KINETIC MAPPING OF ANTIBODY BINDING SITES BY CHEMICAL RELAXATION SPECTROSCOPY

Israel PECHT, David HASELKORN and Susan FRIEDMAN
Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

Received 13 June 1972

1. Introduction

The high specificity exhibited by the binding sites of enzymes and antibodies is a result of a particular spatial arrangement of different interacting groups within the site and within the ligand. In order to probe the dimensions of the site and to determine the nature of the different attracting forces, equilibrium binding constants to a protein were correlated with the structure of a series of systematically varied ligands [1–5]. These reversible binding reactions may be represented by the following scheme:

$$\text{(I)} \qquad P + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} PL,$$

where P and L are a protein and a ligand, respectively, k_{12} is the bimolecular specific rate of binding and k_{21} the monomolecular dissociation rate constant. Since the equilibrium constant, K, is given by:

$$\text{(II)} \qquad K = k_{12}/k_{21} = \frac{[PL]}{[P] \cdot [L]},$$

it is evident that variations in K may be a result of changes in either k_{12} or in k_{21} or in both. Thus, correlating the variation in the structural parameters of the ligand with the changes in the equilibrium constant will provide only a limited amount of information.

Using the chemical relaxation method, both specific rate constants constituting the reaction equilibrium can be measured directly. Hence the structure-dependent variation observed in the equilibrium constants may be resolved in a detailed manner. This approach of kinetic mapping [6] is based on i) that the variation observed in the equilibrium constant (K) may be analysed in terms of the changes in the specific rates, ii) that the extent and properties of a binding site can be deduced from the correlation between the structural features of a homologous series of ligands (differing in size, charge etc.) and their specific rates of interaction with the site, and iii) that the analysis of the relation between structure and dynamics enables us to distinguish between the specific interactions of protein and ligand within the site and on the protein surface.

Several antibody–hapten systems are being investigated by the kinetic mapping approach. The interaction of a homogeneous IgA protein, MOPC-315, possessing specificity towards the 2,4-dinitrophenyl (DNP) group, was the first to be studied over an extended range of ligands. Several series of DNP derivatives were synthesized and their specific rates of binding and dissociation were determined. Among them were 1-amino derivatives of 2,4-dinitrophenyl in which (a) one or both of the hydrogens were substituted by methyl groups (b) one of the hydrogens was substituted by alkyl chains differing in length and branching and (c) one of the hydrogens was substituted by alkyl chains carrying terminal carboxylate or ammonium groups.

Important points emerged from the comparison between the structural variation of the haptens and the measured kinetic parameters. First, the structure dependent variation of both k_{12} and k_{21} indicated that any explanation of the binding process in terms of only the equilibrium constant K is insufficient.

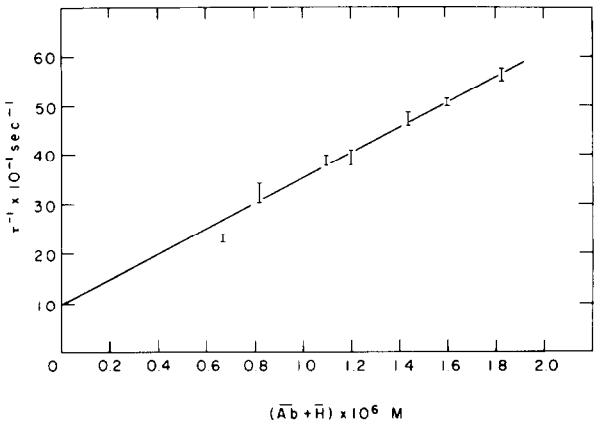


Fig. 1. The dependence of the reciprocal of the observed relaxation times on the sum of concentrations of free protein-315 and free hapten (2,4-dinitrophenyl-n-propylamine) at equilibrium. Measurements were carried out at 25° in 0.1 M NaCl–0.05 M sodium phosphate buffer, pH 7.0.

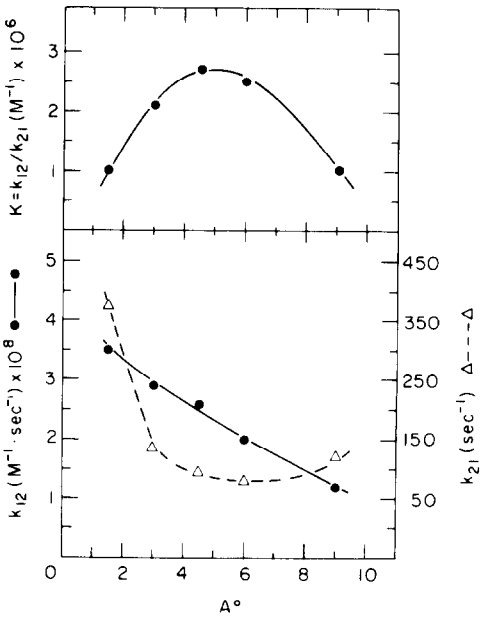


Fig. 2. A plot of the values of k_{12}, k_{21} and $K = k_{12}/k_{21}$, as a function of the length of the $-N$-alkyl groups attached to 2,4-dinitroaniline. The length of the side chain is given in its extended form. The value used for the inter-carbon distance is 1.54 Å and for the C–N bond 1.47 Å. The general formula of these ligands is $R-NH-(CH_2)_n-CH_3$ where R = 2,4-dinitrophenyl. The compounds listed from left to right are: methyl, ethyl, n-propyl, n-butyl and the n-hexyl derivatives.

Second, the observed values of k_{12} were smaller than the value expected for a diffusion-controlled process, moreover they exhibited a structure dependence which could not be accounted for by changes in the diffusion rates, and so values of k_{12} may therefore be a criterion for the ease with which the binding site is recognized. Thus by juxtaposition k_{21}, being the reciprocal life time of the antibody–hapten (protein–ligand) complex, is considered to measure the affinity of the hapten (ligand) to its binding site. Third, in the binding site of the MOPC-315 protein, subsites with specificity to the different groupings of the hapten molecule, supplementary to the DNP ring, could be identified in terms of both their nature and their approximated distances from the DNP subsite.

2. Materials and methods

MOPC-315 protein in its reduced and alkylated monomeric form was prepared as previously described [7]. The plasmacytoma bearing mice were generously provided by Drs. H.N. Eisen and M. Potter.

The series of 2,4-dinitroaniline derivatives was synthesized in this laboratory. Most of these compounds were prepared by reacting 1-fluoro-2,4-dinitrobenzene with the respective amino derivatives. The products were purified by repeated crystallization and checked for purity by thin-layer chromatography and elementary analysis [8].

Kinetic measurements were carried out by following the fluorescence change of the protein-315 upon binding its ligands. The experimental procedure using the temperature-jump spectrofluorimeter has been previously described [7]. The specific rate constants were then calculated by a computer program assuming a one step-mechanism. No initial guess of K,k_{12} or k_{21} is required. The program generates an accurate guess that then permits a rapid minimization scheme [9].

3. Results and discussion

The pertubation, induced by the temperature-jump effected only a single relaxation for all the investigated ligands. The extensive variation of both protein and

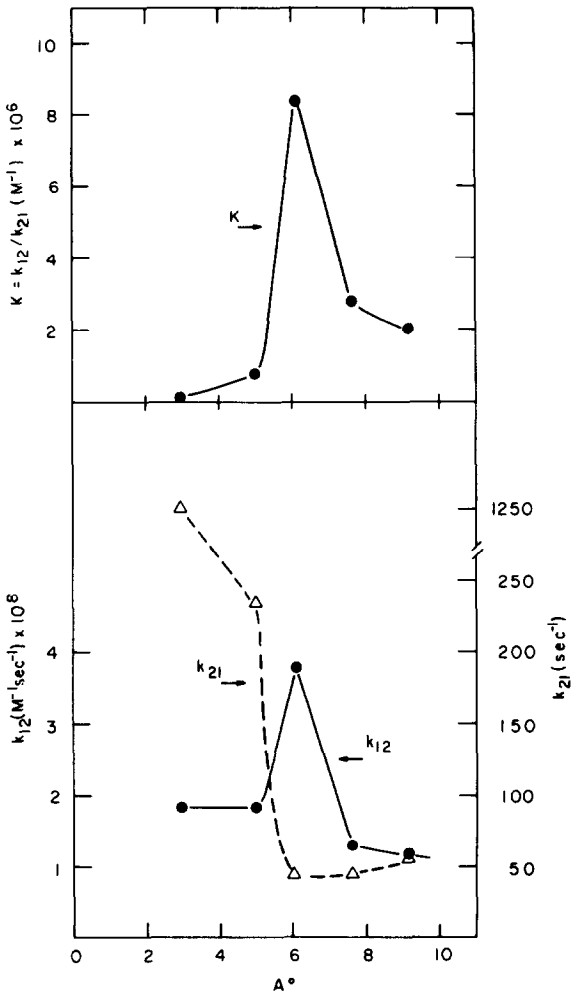
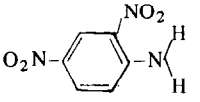
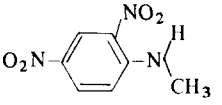
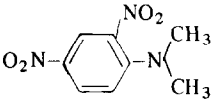


Fig. 3. A plot of the values of k_{12}, k_{21} and $k_{12}/k_{21} = K$ as a function of the distance of the carboxylate group from the 1-amino group of 2,4-dinitroaniline. The general formula of these compounds is $R-NH-(CH_2)_nCOO^-$, were R = 2,4-dinitrophenyl. The listed compounds from left to right are: glycine, β-alanine, γ-amino butyric, δ-amino valeric, ε-amino caproic acid derivatives.

ligand concentrations has shown that the reciprocal relaxation time (τ^{-1}) depended linearly on the concentration of free reactants.

A plot of the data obtained for the binding of 2,4-dinitrophenyl-n-propylamine to protein-315 is shown in fig. 1, and similar behaviour was observed with all the other DNP derivatives that were investigated. The observed concentration dependence of the

Table 1
Rate and equilibrium constants for three derivatives of 2,4-dinitroaniline.

Hapten	k_{12} ($M^{-1}sec^{-1}$)	k_{21} (sec^{-1})	k_{12}/k_{21} (M^{-1})
	4.8×10^8	927	5.1×10^5
	3.5×10^8	373	0.9×10^6
	3.3×10^8	121	2.7×10^6

Measurements were carried out at 25° in 0.1 M NaCl, 0.05 M sodium phosphate buffer, pH 7.0.

relaxation times is in accord with the single step binding mechanism, (equation I) namely: $\tau^{-1} = k_{21} + k_{12}(\overline{Ab} + \overline{H})$, where $\overline{Ab}$ and $\overline{H}$ are the concentrations of free antibody and free hapten, respectively [10–13]. From the lines computed to fit the experimental data for the different ligands the specific rates of binding and dissociation (slope (k_{12}) and intercept (k_{21}), respectively) were evaluated.

It was shown [8] that the substitution of the 1-amino group, by a methyl group caused a decrease in the specific rate of binding and an increase in that of dissociation leading to a 3-fold reduction in the equilibrium constant. Substituting the 1-amino group by a hydroxyl or an ether group ($-OCH_3$ or $-OCH_2-CH_3$) caused a far greater reduction of K, (to $\sim 10^4$ M

These effects (establishing the importance of the nitrogen bound at position-1 of the ring) result mainly from changes in the electron distribution in the aromatic ring and are therefore liable to ambiguous structural interpretations.

Table 1 contains the data for three derivatives of 2,4-dinitroaniline, in which one or both amino hydrogens are substituted by a methyl group. The major effect of these substitutions is a pronounced decrease in k_{21} following the increased hydrophobic nature of the hapten. These data also eliminate the possible

requirement of a hydrogen bond formation between the hapten's amino group and its binding subsite.

The presence of a defined hydrophobic subsite in the MOPC-315 binding site is further shown by measurements with other compounds, (fig. 2). The optimum length of the aliphatic side chain attached to the 2,4-dinitroaniline is reflected in the minimum value of k_{21} observed for a length of three to four methylene groups. The minimum in k_{21} and the gradual decrease in k_{12}, resulting from the elongation of the alkyl side chain, produced a well defined maximum in the equilibrium constant. Measurements of the binding dynamics of 2,4-dinitrophenyl derivatives of branched alkylamines [8] have given further support to the presence of a hydrophobic subsite, 3–6 Å distant from the 2,4-dinitrophenyl binding subsite.

In fig. 3, the changes in k_{12} and k_{21} as a function of the distance of terminal carboxyl groups from the 2,4-dinitrobenzene ring differing only in the length of the aliphatic chain, are shown. The electrostatic forces involved in the binding interactions are clearly illustrated by these results. The rather high values of k_{21} observed when the negative carboxylate group is close to the aromatic ring are probably a result of an electrostatic repulsion.

The steep decrease in k_{21} upon increasing the distance of the carboxylate group from the ring may result from the presence of charged attracting groups in the site at this distance. A parallel series of derivatives carrying a positively charged ammonium group at increasing distances from the ring are being investigated. Preliminary results are in good agreement with this picture. The reported data enables us to divide the MOPC-315 binding site into at least four main subsites: the 2,4-dinitrophenyl subsite, the hydrophobic subsite, the negative subsite and the positive subsite. These data demonstrate also the capacity of kinetic mapping in resolving fine details of the interactions between ligands and their binding sites in molecular terms.

Acknowledgement

The financial support by the Volkswagen foundation is gratefully acknowledged.

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