relatively large number of iodine (about 30 atoms per molecule) have been incorporated into antibody.

Tetranitromethane (TNM) was recently introduced as a mild specific reagent for nitration of tyrosyl residues of proteins [5] and it has been demonstrated that in certain cases TNM reacts selectively with unique tyrosyl residues [6,7]. We report here the effect of TNM on anti-DNP antibodies. Nitration of an average of two tyrosyl residues per anti-DNP molecule destroyed about 50% of the antibody combining sites. In addition the precipitation of the nitrated antibody with DNP-ovalbumin became dependent on the ionization of the nitrotyrosyl residues. Both effects could be prevented by the presence of hapten during the nitration.

2. Materials and methods

Rabbit anti-DNP antibodies were isolated from the pooled immune serum using a trinitrophenyl-bovine gamma globulin-bromoacetylcellulose immunoadsorbent as described by Jaton et al. [8]. Antibodies to poly-D-alanine [9] were a gift from Dr. J.Haimovich. Equilibrium dialysis was performed according to Eisen [10] using α,N-[^{3}H]-acetyl-ϵ-DNP-lysine (22,000 cpm/mμ mole) as hapten. Samples were counted in 10 ml of Bray's solution in a Packard Tricarb liquid scintillation spectrophotometer. Nitration was carried out by the addition of TNM (Fluka) to the antibody solution (1.2 mg/ml in 0.07 M NaCl, 0.1 M Tris buffer pH 8.2). The concentration of ethanol never exceeded 5% and similar amounts of ethanol were added to the control. After 1 hr of incubation at room temperature the solutions were dialyzed against 0.15 M NaCl.

Quantitative determination of nitrotyrosine was obtained from the absorption at 428 mμ [6]. Routinely the absorption spectrum of the nitrated samples was measured at acid (pH 3) and alkaline (pH 11) conditions and the amount of nitrotyrosine was calculated from the difference in the absorbance at 428 mμ using 4100 as the molar extinction coefficients of nitrotyrosine [6]. The molar absorbance of the antibodies at 280 mμ was taken as 210,000.

3. Results

3.1. *Effect of nitration on the precipitability of antibody*

Table 1 gives the nitrotyrosine content of anti-DNP antibodies after nitration with increasing molar ratios of TNM to antibody. The results of precipitin analysis of the nitrated antibody preparations (fig. 1) show

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Table 1
Nitration of Anti-DNP antibody with TNM.

TNM/antibody (M/M)	Nitrotyrosine/antibody (M/M)
5	1.1
10	2.0
15	2.9
20	3.8
30	5.0

TNM (4 μmole/ml in ethanol) was added to anti-DNP antibodies (1.2 mg/ml in 0.07 M NaCl, 0.1 M Tris buffer pH 8.2). After 1 hr of incubation at room temperature the solutions were dialyzed against 0.15 M NaCl and the nitrotyrosine content of the antibody was determined spectrally as described in section 2.

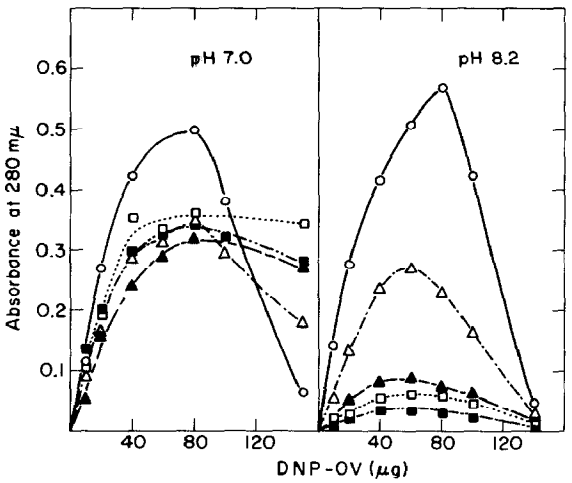

Fig. 1. The effect of nitration of anti-DNP antibodies on the precipitin reaction at pH 7.0 and pH 8.2. 0.5 mg of intact or nitrated anti-DNP antibodies were incubated with increasing amounts of DNP-ovalbumin in 1 ml of either 0.1 M NaCl, 0.04 M phosphate buffer pH 7.0, or 0.1 M NaCl, 0.04 M Tris buffer pH 8.2. After incubation for 1 hr at 37° and 16 hr at 4° the precipitates formed were centrifuged, washed twice with cold 0.15 M NaCl and were dissolved in 1 ml of 0.1 M NaOH. The absorbance of the dissolved precipitates was determined at 280 mμ. ○, intact antibody; △, antibodies containing 1.1 nitrotyrosyl residues; ▲, antibodies containing 2.0 nitrotyrosyl residues; □, antibodies containing 2.9 nitrotyrosyl residues; ■, antibodies containing 3.8 nitrotyrosyl residues per molecule.

marked difference between the amount of immune precipitate formed at pH 7.0 and pH 8.2. At pH 8.2 the precipitability of nitrated antibody decreased markedly with increasing degree of nitration. Thus only 50% and 15% of the immune precipitate was formed after nitration of 1.1 and 2.0 tyrosyl residues, respectively, per antibody molecule. The difference between the precipitability of the nitrotyrosyl antibody at pH 7.0 and pH 8.2 (fig. 1) suggested that the ionization of the nitrotyrosyl residues plays a role in this behaviour. The pH dependence of the formation of immune precipitate by the antibodies containing two nitrotyrosyl residues as compared with intact antibodies is demonstrated in fig. 2. It is clear that there is a correlation between the ionization of nitrotyrosyl residues, exhibited by the absorbance at 428 mμ (λ_{max} for the ionized form of nitrotyrosine), and the amount of precipitate formed.

Fig. 3A shows the precipitin analysis of antibodies that were nitrated in the presence or absence of the hapten 2,4-dinitrophenol (excess hapten was removed

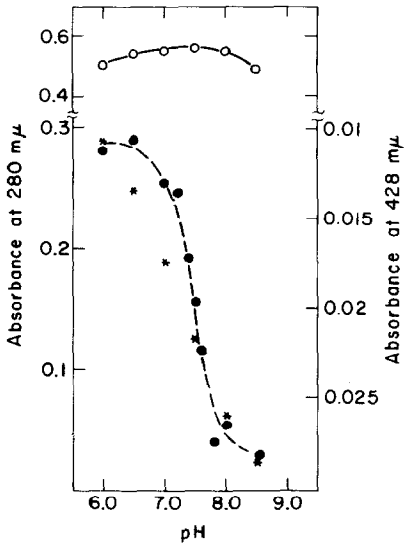

Fig. 2. The pH dependence of the immune precipitation of modified antibodies. 0.5 mg of antibodies were reacted with 60 μg DNP-ovalbumin in 0.1 M NaCl, 0.04 M phosphate buffer at different pH values. The precipitates formed were analyzed as described in fig. 1. ○, intact antibodies; •, antibodies containing 2.0 nitrotyrosyl residues; ⋆, the absorbance of the nitrated anti-DNP antibodies (0.5 mg/ml) at 428 mμ.

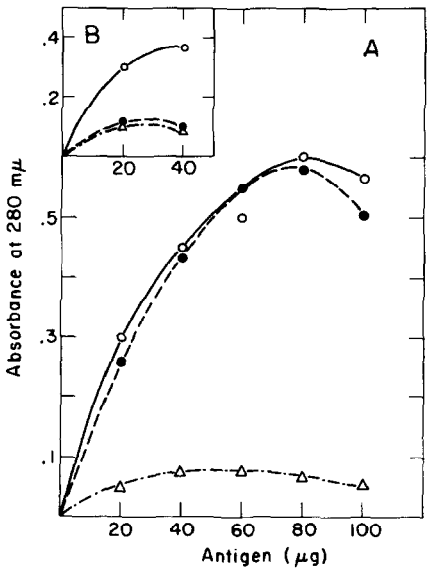

Fig. 3. Protection by hapten against the effect of nitration. Precipitin reaction was performed at pH 8.2 as described in fig. 1. ○, control, antibodies that were incubated with 0.005 M DNPOH; ●, antibodies nitrated in the presence of 0.005 M DNPOH; △, antibodies nitrated in the absence of DNPOH. A: anti-DNP antibodies. The nitrated antibodies contained 2.2 nitrotyrosyl residues per molecule. B: anti-poly-D-alanine antibodies. These nitrated antibodies contained 3.0 nitrotyrosyl residues per molecule. The antigen used was poly-D-alanyl rabbit serum albumin.

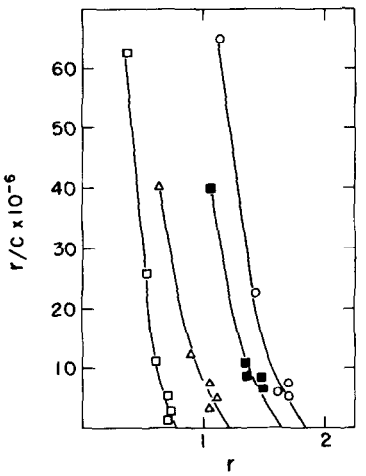

Fig. 4. Binding curves of α,N-[^{3}H]-acetyl-ε-DNP lysine by intact and nitrated anti-DNP antibodies as measured by equilibrium dialysis. The antibody samples were at a concentration of 35 μg/ml. C is free hapten concentration and r is moles of hapten bound per mole of antibody (assuming an antibody molecular weight of 150,000). Dialysis with mixing by gentle rotation was performed in dialysis bags (2 ml) against various concentrations of hapten solutions (2 ml) at pH 7.0, 25° for 10 hr. Aliquots of both the inside and outside solutions were counted. ○, intact antibody; △, antibody nitrated with 5-fold molar excess of TNM; □, antibody nitrated with 10-fold molar excess of TNM; ■, antibody nitrated with 10-fold molar excess of TNM in the presence of α,N-[^{3}H]-acetyl-ε-DNP lysine (20-fold molar excess over antibody).

by exhaustive dialysis against 0.15 M NaCl containing some Dowex-1 × 8). It is demonstrated that the hapten protects the antibody against the effect of nitration. This protection seems to be specific since dinitrophenol did not protect an unrelated antibody (anti-poly-D-alanine antibodies) against the effect of nitration (fig. 3B).

3.2. *Effect of nitration on binding capacity of antibody*

This was studied by means of the equilibrium dialysis technique. Fig. 4 demonstrates that the reaction of anti-DNP antibodies with 5 and 10-fold molar excess of TNM caused the destruction of about a 30% and 55% of the antibody combining sites, respectively. When antibodies were nitrated in the presence of the same hapten (20-fold molar excess of hapten over antibody) that was used for equilibrium dialysis there was a loss of only 10% of the antibody sites (fig. 4). Thus the hapten protects the antibody combining site against destruction by nitration. Contrary to the pH dependence of the precipitability of nitrated antibodies (fig. 2), the binding of hapten as measured by equilibrium dialysis was identical at pH 6.0, 7.0 and 8.5.

4. Discussion

The results presented in this communication demonstrate that limited nitration of only two tyrosyl residues in anti-DNP molecule caused two major effects: 1) destruction of about 50% of the antibody combining sites; 2) modification of the immune precipitability of the remaining active antibodies such that their precipitation (but not binding) with antigen became inversely correlated with the ionization of the nitrotyrosyl residue. Both effects of nitration could

be prevented by the presence of hapten during the reaction of the antibody with TNM.

The protection by hapten suggests that at least in the first effect the modification took place at the antibody combining site, probably by nitration of a tyrosyl residue. The effect of nitration on the precipitability of the antibody could be due to nitration in a place other than the combining site. However, the marked effect of only two nitrotyrosyl groups on the formation of immune precipitate and the protection by hapten against this effect might indicate that these tyrosyl residues are located in a unique position in the molecule.

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