EVIDENCE FOR POSITIVE COOPERATIVITY IN ANTIGEN–ANTIBODY REACTIONS

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Received 30 November 1974

1. Introduction

During the course of investigations on the radioimmunological assay of 3,5,3′-triiodo-L-thyronine (T_3) and L-thyroxine (T_4), we have studied several T_3- or T_4-specific antisera [1]. For very low concentrations of hormone, it was observed that the binding of T_3 or T_4 to their specific antibodies increased with hapten concentration, results which did not fit with the currently accepted hypothesis of antigen binding occuring independently on antibody binding sites [2–6]. Zimmering et al. [7] observed abnormal binding of certain antibodies to steroid haptens and suggested that cooperative interactions, such as those observed in oxygen binding to hemoglobin or in regulatory enzymes [8, 9] might explain the results. Matsukura et al. [10] and Matsuyama et al. [11] also described a similar phenomenon in the assay of ACTH but the authors did not investigate the mechanism of the reaction. While this work was in progress, Weintraub et al. [12] reported that, for minute amounts of human chorionic gonadotropin the percent of labeled hormone increased with increasing native hormone concentration. This effect was also observed with the divalent $F(ab)_2$ fragments but not with univalent Fab fragments. This observation lent support to the view that the enhanced binding reaction was consistent with positive cooperativity between the two binding sites of certain 7S antibodies.

In this letter, we report the results of our studies on the development of a theoretical model based on the assumption of non-independent binding sites existing on the antibody molecule. The fit of the experimental results with the predictions of the model strongly suggests that a positive cooperative interaction in the binding of antigen on a two binding site antibody is operative.

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2. Materials and methods

2.1. *Materials*

Antisera were raised by immunization of rabbits with T_3- and T_4-bovine serum albumin conjugates. Maximum titers, i.e. the dilution of antisera which allows 50% binding of 100 pg of labeled hormones, were 1:15000 for anti-T_3 antiserum (LaT_3) and 1:10000 for anti-T_4 antiserum (LaT_4). Two other anti-T_3 and anti-T_4 antisera of lower titers were also used.

[^{125}I]T_3 and [^{125}I]T_4 of very high specific radioactivities (400–600 mCi/mg) were obtained from Abbott (Chicago, Ill., USA) and purified by chromatography on Sephadex G-25 (Pharmacia Uppsala, Sweden) equilibrated with 0.02 N NaOH.

Charcoal Norit A and bovine serum albumin (BSA) were provided by Sigma (St. Louis, Mo., USA) and Dextran T40 by Pharmacia.

All reagents were diluted in 0.08 M veronal buffer pH 8.4 containing 0.1% BSA.

2.2. *Experimental procedure*

Increasing amounts of [^{127}I]T_3 in the presence of

1 pg [^{125}I] T_3 or increasing amounts of [^{125}I] T_3 were incubated for 3 to 4 days at 4°C with high concentrations (10-fold the titer) of anti-T_3 antiserum in ml of buffer. Reactions between T_4 and anti-T_4 antisera were studied in the same way. After the equilibrium was reached, free and antibody bound hormones were separated by incubating the reaction mixture (10 min at 4°C) with 1 ml of a slurry of charcoal–dextran–albumin (10mg/ml in the same buffer) and centrifuging for 10 min at 5000 *g*. Radioactivity of the supernatant and precipitate was estimated in a Packard Autogamma scintillation spectrometer for enough time to reach 10000 counts. Results were corrected for background and non specific binding by using appropriate controls.

Each figure is the mean of triplicate or quadruplicate assays with maximum deviation of 7% for counts lower than 500 cpm and 2 to 3% for counts higher than 2000 cpm.

3. Results

Addition of small increments of [^{127}I]- or [^{125}I] T_3 to antisera against T_3 results in a progressive sigmoidal increase of the percent of hormone bound to antibodies (*B/T*) until a maximum value is reached (fig. 1).

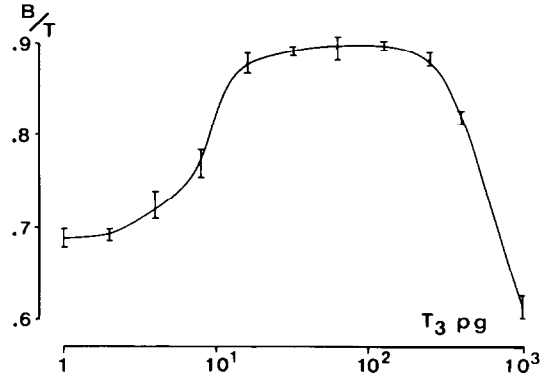

Fig. 1. Binding of T_3 to anti-T_3 antibodies. Antibody bound to total radioactivity (*B/T*) *versus* T_3 concentration is shown. For a given antiserum at the same dilution, the curves obtained using increasing amounts of [^{125}I] T_3 or 1 pg [^{125}I] T_3 and increasing amounts of [^{127}I] T_3 are superimposable. Such curves have been observed with 3 different anti-T_3 antisera. Bars indicate mean ± SEM of assays in triplicate.

A steeper increase of percent T_4 bound is observed with anti-T_4 antisera (fig. 2). In both cases a further increase in hormone concentration results in a decrease of the percent hormone bound.

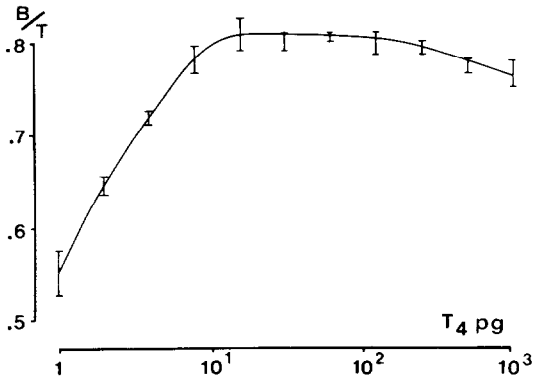

Fig. 2. Binding of T_4 to anti-T_4 antibodies. Same observations as in fig. 1.

These data have been analyzed according to the coordinate system introduced by Scatchard [13] by plotting the concentration of hormone bound to antibodies (*B*) as a function of the bound to free labeled hormone ratio (*B/F*). The plots are curvilinear with a convexity for positive *B/F* and show two values of *B* for one *B/F* (figs. 3 and 4).

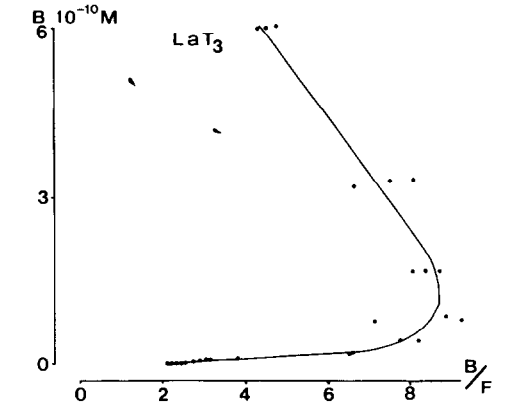

Fig. 3. Scatchard plot of the binding of T_3 to anti-T_3 antibodies. Antibody bound T_3 (*B*) *versus* antibody bound to free radioactivity (*B/F*) is shown. Results of fig. 1 have been used for calculations. Note the convexity of the curve towards positive *B/F* when *B* is very small (1–5 × 10^{-10}M).

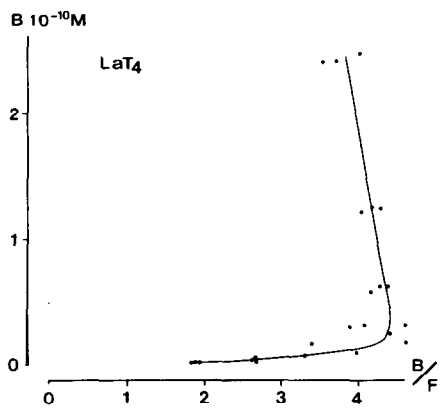

Fig. 4. Scatchard plot of the binding of T_4 to anti-T_4 antibodies. Same observations as in fig. 3.

Similar curves were obtained with all the antisera assayed. However the magnitude of the phenomenon varied greatly with the titer of the antisera and the dilution at which they were tested.

4. Theoretical study

The hypothesis of the independence of the two binding sites of antibodies [2–6] as represented in scheme *a* does not allow an interpretation of the obtained experimental curves.

(a) $\mathrm{Ab} + 2\mathrm{Ag} \overset{K}{\rightleftharpoons} \mathrm{Ab \cdot Ag_2}$.

An alternate hypothesis can be based on the assumption that the two sites are not independent (scheme *b*):

(b)
$$\mathrm{Ab} + \mathrm{Ag} \overset{K_1}{\rightleftharpoons} \mathrm{Ab \cdot Ag}.$$
$$\mathrm{Ab \cdot Ag} + \mathrm{Ag} \overset{K_2}{\rightleftharpoons} \mathrm{Ab \cdot Ag_2}$$

where K_1 is the equilibrium association constant of binding of the first antigen molecule and K_2 that of binding of a second antigen molecule to the remaining free site.

If q is the initial concentration of antibody, B the equilibrium concentration of antigen bound to antibody and F the equilibrium concentration of free antigen, an equation giving B can be written:

$$B = q\,\frac{K_1 F + 2K_1 K_2 F^2}{1 + K_1 F + K_1 K_2 F^2}$$

Giving to K_1, K_2 and q arbitrarily chosen values, one can plot the curves representing the percentage of antigen bound to antibody (B/T) as a function of antigen concentration (fig. 5).

In the case of positive cooperativity i.e. $K_2 > K_1/4$, the curves show a convexity towards positive B/T. This convexity disappears in the cases of independent antigen binding ($K_2 = K_1/4$) or negative cooperativity ($K_2 < K_1/4$). The ligand binding curves can also be plotted according to Scatchard relating the amount of antigen bound to antibody (B) to the B/F ratio of antigen–antibody complex to free antigen (fig. 6). When $K_2 > K_1/4$, the shape of the Scatchard plot is the same as that described by Changeux and Rubin [14] for the evaluation of the parameters of the allosteric model of Monod et al. [8] and by Cook and Koshland [15] in the case of positive cooperativity for the induced-fit model. Linear graphs are obtained in the case of antigen binding to independent sites. When $K_2 < K_1/4$ the Scatchard plots disclose a concavity towards positive B/F as in the case of negative cooperativity in the induced-fit model [15].

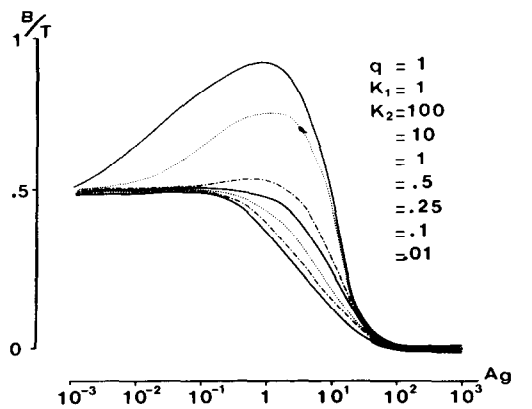

Fig. 5. Binding of an antigen on an homogeneous class of antibodies with two non-independent binding sites. The amount of antibody (q) and K_1 are settled as 1. K_2 variable from 100 to 0.01 (curves from top to bottom in that order). A convexity towards positive B/T (positive cooperativity) is observed for K_2 equal to 100, 10, 1 and 0.5. For K_2 = 0.25, 0.1 and 0.01 (no or negative cooperativity) no convexity is observed.

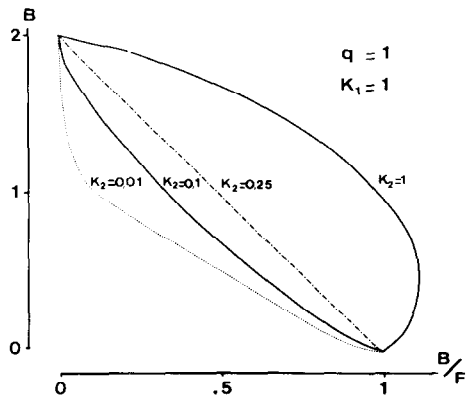

Fig. 6. Binding of an antigen to an homogeneous class of antibodies with non-independent bindings sites (Scatchard plot). Fixing $q = 1$ and $K_1 = 1$, K_2 variable. For $K_2 > 0.25$, the curves show a convexity towards positive B/F (positive cooperativity). For $K_2 = 0.25$ a linear curve is obtained (independent sites). For $K_2 < 0.25$ the curves show a concavity towards positive B/F. In this case the form of the curve does not allow a choice between homogeneous antibodies presenting negative cooperativity and heterogeneous antibodies.

5. Discussion and conclusions

According to the model of scheme *b* and as shown in fig. 5, when antigen concentration is very low the percent antigen bound to antibody is independent of antigen concentration or increases very slowly for increasing antigen concentrations. This fits with the experimental curves (fig. 1). Theoretically, the percent of antigen bound then increases more rapidly with increasing antigen concentrations up to a maximum, and then decreases (fig. 5) which is also observed for the experimental curves (figs. 1 and 2).

The fit between the model and the experimental results allows the following interpretation: at low antigen concentrations, all (B/T = constant) or almost all antigen molecules are bound to one and only one of the two different binding sites of the antibody. For a given antigen concentration, binding on the second site begins and B/T rapidly increases up to a maximum (figs. 1, 2, 5 and 6). Then, antigen binding increases less rapidly than the increase in antigen concentration and B/T decreases. Therefore a positive cooperativity in the binding of antigen to two different sites of the antibody explains the experimental results. This phenomenon can only be observed for an antigen concentration lower than that of antibody.

From our phenomenological studies and from the results of Weintraub et al. [12] a mechanism at the molecular level of the positive cooperativity showed by antibodies can be proposed. The 7S antibody molecule can be envisaged as an intramolecular dimer since it is formed of two parts, each resulting of the association of one heavy and one light chain. In the absence of antigen the molecule would be symmetrical; binding of one molecule of antigen to the first binding site would break the symmetry and induce an increased affinity of the second site. Another possibility woud be that the antibody molecule preexists in two different conformational states as assumed in the model of Monod et al. [8].

In fact, only a part of the specific antibodies seems to participate to the cooperative phenomenon. The Scatchard plots of the theoretical data show B/F maximum occuring for a B value of the order of magnitude of q, and intersection of the curve with the B axis for $B = 2\,q$ (fig. 6). Actually the experimental curves (figs. 3 and 4) show a B/F maximum occuring for a B value which is 10- to 100-times smaller than the value of the intersection with the B-axis obtained by extrapolation. Two possibilities might explain the results: (1) only a given class of specific antibody is able to undergo trans-conformation or exists in two conformational states, or (2) all the antibody molecules can loose symmetry by ligand binding but only a part of them would show a detectable increase in affinity for the second antigen molecule. Study of appropriate theoretical models will perhaps allow a choice between these two possibilities.

Our observations can be applied to the development of radioimmunoassays of high sensitivity. However, optimum conditions for an assay based on antibody cooperativity certainly differ from those which have been proposed for the optimization of the currently practised radioimmunoassay methods. Such a development is presently under study.

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