PREPARATION OF ACTIVE IODINATED SPECIFIC ANTIBODIES

Jean-Marie PAGÈS, Daniel LOUVARD and Claude LAZDUNSKI
Centre de Biochimie et de Biologie Moléculaire du CNRS, 31, Chemin Joseph Aiguier, 13274 Marseille Cédex 2, France

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1. Introduction

Lactoperoxydase-catalyzed iodination of antibodies is very convenient since it is simple to perform and can be carried out in a short-time as previously pointed out by Morrison et al. [1]. Furthermore the radioisotopes of iodine are inexpensive, easy to detect, and iodinated proteins of high specific radioactivities can be obtained. However, it has been shown [1,2] that iodination of antibodies results in the partial inactivation of IgG molecules.

Many problems in molecular biology are currently approached by immunochemical techniques. In most instances inactivated antibodies must be discarded since active iodinated antibodies are required to obtain quantitative results. In this regard, we have recently described the use of immunochemical techniques in investigations of protein conformational change [3] and in topological studies on membrane bound enzymes [4].

Trace iodination of immunoglobulins, catalyzed by lactoperoxydase has been described by Marchalonis [5]. However, in our hands, using rabbit immunoglobulins, we obtained very little iodine incorporation in the conditions used by this author.

In this report we describe a method that allows the obtention of iodinated active antibodies of high specific radioactivity. This technique involves a lactoperoxydase-catalyzed iodination followed by the reisolation of specific antibodies on immuno-adsorbent columns. By this way all the IgG molecule that have been inactivated in the process of iodination are eliminated. Thus, we have been able to evaluate precisely the extent of inactivation as a function of the number of iodine atoms incorporated.

2. Materials and methods

2.1. *Materials*

[^{125}I] INa was obtained from Amersham (15 mCi/μg). Lactoperoxydase (EC 1.11.1.7) was purchased from Calbiochem. Indubiose Ac A-3/4 (a Biogel P 300 substitute with better flow properties) was from Industrie Biologique Française. Glutaraldehyde was a TAAB Laboratory product. All other products were reagent grade.

2.2. *Protein antigens*

Alkaline phosphatase was prepared from *E. coli* C 90 [6] and aminopeptidase was purified from pig intestinal mucosa as previously described [7].

2.3. *Protein concentrations*

This was routinely determined from absorbance measurements: for alkaline phosphatase at 278 nm $A^{1\%}_{1\,cm} = 7.7$ [8]; for aminopeptidase at 280 $A^{1\%}_{1\,cm} = 15.6$ [7]; and for IgG immunoglobulins at 280 nm $A^{1\%}_{1\,cm} = 13.5$.

2.4. *Preparation of polyacrylamide-protein derivatives*

Alkaline phosphatase, as well as aminopeptidase were coupled to glutaraldehyde activated beads of acrylamide-agarose gel (Indubiose) as described by Ternynck and Avrameas [9].

2.5. *Antibodies*

Carefully standardized conditions were used for immunization of rabbits. As a rule, each animal received a total of 6 mg of pure antigen. Rabbits were injected subcutaneously with 1 mg of antigen incorporated into complete Freund's adjuvant. Six weeks

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later, 1 mg was injected intramuscularly and 2 X 2 mg were injected the next two days. After one week, the animals were bled, and the antisera collected were filtered through 0.45 μm Millipore filters and kept at 4°C in the presence of (0.01%) NaN_3 until use. The antibodies were purified to homogeneity in a single step by affinity chromatography. Antisera were run continuously for two hours through specific immunoadsorbent gel columns. The unretarded proteins were washed with (PBS) phosphate saline buffer (10 mM phosphate, 0.15 M NaCl), until the optical density at 280 nm of the effluent was below 0.01. Antibodies were then eluted with a 0.2 M HCl–glycine buffer pH 2.2. The eluate was neutralized with 1 M dipotassium hydrogen phosphate, dialyzed against PBS buffer, concentrated to 5 mg/ml by vacuum dialysis, filtered through 0.22 μm Millipore filters and stored in small aliquots at −80°C until use. Antibodies purified by this technique were found to be homogeneous in SDS-gel electrophoresis and ultracentrifugation ($S_{20,w}$ 6.7).

2.6. *Determination of the number of iodine per mole of antibody*

This was computed from the specific radioactivity of iodine and the molar concentration of IgG immunoglobulins. Specific radioactivity of iodine was determined from an aliquot of the incubation mixture. Counting were carried out in a Packard auto gamma scintillation spectrometer. Routinely we used samples of [^{125}I] INa with a specific radioactivity of 10^9 cpm/μmol.

3. Results

In the incubation mixture (1–2.5 ml) of iodination the molar ratio of the iodide to the immunoglobulin G (IgG) was kept equal to 40. A ten fold molar excess of H_2O_2 over the concentration of the iodide was used (and the reaction was catalyzed by 22–75 μg of lactoperoxydase. Under these conditions the concentration of the anti-alkaline phosphatase antibody was varied. A typical experiment is described in fig.1. The incubation was carried out for 15 min at 20°C in PBS buffer at pH 7.4. The H_2O_2 was added by aliquots every 1 min. After 15 min the excess H_2O_2 was destroyed by addition of 5.5 mM L-cystein. The excess

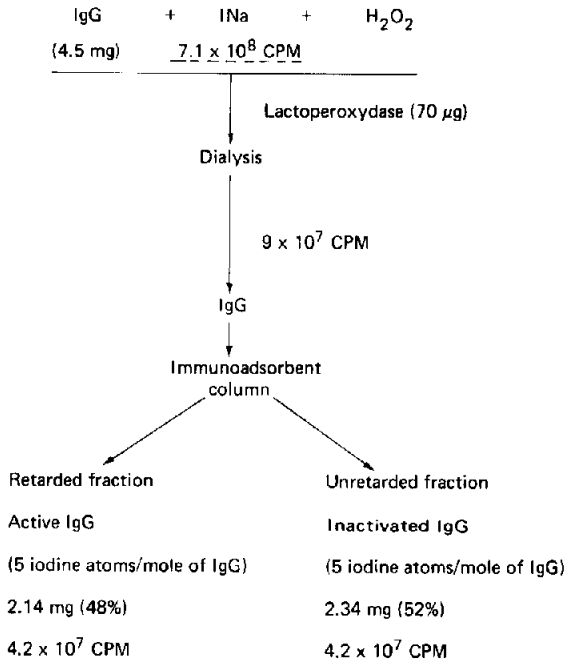

Fig.1. Diagram of the preparation of active iodinated IgG immunoglobulins.

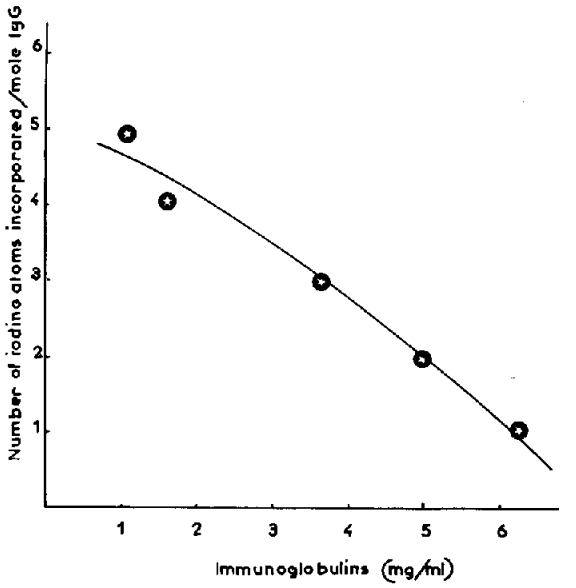

Fig.2. Effect of the concentration of IgG immunoglobulins in the reaction mixture, on the number of iodine atoms incorporated per mole of IgG.

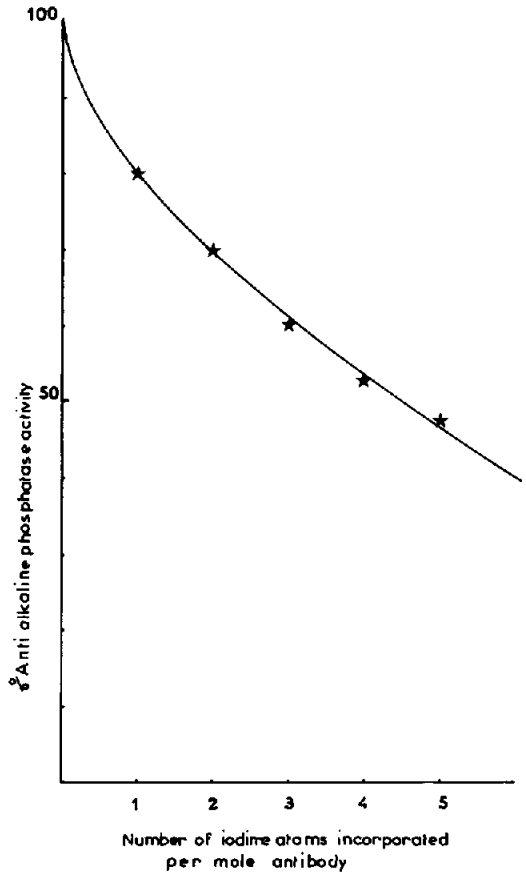

Fig.3. Effect of the extent of iodination on the activity of anti-alkaline phosphatase antibodies. IgG immunoglobulins were iodinated as described in Materials and methods and the percentage of inactivation was calculated from the fraction of the total amount applied that is retarded on immunoadsorbent column.

of iodide was eliminated by extensive dialysis against PBS buffer.

It was possible to control the number of iodine atoms incorporated per mole of antibody by varying the concentration of IgG in the incubation mixture (fig.2). Over a concentration range of 6 fold, that is when IgG concentration is increased from 1.1 mg/ml (final concentration) to 6.3 mg/ml the number of iodine atoms incorporated was decreased from 5 to 1.

The extent of inactivation of the iodinated antibodies was determined by applying this material on an immunoadsorbent column of alkaline phosphatase coupled to indubiose. Then, only active iodinated antibodies were retained whereas those antibodies inactivated by iodination were not retarded. After washing of the column with PBS buffer, active antibodies were eluted at acidic pH as described under Materials and methods. Thus, this technique allows the isolation of active iodinated antibodies. Furthermore, as shown in fig.3, it is possible to determine the extent of inactivation as a function of the number of iodine atoms incorporated. In this figure, the mean number of iodine atoms incorporated per mole of antibody has been plotted. However, in several experiments we found that the number of iodine incorporated was not exactly the same in the active antibody fraction retarded on the immunoadsorbent column and in the inactivated antibody fraction. One can find 10 to 20% difference of incorporation between the two antibody fractions and the higher incorporation is sometimes found in the active fraction.

4. Discussion

Iodination has long been used as a relatively specific reagent for determining the role of certain functional groups in proteins [10]. Iodine is primarly incorporated into tyrosine although histidine can also be iodinated. Since tyrosine residues have been shown to be part of the binding site of various antibodies [11,12], it is not surprising that a significant inactivation of these molecules occurs when more than 1 mol of iodine is bound per mol of IgG. Iodination of available tyrosine residues depends upon accessibility and environment and, only the IgG molecules that have been iodinated in tyrosine residues involved in the binding site, are inactivated.

The effect of the variation of rabbit IgG immunoglobulin concentration on the extent of iodination is not easy to explain. Indeed, an increase in IgG concentration results in a decrease in the amount of iodine incorporated per mol of IgG in 15 min. However, this has been always observed in the range of 1 to 10 mg/ml of rabbit IgG immunoglobulins, and we can assume that mostly tyrosine residues are iodinated by lactoperoxydase since this results in a decrease of binding activity as shown in fig.3.

An early report by Johnsen et al. [2] indicated that incorporation of less than two atoms of iodine resulted in no loss of activity. More recently, Morrison

et al. presented similar results [1]. However, with the availability of immunoadsorbent columns the extent of inactivation can be more precisely determined and we find that 20% inactivation occurs if only one atom of iodine is incorporated per mole of antibody. Moreover the extent of inactivation as a function of the number of iodine atoms incorporated is not as pronounced as that found by Morrison et al. These discrepancies might be due either to the fact that the loss of activity was assayed differently or to the fact that the antigen is totally different.

The technique described in this report is particularly useful when active iodinated antibodies are required for quantitative determinations of their binding to antigenic determinants of proteins [3,4]. This technique has been also tested with the anti-aminopeptidase antibody, from intestinal brush border membrane, purified as described previously [7] on immunoadsorbent column of aminopeptidase coupled to indubiose. Identical results were obtained although the number of antigenic determinants is twice that of the alkaline phosphatase of *E. coli* [3]. This suggest that the applicability of the method described is quite large.

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References

[1] Morrison, M., Sisco Bayse, G. and Webster, R. G. (1971) Immunochemistry 8, 289–297.
[2] Johnsen, A., Day, E. D. and Pressman, D. J. (1960) J. Immun. 84, 213–218.
[3] Lazdunski, C., Pagès, J. M. and Louvard, D. (1975) J. Mol. Biol., in press.
[4] Louvard, D., Maroux, S. and Desnuelle, P. (1975) Biochim. Biophys. Acta 389, 389–400.
[5] Marchalonis, J. J. (1969) Biochem. J. 113, 299–305.
[6] Lazdunski, C. and Lazdunski, M. (1967) Biochem. Biophys. Acta 147, 280–288.
[7] Maroux, S., Louvard, D. and Baratti, J. (1973) Biochem. Biophys. Acta 321, 282–295.
[8] Schlesinger, M. J. and Barret, K. (1965) J. Biol. Chem. 240, 4284–4292.
[9] Ternynck, Th. and Avrameas, S. (1972) FEBS Lett. 23, 24–28.
[10] Vallée, B. L. and Riordan, J. F. (1969) Ann. Rev. Biochem. 38, 733–766.
[11] Pressman, D. and Roholt, O. (1961) Proc. Nat. Acad. Sci. USA 47, 1606–1610.
[12] Wofsy, L., Metzger, H. and Singer, S. J. (1962) Biochemistry 1, 1031–1039.