EFFECTS OF SELF-INTERACTION OF LIGANDS ON BINDING PROCESSES: STUDIES WITH THE ACCEPTORS FIREFLY LUCIFERASE AND THYROGLOBULIN

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Abstract

Previously presented binding theory (Nichol, Smith, and Ogston 1969), describing the effects of ligand self-interaction on binding processes, is applied to the description of two experimental systems, of different type. First, the theory is used to re-examine the sigmoidal kinetic results obtained by other workers (Denburg and DeLuca 1968) with the enzyme firefly luciferase. Denburg and DeLuca attributed the pseudo-allosteric behaviour of the enzyme to the reversible dissociation of the substrate: the present treatment supports the general conclusions drawn by these workers, but considers both substrate self-interaction and binding of the substrate to the enzyme. Secondly, the theory is used to describe the binding of the dimerizing thyroid acid proteinase to its natural substrate, thyroglobulin. In this case the enzyme is regarded as the ligand. It is suggested that this approach may prove useful in describing the binding of other self-associating enzymes to their macromolecular substrates.

I. INTRODUCTION

Several studies on the binding of ligands to acceptor molecules have been concerned with the effects of self-interactions (isomerizations and associations) of the acceptor molecules, particularly with regard to the description of allosteric binding results (Monod, Wyman, and Changeux 1965; Koshland, Nemethy, and Filmer 1966; Nichol, Jackson, and Winzor 1967). Nichol, Smith, and Ogston (1969) showed that self-interactions of the ligand would also influence binding behaviour and presented a general theoretical treatment to describe systems in which both ligand and acceptor, or ligand alone, self-interacted. The present work describes the application of the theory to two experimental systems.

First the theory is used to curve-fit the kinetic data obtained by Denburg and DeLuca (1968) with the enzyme firefly luciferase, the substrate of which, magnesium pyrophosphate, dissociates in solution. The previous analysis of the kinetic results assumed that negligible amounts of the substrate were bound to the enzyme. The purpose of the present treatment is to examine the validity of this assumption and to provide a direct comparison of the experimental results obtained by Denburg and DeLuca with a curve computed on the basis of their model involving substrate self-interaction and utilizing all available kinetic parameters. Secondly, the equations are used to interpret results of a study involving binding of the dimerizing enzyme, thyroid acid proteinase (Smith *et al.* 1969) to its natural substrate, thyroglobulin.

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II. Materials and Methods

Hog thyroid acid proteinase was prepared by the method of Smith *et al.* (1969) and was stored as a lyophilized powder until required. Solutions were prepared by weighing a sample to constant weight over P_2O_5 and then adding the desired volume of buffer. Enzymic assays were carried out by the method of Menzies and McQuillan (1967). Solutions of enzyme were diluted into a concentration range where the enzymic activity proved to be directly proportional to the weight concentration.

Thyroglobulin was prepared by the method of Salvatore *et al.* (1964), and was twice passed through a column of Sephadex G200. Sedimentation analysis of the purified sample revealed approximately 95% of 19S material and 5% of a 27S contaminant. The protein was stored frozen at a concentration of 8 g/l. When required for binding experiments, the solutions were thawed and dialysed against buffer. Concentrations were determined from optical density readings; the specific extinction coefficient at 280 nm ($E^{1\%}_{280}$) was taken as 10 (Salvatore *et al.* 1964).

Binding experiments were carried out by the frontal-analysis method described by Nichol and Winzor (1964), and Cooper and Wood (1968), which was uniquely suited to the present study because the high molecular weight of the enzyme ligand precluded the use of methods such as equilibrium dialysis. A Sephadex G100 column (60 by 1 cm) was equilibrated with diethylbarbiturate buffer (pH 7·5, ionic strength 0·1) at 1°C. In preliminary experiments, thyroglobulin and (separately) proteinase were chromatographed by frontal analysis; neither protein irreversibly adsorbed to the column and nor did the enzyme lose activity. The elution volume of thyroglobulin proved to be the void volume of the column, while the proteinase eluted at a larger volume, as required by the frontal analysis method. In all experiments a fixed total concentration ($\bar{c}_A$) of thyroglobulin ($5{\cdot}77\times10^{-1}$ g/l) was employed, the total proteinase concentration ($\bar{c}_L$) being in the range $1\times10^{-2}-1{\cdot}3\times10^{-1}$ g/l. Mixtures (25 ml) were applied to the column and eluted at a constant flow rate of 14 ml/hr. Fractions of approximately 0·5 ml were collected and it was shown that on the trailing side of the elution profile, the plateau of original composition was followed by one containing the enzyme alone. The concentration of enzyme ($\bar{c}$) in the latter plateau was estimated using several separate fractions (after suitable dilution) in terms of enzymic activity, from which its weight concentration could be determined. The arithmetic mean of these values of $\bar{c}$ found in each experiment was employed to evaluate the binding function, r, as $(\bar{c}_L-\bar{c})/\bar{c}_A$, the weight of ligand bound per unit weight of acceptor. The scatter about the mean was used to assess probable errors in r values, shown as error bars in Figure 2.

III. Theory

Nichol, Smith, and Ogston (1969) have presented binding equations which describe systems in which several ligand species multiply bind to a single acceptor molecule. It was shown that if x ligand species bind at p equivalent and independent sites on an acceptor molecule, A, then at chemical equilibrium the molar concentration of the kth ligand species, $[k]$, is related to a binding function, r, by

$$r = p\sum_{k=1}^{k=x} M_k\tau_{k,A}[k] \Big/ M_A\left(1+\sum_{k=1}^{k=x}\tau_{k,A}[k]\right), \qquad (1)$$

where M_A is the molecular weight of acceptor, M_k is the molecular weight of the kth species, and $\tau_{k,A}$ is the intrinsic association binding constant (Klotz 1953; Nichol, Smith, and Ogston 1969) relevant to the binding of the kth species to A. These ligands may be unrelated to each other, or alternatively may be in chemical equilibrium with each other. In the latter case the concentrations of the various species are related by the appropriate equilibrium constants. In either case it can be shown

(cf. Nichol, Smith, and Ogston 1969) that the total (bound and unbound) weight concentrations of acceptor ($\bar{c}_A$) and ligand ($\bar{c}_L$) are given by

$$\bar{c}_A = M_A[A]\left(1+\sum_{k=1}^{k=x}\tau_{k,A}[k]\right)^p, \tag{2}$$

$$\bar{c}_L = p[A]\left\{\left(1+\sum_{k=1}^{k=x}\tau_{k,A}[k]\right)^{p-1}\sum_{k=1}^{k=x}M_k\tau_{k,A}[k]\right\}+\sum_{k=1}^{k=x}M_k[k], \tag{3}$$

where $[A]$ is the molar equilibrium concentration of unbound A. If one or more of the ligands do not bind, equations (1)–(3) can be simplified accordingly by substitution of the binding constants for those ligands as zero.

IV. Results

(a) *Firefly Luciferase*

The enzyme has been shown to display sigmoidal kinetics in one of its reactions involving the substrate, Mg^{2+}–PP, a magnesium pyrophosphate complex, when the initial velocity of the reaction, v, was plotted against total pyrophosphate concentration (Denburg and DeLuca 1968). The effect is shown in Figure 1 where the experimental points (solid circles) are those obtained by Denburg and DeLuca.

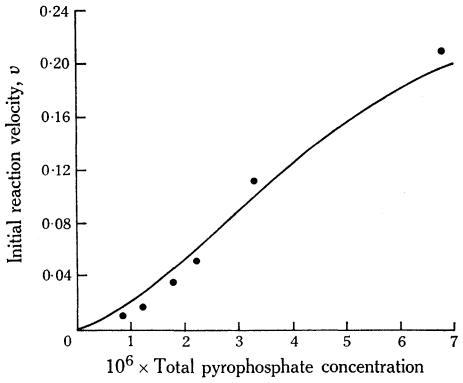

Fig. 1.—The sigmoidal kinetics of firefly luciferase. The experimental values of Denburg and DeLuca (1968) are shown as solid circles. The curve represents theoretical values calculated with the following values of the parameters defined in the text: $p = 1$, $K_A = 1\times10^5\text{M}^{-1}$, $[\overline{Mg}^{2+}] = 2\cdot5[\overline{PP}]$, $K_D = 4\times10^{-6}\text{M}$, $V = 0\cdot6$ units, and $[\bar{A}] = 1\cdot07\times10^{-7}\text{M}$.

These workers reasoned that the observed sigmoidality arose as a consequence of selecting the concentration of total pyrophosphate as the abscissa rather than that of the actual substrate, Mg^{2+}–PP, and examined this postulate in the following way. The dissociation constant describing the equilibrium between the complex, free magnesium, Mg^{2+}, and free pyrophosphate ions, PP, was defined as

$$K_D = [Mg^{2+}][PP]/[Mg^{2+}\text{–}PP]. \tag{4}$$

Equation (4) was written in terms of the *total* concentrations of Mg^{2+} and PP and the resulting quadratic equation was solved to give estimates of the equilibrium concentration of the complex, $[Mg^{2+}$–PP], as a function of total pyrophosphate concentration. The values of $[Mg^{2+}$–PP] were plotted against the experimentally observed initial velocities, whereupon sigmoidality was no longer observed, the plot being

essentially linear over the substrate concentration range investigated. Although the workers commented that this behaviour was expected of an enzyme obeying Michaelis–Menten kinetics, no further quantitative test of the model was made. Moreover, it was implicitly assumed, through the sole use of equation (4) in the computation of values of [Mg^{2+}–PP], that negligible amounts of complex were bound to the enzyme. One purpose of the present re-examination of the results of Denburg and DeLuca (1968) is to test the validity of this assumption by employing equations (1)–(3), which account both for ligand self-interaction and binding to the acceptor.

Considerable simplification of equations (1)–(3) results on noting that for the system under discussion $x = 3$ and only the intrinsic binding constant, K_A, need be considered, since neither Mg^{2+} nor PP alone function as substrates ($\tau_{Mg^{2+},A} = \tau_{PP,A} = 0$). Moreover, Denburg and DeLuca showed that with excess Mg^{2+} essentially all PP was converted to the substrate complex, whereupon normal Michaelis–Menten kinetics were observed. This permitted the direct determination of $p = 1$, K_A (taken as the reciprocal of the Michaelis constant) $= 1 \times 10^5 M^{-1}$, and the maximum initial velocity $V = 0{\cdot}6$ units. In these terms, equation (1) becomes

$$r_M = K_A[Mg^{2+}\text{–PP}]/(1+K_A[Mg^{2+}\text{–PP}]), \tag{5}$$

where r_M is the number of moles of complex bound per mole of acceptor. Similarly, equations (2) and (3) may be written for the *total* molar concentrations, $[\bar{A}]$, $[\overline{Mg}^{2+}]$, $[\overline{PP}]$:

$$[\bar{A}] = [A](1+K_A[Mg^{2+}\text{–PP}]), \tag{6}$$

$$[\overline{Mg}^{2+}] = [Mg^{2+}]+[Mg^{2+}\text{–PP}]+[A]K_A[Mg^{2+}\text{–PP}], \tag{7}$$

$$[\overline{PP}] = [PP]+[Mg^{2+}\text{–PP}]+[A]K_A[Mg^{2+}\text{–PP}]. \tag{8}$$

The values given by Denburg and DeLuca (1968) for $K_D = 4 \times 10^{-6} M$, $[\bar{A}] = 1{\cdot}07 \times 10^{-7} M$ (personal communication), $[\overline{Mg}^{2+}]$ and $[\overline{PP}]$ (from their Figure 1) were used to solve the simultaneous equations (4), (6), (7), and (8) to obtain values of [Mg^{2+}–PP]. It was found for each value of $[\overline{PP}]$ that the values of [Mg^{2+}–PP] calculated in this way were less than those found by the method of Denburg and DeLuca employing only equation (4). However, the maximum discrepancy at $[\overline{PP}]$ of $7 \times 10^{-6} M$ was only 1% and accordingly the previously made assumption, that negligible amounts of complex are bound to the enzyme, is shown to be entirely reasonable.

It is now possible to obtain from equation (5) values of r_M corresponding to each computed value of [Mg^{2+}–PP]. Moreover, if it is assumed that the catalytic step is rate-determining rather than that involved in the formation of the enzyme–substrate complex, corresponding values of v follow directly from the relation $v = Vr_M/p$ (Frieden 1967). It is noted that the same assumption was made in relating the binding constant, K_A, to the observed Michaelis constant. Values of v, obtained in this way, were plotted against corresponding values of $[\overline{PP}]$ and the result is shown as the solid line in Figure 1, which permits direct comparison with the experimental results (solid circles).

(*b*) *Thyroid Acid Proteinase–Thyroglobulin*

A study of the binding of thyroid acid proteinase (Smith *et al.* 1969) to thyroglobulin was undertaken as a step towards an understanding of the mechanism by

which the thyroid hormones are released from their bound form (McQuillan and Trikojus 1966; Smith *et al.* 1969). In order that this preliminary study should not be complicated by any degradation of the thyroglobulin, an environment was sought in which the enzyme was catalytically inactive but in which binding of substrate still occurred. Such an environment was provided by diethylbarbiturate buffer (pH 7·5, ionic strength 0·1) and temperature 1°C. In this environment, also, the enzyme has been shown (Smith *et al.* 1969) to exist as monomer, M, in rapid reversible equilibrium with dimer, D, the equilibrium constant being given by $K = [D]/[M]^2$. The weight concentration of unbound enzyme is given by $\bar{c} = M_M([M]+2[D])$, where M_M is the molecular weight of monomer. Values of $\bar{c}$, in the presence of thyroglobulin, were determined as described in the experimental section and corresponding values of r (defined in this case as the weight of total enzyme, M and D, bound per unit weight of thyroglobulin, A) were calculated directly from the known total concentrations of enzyme, $\bar{c}_L$, and thyroglobulin, $\bar{c}_A$. The results are presented (solid circles) as a binding curve of r versus $\bar{c}$ in Figure 2, the estimated accuracy of points being indicated by horizontal ($\bar{c}$) and vertical (r) error bars.

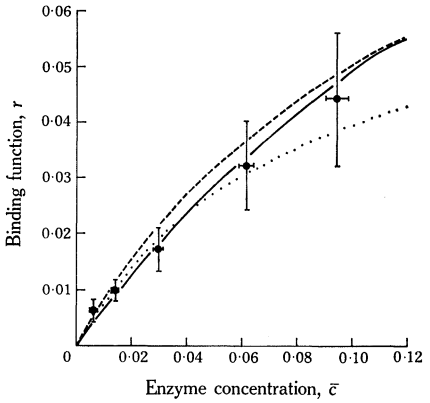

Fig. 2.—Binding curve of hog thyroid acid proteinase to thyroglobulin in diethylbarbiturate buffer, pH 7·5 and ionic strength 0·1, at 1°C. Experimental values are shown as solid points, their accuracy being indicated by the error bars. The curves are theoretically calculated with values of the parameters specified in the text.

A double reciprocal plot of the mean values of (r, $\bar{c}$) points shown in Figure 2 exhibits downward curvature as $1/\bar{c}$ increases, the limiting tangent intersecting the ordinate axis at a value corresponding to three or four binding sites, when the ordinate intercept is taken as M_A/pM_M. This treatment of the results must be regarded as approximate, not only because of the large experimental scatter, but also because the binding equation approaches the form of a rectangular hyperbola only as K tends to zero. It is, however, possible to account for the ligand dimerization ($K \neq 0$) by employing equations (1)–(3) with $x = 2$ and A representing thyroglobulin and thereby to compute theoretical binding curves based on the values of the parameters $M_A = 650{,}000$, $M_M = 21{,}000$, and $K = 5$ litres/g (Smith *et al.* 1969). The purpose of constructing such curves is to illustrate that the postulate of three or four binding sites per molecule of thyroglobulin is consistent with the experimental results. The lines in Figure 2 were computed in this way with $p = 4$, $K_A = L_A = 1{\cdot}2\times10^5\text{M}^{-1}$ (continuous line); $p = 3$, $K_A = L_A = 2\times10^5\text{M}^{-1}$ (dashed line), and $p = 3$, $K_A = 2\times10^5\text{M}^{-1}$, $L_A = 0$ (dotted line), where K_A and L_A are arbitrarily selected

values of the intrinsic constants describing the binding to thyroglobulin of the monomer and dimer of the proteinase, respectively. Clearly, with the large experimental uncertainty, it is not possible to evaluate unique values of the intrinsic binding constants, but the consistency of the theoretical lines in Figure 2 with the experimental results does support the hypothesis that $p = 3$ or 4 in the environment specified. The development of a method for estimating $\bar{c}$ with greater accuracy and additional binding experiments (presently limited due to the scarcity of the enzyme) would be necessary to allow a more precise description of the system.

V. Discussion

In relation to the firefly luciferase–magnesium pyrophosphate system, it is evident from Figure 1 that the sigmoidal distribution of the experimental points may reasonably be explained in terms of the dissociation reaction of the substrate complex. A knowledge of the uncertainty in the experimental points would be necessary to allow further comment on the closeness of the fit between the theoretical curve and the experimental results. It is also clear from equation (5) that a plot of v ($= Vr_M/p$) against [Mg^{2+}–PP] would assume the form of a rectangular hyperbola as indicated by Denburg and DeLuca (1968), their approximation involving neglect of the complex bound to the enzyme being reasonable with the experimental conditions selected. The important point is confirmed that it is essential in attempting to interpret experimentally obtained sigmoidal curves to consider possible self-interactions of the ligand (substrate). Firefly luciferase is not unique in catalysing the reaction of the substrate magnesium pyrophosphate. It has been shown that mouse duodenal alkaline phosphatase displays sigmoidal kinetics with this substrate (Nayudu and Miles 1969) and again substrate dissociation appears to be responsible. Moreover, magnesium ions are known to associate with a number of biologically important phosphate compounds, the resulting complexes interacting with a variety of enzymes. An example is provided by the enzyme muscle phosphofructokinase which binds magnesium–ATP (Kemp 1969): in this case, the observed allosteric behaviour of the enzyme was discussed in terms of the properties of the acceptor (enzyme) with no detailed discussion on the possible contribution of ligand self-interaction.

The general applicability of the binding equations (1)–(3) is further illustrated by the studies with the proteinase–thyroglobulin system, where the self-interacting ligand (proteinase) is macromolecular. This system is not an isolated example of the relevance of the present theoretical treatment to the study of proteolytic enzymes, for trypsin (Cunningham *et al.* 1953; Guinand 1957), α-chymotrypsin (Rao and Kegeles 1958), and carboxypeptidase (Bethune 1965) are also known to self-associate in solution. With such systems the theory predicts a pronouncedly sigmoidal binding curve (or a double reciprocal plot exhibiting *upward* curvature) in cases where the higher polymeric form of the ligand binds exclusively to the acceptor [cf. Fig. 1(*b*) of Nichol, Smith, and Ogston 1969]. The results presented in Figure 2, which are associated with a double reciprocal plot exhibiting downward curvature, do not appear to conform to this behaviour and indeed no calculated curve compatible with the results could be found when it was assumed that the dimeric form of the proteinase alone bound to thyroglobulin ($K_A = 0$). However, the results in Figure 2 are con-

sistent with the alternate possibilities that *either* monomer and dimer *or* only monomer bind to thyroglobulin. It is of interest that binding does occur at an estimated three or four sites in an environment (pH 7·5, ionic strength 0·1) where the enzyme is catalytically inactive and that the results of *N*-terminal analysis (Dopheide and Trikojus 1964; McQuillan and Trikojus 1966) indicate the existence of three or four polypeptide chains per mole of hog thyroglobulin of molecular weight 650,000. It is not claimed, however, that the number of binding sites indicated in this study need necessarily pertain at lower pH values where proteolysis occurs. Nevertheless, the present experimental approach of frontal analysis illustrates a method by which binding parameters may be estimated in a variety of media for systems involving macromolecular ligands and acceptors. Although a medium must be selected where complications due to breakdown products do not arise, in cases where the ligand is an enzyme, the relevant equilibrium concentration, $\bar{c}$, may still be estimated by enzymic assay, after diluting aliquots into a medium in which it is active. The potentiality of investigating binding over a wide range of ligand concentration is apparent.

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