IMMUNOCHEMICAL STUDY OF PHAGE T4 LYSOZYME

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1. Introduction

Quantitative immunochemical methods based on [1] and initiated [2–4], have been developed and applied successfully to study the refolding of several proteins, among them, serum albumin [5–8], ribonuclease [9–12], elastase [13,14] and bovine pancreatic trypsin inhibitor [15].

When injected into rabbit or goat, native proteins generally elicit antibodies directed against antigenic determinants located at the surface of a protein, and specific to the native conformation. Antibody probe is very sensitive to variations in protein conformation and thus may be a useful tool to detect any modification which affects the surface of the protein.

For a quantitative immunochemical approach, it is necessary to know precisely the immunological properties of the antigen. Here, we describe the purification of anti-phage T4-lysozyme antibodies, and study antigen–antibody interactions. The refolding of T4-lysozyme is studied by immunoassay.

2. Materials and methods

2.1. *Enzyme*

Phage T4 lysozyme was prepared and purified according to [16], as modified [17,18] with further minor changes.

2.2. *Enzyme assay*

Enzyme assays were performed on a suspension of 0.4 mg/ml lyophilized *Escherichia coli* in a 0.05 M Tris buffer (pH 7.4) containing 1 mM β-mercaptoethanol and 1 mM $MgCl_2$. Enzyme final concentration was 10^{-10}–10^{-12} M. The decrease of turbidity at 450 nm was recorded on a Cary 219 or on a Cary 14 spectrophotometer.

Abbreviations: Ab, antibody; Ag, antigen

2.3. *Immunization of rabbits*

Anti-T4 lysozyme serum was obtained by hyperimmunization of rabbits by intradermal injections of 0.6 mg T4 lysozyme emulsified within an equal volume of complete Freund's adjuvent. Subsequent booster injections were administrated at intervals of 3 weeks under the same conditions but using incomplete Freund's adjuvent. Rabbits were bled 1 week after each booster injection.

2.4. *Purification of anti-T4 lysozyme antibodies*

Serum was treated twice with 45% saturation $(NH_4)_2SO_4$ and centrifuged; the precipitate was dissolved and dialyzed against saline; this solution was kept in sealed tubes at −25°C.

Two different kinds of fractionation were performed, a fractionation on DEAE–Sephadex A-50 column as described for elastase [12], and a fractionation on affinity column. To obtain specific anti-T4 lysozyme antibodies, a column of Sepharose to which T4 lysozyme was covalently bound was prepared as in [18], but with milder conditions of coupling (pH 7.0) because of the large number of amino groups in T4 lysozyme. Elution was achieved with 0.1 M glycine–HCl, 0.5 M NaCl buffer (pH 2.8).

2.5. *Preparation of F_{ab} fragments*

F_{ab} fragments were prepared as in [19]; they were separated from F_c fragments by chromatography of the total antibodies on a Sepharose–protein A column (Pharmacia, Uppsala). Specific anti-T4 lysozyme F_{ab} fragments were obtained by affinity chromatography on a T4 lyzozyme–Sepharose column and eluted as the specific antibodies.

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Concentration of ^{125}I-labeled F_{ab} fragments was determined from Mancini's radial immunodiffusion technique [20] using goat antiserum to rabbit 7 S globulin (Hyland).

2.6. *Labelling of F_{ab} fragments*

F_{ab} fragments were iodinated (^{125}I) by enzymatic solid-phase method using immobilized glucose oxidase and lactoperoxidase (Biorad product) according to the technique in [21]. Na^{125}I (16 mCi/μg iodine) was an Amersham product. The labeled fragments had spec. act. 0.5×10^3 cpm/pmol. Radioactivity was measured on an Intertechnique counter.

2.7. *Immunological assays*

Quantitative precipitin analysis [22] was used for determination of anti-T4 lysozyme antibodies. Ouchterlony [23] double-diffusion technique and immunoelectrophoresis according to [24] were used to check the purity of antibodies or F_{ab} fragments.

3. Results

3.1. *Inactivation of T4 lysozyme activity upon binding of specific antibodies*

By fractionation on DEAE–Sephadex A-50, two fractions have been separated. Contrary to that observed for elastase [12], both fractions of antibody are able to inhibit enzymatic activity of T4 lysozyme. Fig.1 illustrates the inhibition produced by whole antibody population as a function of incubation time for various Ab/Ag molar ratios. Incubation was done at 4°C and at 20°C. No significant differences were observed at these two temperatures. Inhibition is instantaneous, indicating that Ab–Ag complex rapidly reaches its equilibrium. Similar results were obtained with antibodies fractioned on DEAE–Sephadex.

In fig.2, T4 lysozyme (final conc. 9×10^{-8} M) was incubated in the presence of increasing antibody concentration (from 0–4.5×10^{-7} M IgG) in saline solu-

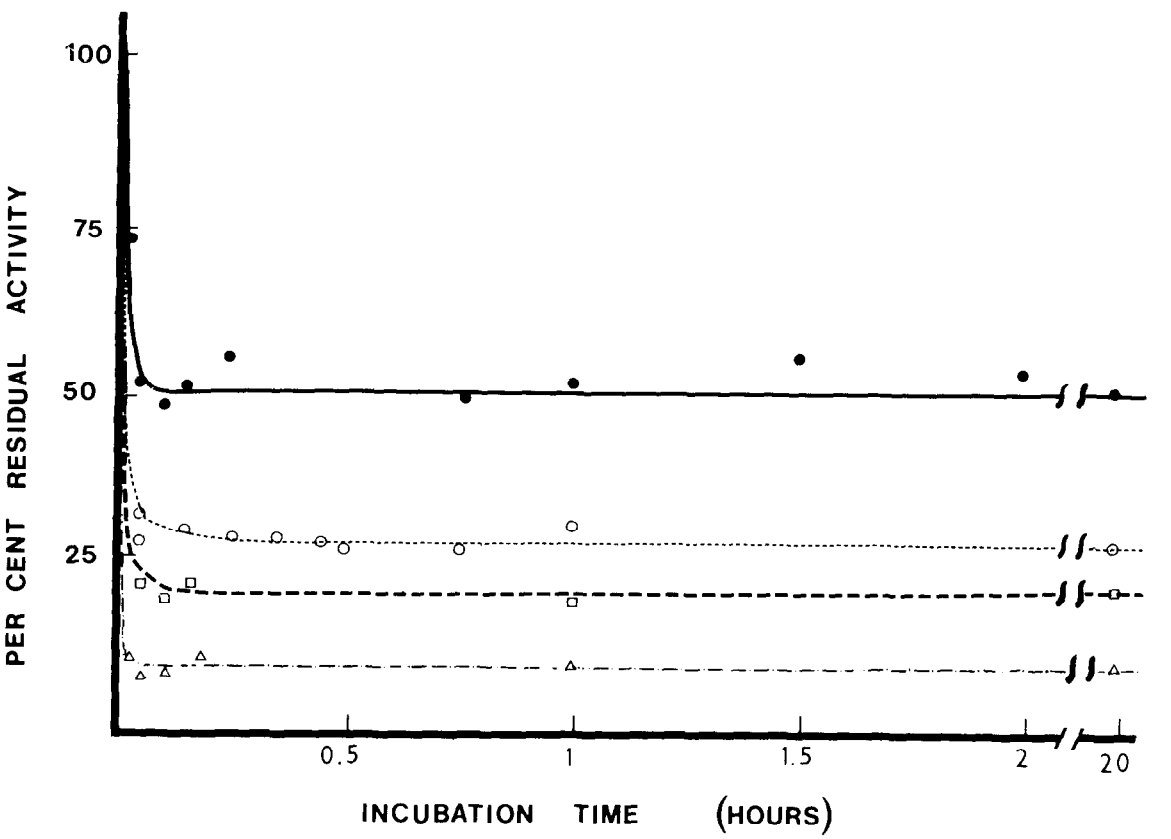

Fig.1. Inhibition of T4 lysozyme activity by anti-lysozyme antibodies *vs* incubation time. Molar ratio Ab/Ag: (●) 0.25; (○) 0.50; (□) 0.75; (△) 1.00.

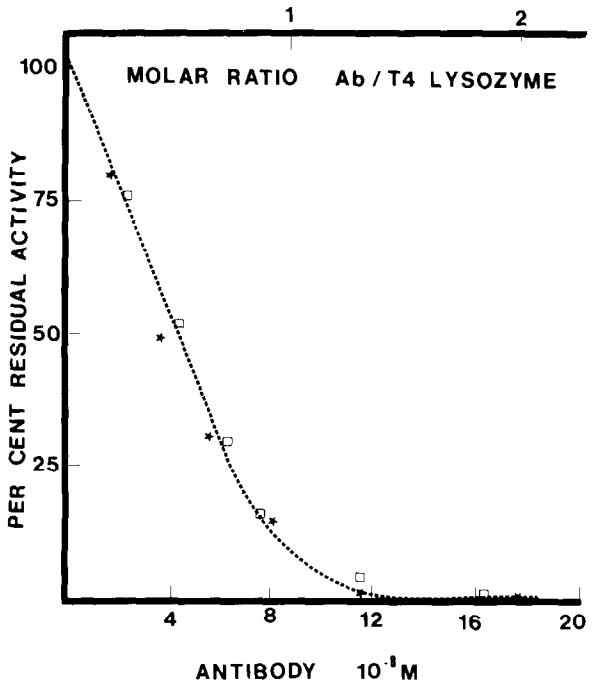

Fig.2. Effect of antibodies on enzymatic activity. Each sample contained 9×10^{-8} M final concentration of T4 lysozyme, the specified final concentration of immunoglobulins G anti-T4 lysozyme and 20 μl (10 mg protein) of non-immunized rabbit serum. Activity was assayed after 12 h incubation at 4°C and results were expressed in % of residual activity, taking 100% as the activity of the sample which did not contain antibodies. Results obtained with antibodies before (⋆) and after (□) fractionation on DEAE–Sephadex.

tion at 4°C. The incubation was performed in the presence of non-immunized rabbit serum to discard any non-specific inhibition. Residual T4 lysozyme activity was measured after 12 h incubation at 4°C.

Inhibition is complete for an Ab/Ag ratio of 1/1. This result was obtained either with total antibodies or with specific anti-T4 lysozyme antibodies. It indicates that total inhibition is provoked by binding of 1 antibody by antigen molecule. Since these antibodies are not monospecific, we can conclude that the inhibition is not specific and not only provoked by an antibody population directed against the active site. It occurs whatever may be the antigenic site occupied by the antibody. The binding of any molecule of antibody on T4 lysozyme prevents the formation of enzyme–substrate complex, probably by steric hindrance. This result may be explained by the size of the substrate. In [25] inhibition of hen egg white lysozyme by specific antibodies was found to be due to steric hindrance.

Similar results were obtained with F_{ab} fragments; however, in this case the complete inhibition required a molar ratio F_{ab}/Ag of 1.5/1; the value of 1.5 rather than 1 may be explained by the presence of a fraction of inactive F_{ab} fragments.

The presence of cell walls in a typical enzymatic run (0.4 mg/ml) protects the enzyme from the action of antibodies added 1 min after the zero time of reaction. Under these conditions, 50% inhibition was reached for an Ab/Ag ratio of 100/1 (not shown).

3.2. *Determination of the number of antigenic sites*

A quantitative technique using ^{125}I-labeled F_{ab} fragments to evaluate the number of antigenic determinants, i.e., the maximum number of specific F_{ab} fragments that can bind to the surface of the antigen, was reported in [26,27]. Here, we have used an indirect procedure. Increasing quantity of T4 lysozyme was added to a fixed amount of ^{125}I F_{ab} fragments (final conc. 10^{-7} M). They were incubated for 24 h at 4°C in saline solution; free F_{ab} was removed by addition of T4 lysozyme immunoabsorbant and centrifugation. Radioactivity of bound F_{ab} was measured in the supernatant. Concentration of both F_{ab} and antigen were kept higher than the dissociation constant to obtain saturation. Further addition of T4 lysozyme immunoabsorbant did not modify T4 lysozyme–F_{ab} complex (fig.3); 4.1 ± 0.2 antigenic determinants in T4 lysozyme were determined (fig.3). A value of 5 ± 1 was evaluated from quantitative immunoprecipitation; although this last method is less accurate, there is an agreement between the two results. The number of antigenic determinants of T4 lysozyme is consistent with its M_r-value according to the relationship proposed in [28].

3.3. *Study of the antigen–antibody equilibrium using enzymatic activity in the immunoassay*

Since the antigen–antibody complex was inactive, the enzymatic activity was used to determine the amount of free antigen, the sensitivity of the assay allowing the detection of as little as 10^{-12} M enzyme. This technique has several advantages. It does not require a separation of the antigen–antibody complex from free antigen and antibody, and no modification of the antigen, which could modify the antibody–antigen interaction, is introduced.

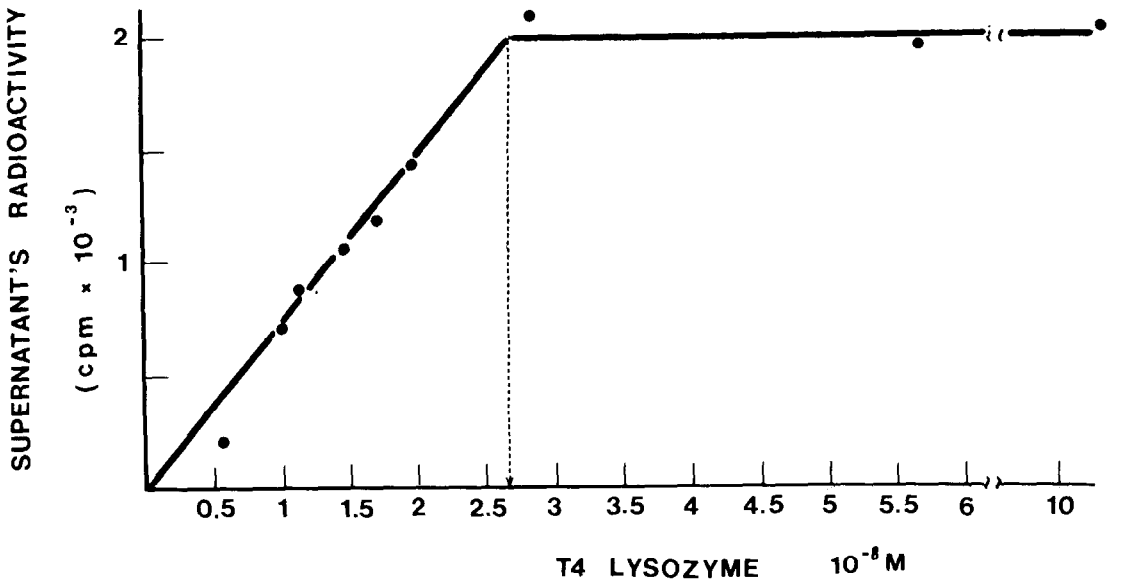

Fig.3. Determination of the number of antigenic sites: 45 pmol (1.8×10^4 cpm) of ^{125}I-labelled F_{ab} fragments (1.13×10^{-7} M final conc.) were incubated for 12 h at room temperature with the specified amount of T4 lysozyme and 20 μl non-immunized rabbit serum. Final volumes were adjusted to 400 μl with 0.9% NaCl. After incubation, 200 μl of a T4 lysozyme-immunoabsorbant suspension were added to remove free F_{ab} and were incubated at room temperature for 30 min with gentle shaking. After centrifugation, bound F_{ab} was determined by the measure of the radioactivity of aliquots of the supernatant. As a control, radioactivity of the pellet after exhaustive washing was also measured in few samples. Inactive F_{ab} fragments were determined by the measure of the radioactivity in an aliquot of the supernatant after incubation of F_{ab} fragments (1.13×10^{-7} M final conc.) with an excess of T4 lysozyme immunoabsorbant. This quantity was subtracted from the radioactivity assayed in the experiments. To determine non-specific fixation of F_{ab} fragment on Sepharose, reference was made with hemoglobin immunoabsorbant instead of T4 lysozyme immunoabsorbant: fixation was observed.

T4 Lysozyme in a saline solution of 5–100 μl (final conc. 10^{-10}–10^{-7} M) was added to 150 μl antibody (final conc. 10^{-9} M) and 20 μl non-immunized rabbit serum. Saline solution was added to obtain a final volume of 1 ml. For each sample, a blank (without antibody) was prepared; the recorded values correspond to 100% activity. Incubation was allowed for 18 h at 4°C. Enzymatic activity of both sample and reference was measured after incubation.

The data were analyzed according to Scatchard [20]; B/F vs B is plotted in fig.4, taking (total activity – residual activity) as bound antigen and residual activity as free antigen. It corresponds to the results of several experiments. It allows us to determine a unique average affinity constant of $(5 \pm 0.5) \times 10^9$ M^{-1}. Since all the data fit on a single straight line, it seems that the various antibody populations specific to different antigenic determinants have very similar affinities for the antigen.

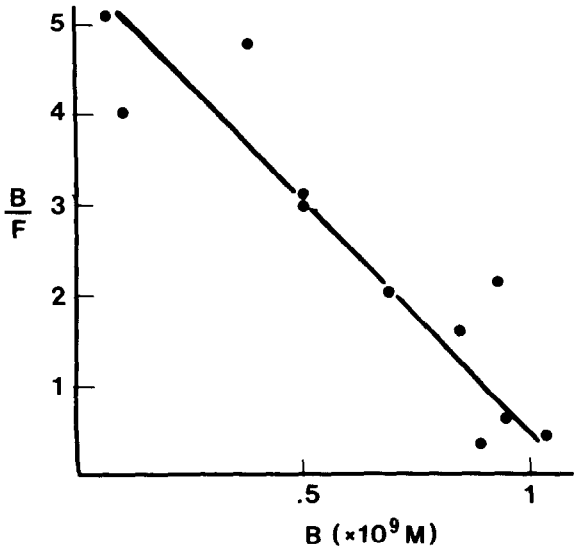

Fig.4. Scatchard analysis of the data obtained from several equilibrium experiments on the inactivation of T4 lysozyme by antibodies. The curve intercepts the abscissa at 10^{-9} M, corresponding at 1 immunoglobulin bound to T4 lysozyme.

4. Conclusion

These results show that T4 lysozyme contains 4 antigenic sites; it is consistent with the size of the molecule. Each site has about the same affinity constant of 5×10^9 M^{-1} for antibodies. The interaction of antibodies with any of these 4 sites induces an inhibition of the enzymatic activity which is not specific, but likely results from a steric hindrance. This inactivation can be prevented by incubation of the enzyme in the presence of the substrate prior to addition of antibodies.

The inhibition of enzymatic activity provides an interesting property which was used to develop the immunoassay. Thus antibody probe can be used to study the folding of T4 lysozyme quantitatively.

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