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Further examination of a "concerted cluster" model of multivalent affinity Heterogeneous adsorption of lactate dehydrogenase to Cibacron Blue immobilised on cellulose

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Abstract

A multivalent affinity model, termed the "concerted cluster" model, envisages interactions between a multisite protein and discrete and permanent sets of single and paired immobilised ligands, requiring a rigid support matrix. Extensive data on the binding of rabbit muscle lactate dehydrogenase to Cibacron Blue immobilised on cellulose, collected at several immobilised-dye concentrations, gave biphasic Scatchard plots, and were interpreted quantitatively in terms of single and paired ligands. The data were fitted by a least-squares method to model equations. The concentrations of single and paired ligands, and the stoichiometric association constant for single ligands, were in agreement with literature values and model predictions. However, the stoichiometric association constant for ligand pairs was considerably smaller than predicted, indicating less cooperativity within a ligand pair than expected. Nevertheless these results support the hypothesis that cellulose (unlike agarose) provides a sufficiently rigid matrix for permanent ligand clusters to exist.

1. Introduction

The interactions of multisite proteins (i.e. proteins having several identical ligand binding sites) with ligands immobilised on insoluble stationary phases have been analysed by the use of several theoretical models [1–7]. Multivalent interaction (interaction of a single protein molecule with several immobilised ligands) requires a pair (or higher-order cluster) of ligands to exist in an accessible geometry for the time required by the protein molecule to make two or more contacts. If the strands of the supporting matrix are highly motile (e.g. hydrophilic and infrequently cross-linked long-chain polymers), such clusters may form only transitorily, reducing the probability of multivalent encounters. If the support matrix is rigid, however, immobilised ligands would be incapable of translatory motion relative to each other, allowing perma-

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nent clusters to exist. Proximity and entropic considerations suggest that there should then be strong cooperativity between ligands within an accessible cluster. Compared with binding to an isolated, single ligand, a multisite protein molecule should bind extremely tightly to a permanent accessible cluster.

To model this behaviour quantitatively, it was proposed [6,7] that (a) concentrations of singlets, pairs and higher-order clusters may be obtained by assuming a localised Poisson distribution of ligands, (b) a factor may be introduced to account for macroscopic non-uniformity and (c) the cooperativity within a cluster is so high that protein-to-cluster binding is concerted. Equations derived from this "concerted cluster" model were tested on the binding of aldolase to phosphocellulose, which showed at least two sets of adsorption sites. Numerical analysis led to realistic estimates of the concentrations of single and accessible paired ligands, the microscopic association constant for ligand binding, and hence the conclusion that cellulose is a sufficiently rigid matrix for the "concerted cluster" model to hold [7].

A difficulty in analysing this and similar systems is the low concentration of paired ligands to be expected, detection of which requires binding data at very low protein concentrations. In the aldolase–phosphocellulose case, there was a relative paucity of such data. In search of supporting evidence, we examined the binding of rabbit muscle lactate dehydrogenase to the biomimetic dye Cibacron Blue immobilised on cellulose. Using a sensitive enzyme assay, we studied the interaction at very low protein concentrations where, in addition to the expected single-ligand binding, at least one additional population of high-affinity adsorption sites is clearly evident. To cover both populations of adsorption sites, we present binding data covering nearly four orders of magnitude of soluble protein. Additionally, the effect of varying the overall concentration of immobilised ligand is examined. These results permit a more thorough examination of the clustering hypothesis than has hitherto been possible.

2. Materials and methods

2.1. Materials

Rabbit muscle lactate dehydrogenase and Reactive Blue 2 were from Sigma, Poole, UK. Cellulose (microcrystalline) was from Whatman, Maidstone, UK. Sephadex G-25 was from Pharmacia-LKB, Milton Keynes, UK. All other reagents and buffers were from Sigma or from BDH, Poole, UK.

Blue celluloses, with varying amounts of immobilised dye, were prepared according to the salting-in method of Dean and Watson [8] with modifications suggested by Stead [9]. The concentrations of immobilised dye were determined after acid hydrolysis [10]. Although there was slow leakage of dye from the darker blue celluloses, the loss during the time course of a partitioning experiment was negligible.

2.2. Enzyme assay

Lactate dehydrogenase was assayed by spectrophotometric monitoring of the oxidation of β-NADH (0.1 mM) in sodium phosphate buffer (20 mM, pH 7.4) containing 2 mM sodium pyruvate. The reaction was monitored at 340 nm in a Pye-Unicam SP500 spectrophotometer interfaced with a BBC Model B microcomputer for computation of reaction rate and enzyme concentration. The reaction rate was linearly proportional to enzyme concentration down to 0.2 nM of lactate dehydrogenase.

2.3. Partitioning experiments

Partitioning experiments were performed in imidazole–chloride buffer, pH 7.5 (0.04 M imidazole and 0.39 M NaCl adjusted with HCl). Immediately prior to these experiments, lactate dehydrogenase (supplied as an ammonium sulphate suspension) was desalted by passage through a Sephadex G-25 column equilibrated with imidazole–chloride buffer. Initially, partitioning of the enzyme between the immobilised dye and free solution was determined by the

column recycling method [11] which allowed continuous monitoring of the unbound protein during the approach to equilibrium. From these experiments an appropriate equilibration time was selected for the remainder of the partitioning experiments, which were performed by the mix-centrifuge method [7,12]. The enzyme concentration in free solution was determined by protein absorbance at 280 nm or by enzyme assay.

3. Data treatment

For statistical and accessibility reasons we consider only single and paired ligands, ignoring higher-order clusters [6,7]. Protein binding to two independent sets of immobilised adsorption sites may be shown [7] to be governed by the following equation, which assumes equilibrium binding:

$$[P_b] = \frac{K_1[X_1][P_s]}{K_1[P_s] + 1} + \frac{K_2[X_2][P_s]}{K_2[P_s] + 1} \quad (1)$$

$[P_b]$ and $[P_s]$ are the experimental variables; $[P_b]$ is the molar concentration of adsorbed protein at equilibrium and $[P_s]$ is the molar concentration of soluble (unadsorbed) protein. The other symbols are model parameters whose values are estimated by least-squares fitting. $[X_1]$ and $[X_2]$ are the molar concentrations of two populations of immobilised protein-binding sites, which in the present analysis are taken to be single and paired ligands, respectively. K_1 and K_2 are the corresponding stoichiometric association constants.

We have fitted the experimental data to Eq. 1 in two ways:

(1) Three-parameter fit: data were fitted to the unchanged concerted-cluster model as detailed in Ref. [7]. The values of $[X_1]$, $[X_2]$, K_1 and K_2 are dependent on three independent parameters: [M] (total accessible ligand), K_M (microscopic or site binding constant) and F (reciprocal of accessible fraction of total reaction volume). Of particular relevance to the present discussion is the dependence of K_2 on these parameters:

$$K_2 = 0.00134 \cdot \frac{K_M^2}{([M]F)^2} \quad (2)$$

This and other relevant equations are given in Ref. [7].

(2) Four-parameter fit: in a second fit to the concerted cluster model we relaxed the constraint on K_2 imposed by Eq. 2, i.e. K_2 was fitted as a fourth independent parameter. Otherwise the procedure was the same as in (1).

The fitting procedure generates parameter values and standard errors by non-linear regression using the simplex method on proportional errors in $[P_b]$ as described previously [7].

4. Results

4.1. Characterisation of blue cellulose

The five dyed cellulose samples used were dark blue and contained 1.62, 3.16, 4.76, 9.46 and 14.3 mM immobilised dye (average concentrations relative to the packed wet volume). These values were determined immediately after the partitioning experiments described below. Although the samples were thoroughly washed prior to use, on storage over several days a slow leakage of dye was observed; however the rate of loss was negligible over the time period of a partitioning experiment, and the leaked dye had negligible affect on the enzyme assay. Partitioning experiments (below) showed that the dye accessible to the enzyme was generally about 0.1% of the total immobilised, in line with previous reports of low accessibility [13,14].

4.2. Evidence for two sets of adsorption sites from Scatchard plots

In tests with unmodified cellulose, no binding of lactate dehydrogenase could be detected with the enzyme at 1 nM and at 5 μM, confirming that binding to the modified (blue) cellulose

involved the immobilised dye. At each of five concentrations of immobilised dye, 30–35 measurements of $[P_s]$ and $[P_b]$ were taken covering total protein concentrations from about 0.2 nM to about 5 μM. The results for three dye concentrations are shown as Scatchard plots in Fig. 1 (two of the five sets are omitted for clarity). The pronounced biphasic curvature is clear evidence of at least two sets of adsorption sites for protein in each case. The set with higher affinity was invariably present at low concentrations, and was only evident at protein concentrations below 10^{-7} M.

4.3. Model fitting and error distribution

The experimental data were fitted to Eq.1 by the simplex method which generates estimates of the model parameters. The quality of fit was assessed by examining the distribution of proportional errors {proportional error = (experimental value of $[P_b]$ − fitted value of [Pb])/fitted value of [Pb]}. The complete set of experimental data covered nearly four orders of magnitude for both $[P_b]$ and $[P_s]$ (Fig. 2A). In Fig. 2B and C the error distributions are shown for two modes of fitting to Eq. 1, as follows:

(1) Data were fitted to the unmodified concerted-cluster model. This fit required only three

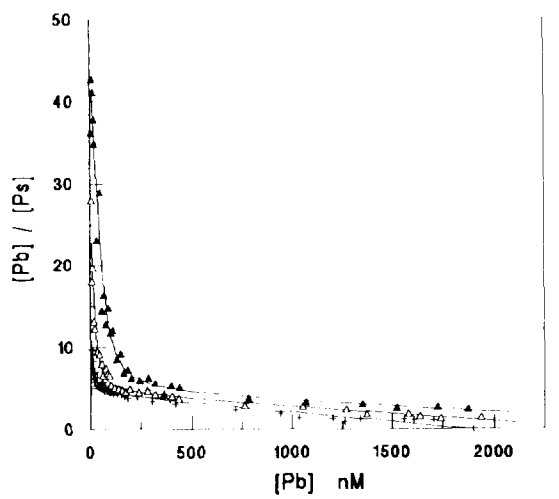

Fig. 1. Scatchard representation of lactate dehydrogenase binding to Cibacron Blue immobilised on cellulose. Data are shown for protein interacting with Cibacron Blue immobilised at total concentrations of 1.62 mM (+), 3.16 mM (△) and 14.3 mM (▲). Data at other dye concentrations are omitted for clarity. The continuous lines represent the best four-parameter fits to Eq. 1.

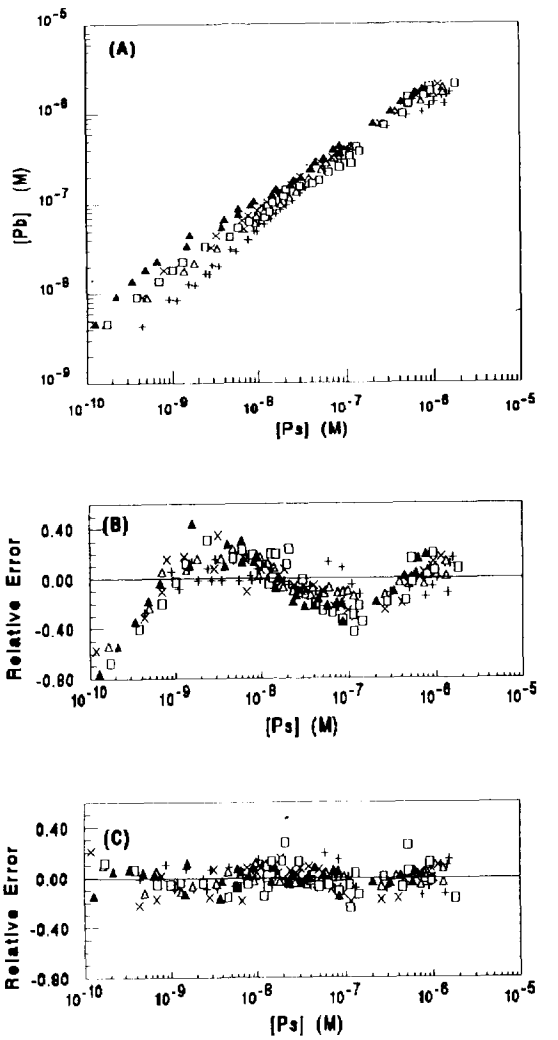

Fig. 2. Experimental data and error distributions. (A) Double-logarithmic plot showing the full range of partitioning data for lactate dehydrogenase interacting with immobilised Cibacron Blue. (B) Error distribution for the best fit of the experimental data to the model, including all constraints (three-parameter fit). (C) Error distribution for the best fit to the model, with constraint on K_2 relaxed (four-parameter fit; see text). Partitioning data are shown for total immobilised dye concentrations of 1.62 mM (+), 3.16 mM (△), 4.76 mM (□), 9.46 mM (×) and 14.3 mM (▲).

parameters, [M], K_M and F. The error distribution is shown in Fig. 2B. The fit is relatively poor; errors are not only non-uniformly distributed, but are worst at the lowest protein concentrations, signifying that the fit to the second (high-affinity) set of sites is particularly bad. The deteriorating fit was very largely due to the extremely high values of K_2 generated by Eq. 2.

(2) The data were refitted to Eq. 1 but the constraint on K_2 (Eq. 2) was omitted. This fit therefore had four independent parameters, [M], K_M, F and K_2. The error distribution is much more uniform (Fig. 2C), and represents a much better fit. This procedure is equivalent to an unrestricted fit to Eq. 1 with $[X_1]$, K_1, $[X_2]$ and K_2 as independent parameters, since there are four independent parameters in each case. Such an unrestricted fit generated identical values for the parameters of Eq.1, and an identical error distribution.

The fitted values of K_2 are generally of the order 10^8 M^{-1}, which is much smaller than predicted by Eq. 2 (which predicts $K_2 \geq 10^{14}$ M^{-1}, i.e. essentially irreversible binding). It is clear therefore that, although a high-affinity set of sites exists such as is predicted by the cluster model, the extremely high level of site–site cooperativity predicted by Eq. 2 does not exist in this system. This arguably signifies that, within an accessible ligand pair, there are interfering factors (possibly steric) that reduce the intrinsic site constant K_M for protein interaction with the second member of the pair compared with that for the first.

4.4. Effects on model parameters of varying the dye concentration

For the remainder of this section we discuss only the four-parameter fit and the associated values of $[X_1]$, $[X_2]$, K_1 and K_2. The best-fit values of these parameters and their standard errors were determined at each of the five total-dye concentrations, and are shown in Fig. 3.

The concentrations of both the single ($[X_1]$) and paired ($[X_2]$) ligands increase hyperbolically with the total-dye concentration, showing a tendency to saturation, as expected (Fig. 3A and B). Depending on total-dye concentration, $[X_1]$ is 50–200 times greater than $[X_2]$. However the *relative* increase in $[X_2]$ is greater than that in $[X_1]$; for example, the increase in total-dye from 1.5 to 15 mM approximately doubles the value of $[X_1]$ but increases $[X_2]$ seven-fold. This observation is consistent with a Poissonian distribution of ligands within the volume-space accessible to the protein, as proposed in the model (see Fig. 1 of Ref. [6]).

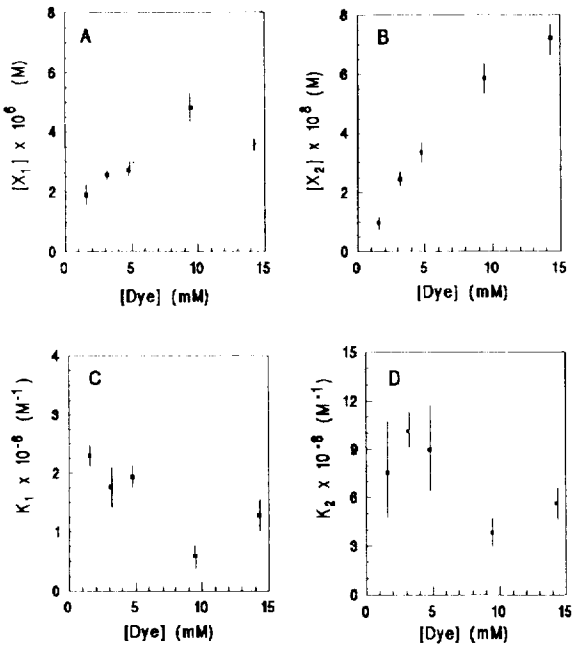

Fig. 3. Dependence of (A) $[X_1]$, (B) $[X_2]$, (C) K_1 and (D) K_2 on the total concentration of immobilised Cibacron Blue. Parameter values are shown with standard error range (vertical bars).

The fitted values of K_1 and K_2 in general have larger standard errors than the estimates of $[X_1]$ and $[X_2]$, hence trends in these parameters are discussed with lower confidence (Fig. 3C and D). Fig. 3C gives K_1 values that are all in the range $(0.6–2.3) \cdot 10^6$ M^{-1}. These compare favourably with published values (given as dissociation constants, here converted to association constants) of $4.3 \cdot 10^6$ M^{-1} [15] and $2.2 \cdot 10^6$ M^{-1} [16] for monovalent binding of lactate dehydrogenase to Cibacron Blue immobilised on Sepharose, and $2 \cdot 10^6$ M^{-1} for binding the dye in free solution

[17]. In terms of the cluster model we would expect K_1 and K_2 to be independent of total dye concentration. In fact both K_1 and K_2 appear to decrease with increasing total-dye concentration, suggesting that at higher dye concentrations there is interference from effects such as dye adsorption to the matrix [16] or dye stacking [18].

5. Discussion

Lactate dehydrogenase has four coenzyme-binding sites [19]; these sites bind the free dye with identical microscopic binding constants, and the interaction of the enzyme with the free dye has been characterised [17,20,21]. Monovalent interaction of the enzyme with the dye immobilised on beaded agarose (Sepharose) has also been characterised [11,14–16]. For the present theory, the enzyme was treated as a sphere of radius 4 nm.

The results reported in the present paper confirm the existence in Cibacron Blue cellulose of a small proportion of high-affinity sites, in addition to the major proportion of lower-affinity sites for binding lactate dehydrogenase. In terms of the concerted-cluster model [6], we interpret these as ligand pairs and single ligands, respectively (the concentrations of three- and four-ligand clusters we assume to be negligible both on statistical and accessibility grounds; see discussion in Ref. [6]). The concentrations of these sites are compatible with the concerted-cluster model which suggests a Poisson distribution of ligands within the matrix space that is accessible to the protein (necessarily less than the total reaction volume [7]). The association constants for single ligands ("monovalent" binding) are close to published figures for both the immobilised and free dye. However the association constants for the presumed ligand pairs are only about 100-fold greater than the constants for single ligands; the model requires that they be at least 10^7 times greater, i.e. it postulates a much higher degree of cooperativity between sites than is observed. The extremely high model value for K_2 is calculated by assuming that only two factors are important in determining this value, namely (1) the intrinsic association constant K_M, assumed to be the same for each member of the ligand pair and for an isolated ligand, and (2) the "apparent local concentration" of ligand within the cluster, which is very much greater than the accessible dye concentration. The calculation ignores the geometrical/steric constraints on the protein achieving a good fit to both ligands simultaneously, and this is the most likely reason for the model failing in its estimate of K_2.

The present results support the suggestion, based originally on studies with aldolase and phosphocellulose [6], that cellulose provides the degree of rigidity in a matrix that is essential for permanent ligand clusters to exist. We speculate that this is a consequence of the micro-crystalline structure of cellulose. In contrast, studies on the lactate dehydrogenase–Cibacron Blue system, at similar low protein concentrations, but with agarose (Sepharose) and other "soft" materials as the support matrix, show no evidence of the low-concentration, high-affinity adsorption sites observed in the present case [14,22], arguably indicating the transient nature of clustering on these "soft" matrices.

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