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Heterogeneous binding of aldolase to phosphocellulose

Interpretation in terms of a "concerted cluster" model of multivalent affinity

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ABSTRACT

It has been suggested that when multi-site protein molecules bind to immobilised ligands on an inflexible matrix, they should encounter discrete sets of single and clustered ligands, binding to clusters being concerted and very tight [R. J. Yon, *J. Chromatogr.*, 457 (1988) 13–23]. To test this model, the aldolase–phosphocellulose interaction was re-examined at low protein concentrations, with and without the presence of soluble ligands. In all cases the data plotted as non-linear (concave upwards) Scatchard plots, indicating at least two populations of adsorption sites, while soluble ligands produced competitive effects as expected. When fitted to a 2-population Langmuir model based on the concerted cluster concept, the data suggested that (a) a very small proportion (about 0.3%) of the total immobilised phosphate was accessible as matrix ligand; (b) of this, about 8% comprises accessible pairs; (c) the matrix ligand was non-randomly distributed within the actual matrix volume; (d) affinity constants for soluble ligands were close to their published values, and (e) the effective matrix ligand is a bisphosphate structure in phosphocellulose. It is suggested that the concerted-cluster model may be valid for an affinity system based on a "hard" matrix such as cellulose.

INTRODUCTION

Analysis of the interactions of multisite proteins (*i.e.*, proteins having several identical ligand binding sites) with immobilized ligands (*e.g.*, in affinity chromatography) have given rise to a number of theoretical models [1–5]. The problem of the expected co-operativity due to proximity that should arise when a multi-site protein molecule encounters a static cluster of ligands was first addressed by Kyprianou and Yon [4] and later extended by Yon [1]. This last paper proposed a "concerted cluster" theory which predicted that, at sufficiently high densities of immobilized ligands on a matrix that prevented translatory motion of these ligands, the protein should encounter a heterogeneous collection of distinct adsorption sites which respectively bound the protein monovalently, bivalently etc. Bivalent and higher-order clusters of immobilized ligands should have very large stoichiometric association constants, but are likely to be present at very low concentrations. As a first approximation the distribution of the immobilized ligand was assumed to be uniformly random, *i.e.*, determined by a Poisson distribution.

Experimental data on the partitioning of rabbit muscle aldolase between free solution and the adsorbent phosphocellulose, in the presence and absence of the soluble ligand phosphate, were used to support a theory of multivalent affinity partitioning based on reacted-site probability theory [3]. Inspection of some of these data when plotted in a

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Scatchard format, showed suggestions of adsorption-site heterogeneity, which was taken by one of us to be evidence of ligand clustering, and accordingly was interpreted in terms of the concerted cluster theory [1,6]. However, it was recognised that the experimental evidence needed re-examining for two reasons: (a) more data at lower protein concentrations were needed (when the clusters might be expected to significantly influence the binding isotherm), and (b) the effect of a multi-phosphate ligand such as hexitol bisphosphate should also be examined for competition, since the heterogeneity might simply reflect two (different) binding sites on aldolase. For reasons of simplicity a bisphosphate ligand (hexitol bisphosphate) was chosen in preference to more complex multi-phosphate ligands of aldolase such as inositol triphosphate. In the present paper we report an extended set of binding data and interpret them in terms of the concerted cluster model. The opportunity is also taken to extend and improve analysis by using a binding equation of the Langmuir type, and to introduce a factor allowing for high local concentrations of the immobilized ligand.

EXPERIMENTAL

Materials

Fructose-1,6-bisphosphate aldolase from rabbit muscle (13 units/mg), fructose-1,6-phosphate, β-NADH, α-glycerophosphate dehydrogenase (165 units/mg), triosephosphate isomerase (1700 units/mg) and phosphocellulose (medium mesh grade) were obtained from Sigma, Poole, UK. Sephadex G-25 was supplied by Pharmacia-LKB, Milton-Keynes, UK. Buffer components and other materials were from Sigma or BDH, Poole, UK. Hexitol bisphosphate was synthesised from fructose-1,6-bisphosphate according to Bragg and Hough [7]. The product was obtained in 72% yield and was essentially pure by paper chromatography [8].

Buffers [3] have a nominal ionic strength of 0.15 *M* and consist of imidazole (0.01 mol/l; pH 7.4 adjusted with hydrochloric acid) with remaining ionic strength provided by sodium chloride with allowances being made, as necessary, for the presence of soluble ligand.

Enzyme assay

Aldolase was assayed by enzyme activity, essentially using the method of Rajkumar *et al.* [9] which couples aldolase to triosephosphate isomerase and α-glycerophosphate dehydrogenase. Linearity of the assay and stability of enzyme activity was confirmed at low enzyme concentration (down to 1 n*M*).

Partitioning experiments

Prior to affinity binding studies, aldolase, as an ammonium sulphate resuspension, was desalted into the relevant buffer by passage through a Sephadex G-25 column. Adsorption of aldolase by phosphocellulose was determined using the mix-centrifuge method of Harris and Winzor [10]. Phosphocellulose was washed [3,8] and a sample resuspended in the appropriate buffer (0.30 g wet cake weight, equivalent to 0.028 g dry weight, in 3.0 ml total volume). An aliquot (20 μl) of aldolase solution was added. This suspension was mixed by gentle rotation for 5 min, followed by centrifugation at 250 *g* for a further 5 min. The concentration of the non-bound aldolase was determined by enzymic assay using an aliquot (20 μl) of supernatant. An isochoric affinity system was maintained by addition of relevant stock enzyme solution. This procedure was repeated to give 15–20 additions of aldolase, with corresponding increases in unbound aldolase. At the end of each experiment the phosphocellulose was washed and dried [3] before being weighed. The phosphate content of the dry matrix was determined after ashing [11].

THEORY AND DATA TREATMENT

Our discussion will use the following symbols for experimental variables: $[P_s]$ and $[P_b]$ denote concentrations of non-adsorbed (including soluble complexes) and adsorbed protein, respectively; [S] denotes the concentration of soluble ligand when this is included. The other terms are model parameters; in the present interpretation they are as follows: [M] = total concentration of accessible matrix ligand, $[X_1]$ = concentration of accessible isolated, single matrix ligands, $[X_2]$ = concentration of accessible immobilized ligand pairs, K_M = microscopic (site) association constant for binding matrix ligand, K_1 = stoichiometric association constant for binding to isolated, single matrix ligand, K_2 = stoichio-

metric association constant for concerted binding to paired matrix ligands, K_S = microscopic constant for binding the soluble competing ligand. Concentrations are in mol/l and association constants are in M^{-1}. Triplet and higher order clusters were neglected. These and other aspects of the concerted cluster theory are discussed in ref. 1.

We choose in the present paper to discuss binding phenomena in terms of a Langmuir-type equation, which is generally more familiar than the R (partitionig ratio) *vs.* $[P_t]$ (total concentration of protein) function used previously [1] and is well suited to batchwise binding experiments (the R *vs.* $[P_t]$ format is better suited to frontal chromatography experiments). Derivation of the appropriate equation (eqn. 1 below) is presented in an Appendix to this paper.

Protein adsorption to two independent sets of adsorption sites, in the presence of soluble ligand, is governed by the equation:

$$[P_b] = \frac{K_1[X_1][P_s]}{K_1[P_s] + 1 + K_S[S]} + \frac{K_2[X_2][P_s]}{K_2[P_s] + (1 + K_S[S])^2} \quad (1)$$

The concerted-cluster model [1] provides expressions for the constants $[X_1]$, $[X_2]$, K_1 and K_2 as follows:

$$[X_2] = \frac{6504([M]F)^3}{F(e^{161.3[M]F} - 1)} \quad (2)$$

$$[X_1] = [M] - 2[X_2]$$

$$K_1 = 4K_M$$

$$K_2 = 0.00134 \cdot \frac{K_M^2}{([M]F)^2} \quad (3)$$

Thus in applying the cluster model to eqn. 1, [M], K_M, K_S and F are treated as independent parameters (to be obtained by the data-fitting procedure), after which K_1, K_2, $[X_1]$ and $[X_2]$ are derived according to the above equations. Eqns. 2 and 3 relate to bivalent adsorption, and are derived from eqns. 5 and 9, respectively, in the previous paper [1]. The numerical factors include the radius of aldolase taken as 4 nm (the mean of the crystallographic unit cell dimensions [12]), and the assumption of four binding sites on aldolase. The factor F requires special comment, since it was not discussed in the original model. The concentration of ligand pairs is calculated on the assumption of a uniformly random (Poisson) distribution of matrix ligands. With $F = 1$ this distribution is calculated relative to the entire reaction volume; use of this factor led to a rather poor fit to the experimental data, especially at the lowest protein concentrations, indicating a substantial underestimation of $[X_2]$. Since the distribution must in practice be non-uniform (partly because the suspended matrix occupies only about 0.4% of the reaction volume, and partly because of unknown non-uniformity within the matrix itself) there will be local concentrations of ligand that are much higher than the concentration averaged over the whole reaction space, increasing the probability of pairs and higher clusters. Implicit in our use of the factor F are the assumptions that (i) the local concentration is F times greater than the average concentration over the whole reaction volume, and (ii) that the Poisson distribution can be applied to this local concentration, *i.e.*, within the local environment the distribution is uniformly random.

The parameters [M], K_M, K_S and F were estimated by non-linear regression using the simplex method [13]. To achieve a more even weighting of residual errors, proportional rather than absolute errors in $[P_b]$ were used (proportional error is defined as the difference between theoretical and experimental values divided by the theoretical value). Four separate fits were made, to assess the internal consistency of the results: (1) [M], K_M and F were fitted to 19 data points in the absence of soluble ligands; (2) [M], K_M, $K_{S(phosphate)}$ and F were fitted to 34 data points in the presence of two concentrations of phosphate; (3) [M], K_M, $K_{S(hexitol\ biphosphate)}$ and F were fitted to 30 data points in the presence of two concentrations of hexitol bisphosphate; (4) a global fit of [M], K_M, $K_{S(phosphate)}$, $K_{S(hexitol\ bisphosphate)}$ and F was made to all the previous 83 data points. Since the simplex method does not provide estimates of standard errors directly, standard errors were obtained by a Monte Carlo method [13], in which 10 sets of simulated data were generated, each with random errors distributed about the theoretical best-fit with the same mean and standard deviation as the experimental data. These provided 10 values of each of the parameters; the standard errors reported in Table I are for these parameter-sets.

TABLE I

P_i = Phosphate; HBP = hexitol bisphosphate.

Parameter	Fit to data in absence of ligands	Fit to data in presence of phosphate	Fit to data in presence of HBP	Global fit (all data)
(a) Fitted parameters, with S.E.M.				
$10^7 \cdot [M]$	6.97 ± 0.24	7.67 ± 0.44	6.79 ± 0.38	7.09 ± 0.23
$10^{-6} \cdot K_M$	1.11 ± 0.10	0.94 ± 0.14	1.34 ± 0.20	1.13 ± 0.09
$10^{-3} \cdot F$	3.16 ± 0.26	3.91 ± 0.43	2.66 ± 0.38	3.20 ± 0.21
$10^{-2} \cdot K_{S(P_i)}$	–	9.19 ± 1.62	–	7.80 ± 0.43
$10^{-6} \cdot K_{S(HBP)}$	–	–	1.25 ± 0.29	1.24 ± 0.05
(b) Calculated parameters				
$[X_1]$	$0.601\ \mu M$			
$[X_2]$	$0.054\ \mu M$			
K_1	$4.52 \cdot 10^6\ M^{-1}$			
K_2	$1.77 \cdot 10^{18}\ M^{-1}$			

RESULTS

Examination of the binding of aldolase to phosphocellulose at low protein concentrations reveal a pronounced heterogeneity of matrix binding sites, as indicated by severely non-linear, concave-upwards Scatchard plots (Fig. 1). Although non-linearity in each plot is due to relatively few points at the lowest protein concentrations, the effect is highly reproducible; in addition to results reported here, a similar effect has been shown using data from an independent laboratory (data from ref. 3, reinterpreted in refs. 1 and 6). Moreover the effect is detected by two assay methods, spectroscopy [3,6] and enzyme assay (present paper).

We examined the fit of the present experimental data to a model postulating that heterogeneous adsorption arises from binding of the multisite protein to accessible singlets and clusters (predominately pairs) of immobilized ligands. In this interpretation, the near-horizontal right-hand limb of each curve (see Fig. 1A) is asymptotic to a straight line denoting a population of sites assumed to be isolated matrix-ligands that bind the protein monovalently. The steeper left-hand limb is asymptotic to an essentially vertical straight line denoting a second population, assumed to be paired immobilized ligands to which the protein binds bivalently, concertedly and extremely tightly (asymptotes indicated in dotted lines in Fig. 1A). Concentrations of triplet and higher-order sites were assumed to be negligible [1].

The fitted parameters are presented in Table I. Independent fits of data in the absence of ligands, and in the presence of phosphate and hexitol bisphosphate, were generally consistent with each other and with a global fit to all the data, by the criterion that their S.E.M. ranges overlap. The only exception is the estimated value of F, which in one subset of data (in the presence of phosphate) is estimated to be significantly different, by the above criterion, from other estimates of F. We shall use the values from the global fit (Table I) is subsequent discussion.

The estimate of [M] suggests a very low value ($0.7\ \mu M$) of total accessible matrix ligand, or $7\ \mu M$ relative to the packed wet matrix. This is a small fraction of the 2.3 mM phosphate in the packed adsorbent, *i.e.*, only 0.3% of the phosphate comprise accessible matrix ligand. The microscopic (site) association constant for binding matrix ligand is $1.13 \cdot 10^6\ M^{-1}$. Initially, attempts to fit the experimental data to eqn. 1 with $F = 1$, led to a rather poor fit which did not model well the steep upwards curvature towards the left, *i.e.*, the fit underestimated $[X_2]$. To improve the fit, F was treated as an additional independent parameter, leading to a much improved fit (continuous line in Fig. 1A). The best fit was obtained with $F = 3200$, *i.e.*, a local concentration of matrix ligand 3200 times greater than the concentration averaged over the whole

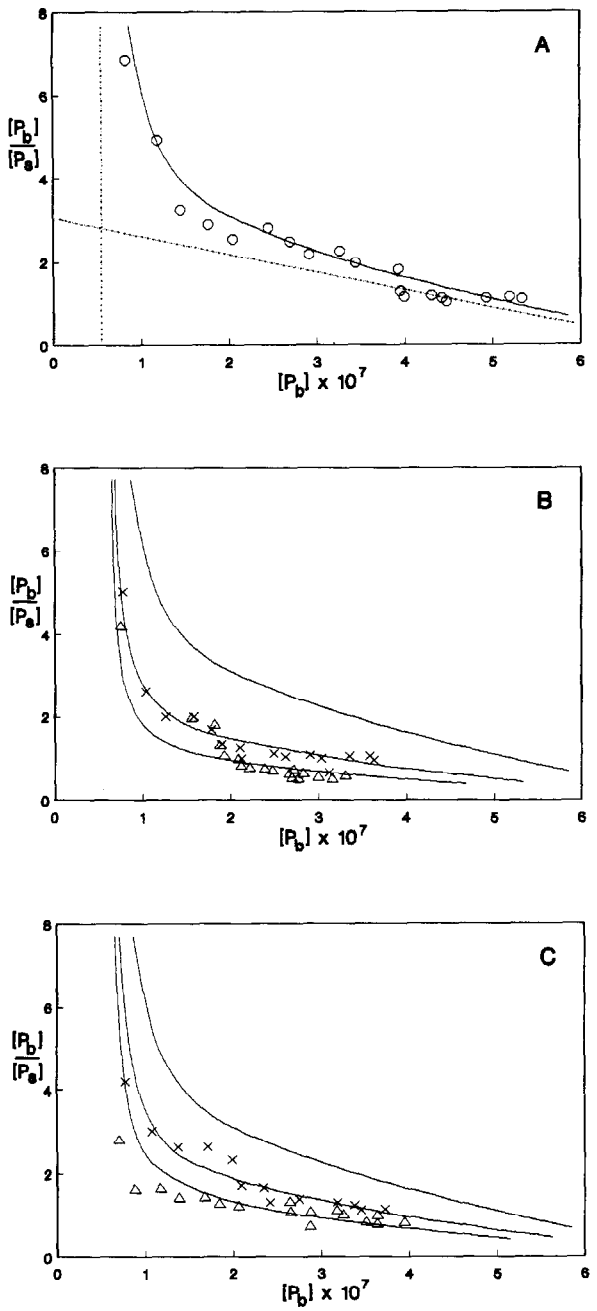

Fig. 1. Scatchard representation of the adsorption of aldolase to phosphocellulose. (A) Data collected in the absence of soluble ligands. (B) Data collected in the presence of 1.43 mM (×) and 2.86 mM (△) phosphate. (C) Data collected in the presence of 0.59 (×) and 1.18 μM (△) hexitol bisphosphate. The curves are theoretical, based on the concerted-cluster model [1] and the parameters from the global fit (Table I). The dotted straight lines in A are asymptotes to the theoretical curve. The theoretical curve from A is overlayed on B and C for comparison.

reaction volume. Volume-displacement experiments with dry phosphocellulose showed that the water-free matrix occupied approximately 4% (v/v) of a packed wet cake, and hence 0.4% (or 1/250) of the reaction volume in our experiments. Purely on comparison of relative volumes, therefore, one may expect a minimum value of 250 for F, not counting further apparent concentration due to non-uniformity within the matrix. The fact that F is well in excess of this minimum value suggests further non-uniformity within the matrix space, *i.e.*, the matrix ligand appears to occupy about 1/12 of the local matrix volume (itself 0.4% of the total reaction volume).

Both phosphate and hexitol bisphosphate are known to be competitive inhibitors of the natural substrate, fructose-1,6-bisphosphate [8,14]. In both cases the expected competition was seen in the displacement of the curves (Fig. 1B, C) at increasing concentrations of soluble ligand, supporting the suggestion that phosphocellulose is an affinity matrix for aldolase. The association constant K_S was found to be 780 M^{-1} for phosphate and $1.24 \cdot 10^6$ M^{-1} for hexitol bisphosphate. These values are somewhat higher, but of the same order as published values of 350 M^{-1} (phosphate) and $8.3 \cdot 10^5$ M^{-1} (hexitol bisphosphate) found in free solution binding studies [8], 350 M^{-1} (phosphate) by competitive inhibition [3] and 400 M^{-1} (phosphate) by batch-wise adsorption of aldolase to myofibrils [15]. The differences may be due a batch-to-batch variations in commercial aldolase.

DISCUSSION

The present experiments, as in previous work [3], support a specific interaction between aldolase and phosphocellulose, since (a) the enzyme is known to bind a number of phosphate-containing ligands in competition with the substrate, fructose bisphosphate; (b) there is clear evidence of competition between the matrix and these ligands; (c) these and earlier experiments [3] were performed in relatively high salt concentration (ionic strength 0.15 M) to suppress non-specific cation-exchange effects; (d) the concentration of accessible immobilized ligand is a small fraction (0.3%) of the total phosphate immobilized. The aldolase–phosphocellulose system may therefore reasonably be used as a model for multivalent affinity interactions.

The pronounced heterogeneity in matrix adsorption sites shown in the present study does not itself provide proof that the high-affinity sites are due to ligand clusters in phosphocellulose. Nevertheless the cluster idea does provide a simple rational explanation for the observed binding heterogeneity, and the quantitative treatment above shows that it is not inconsistent with the observed binding behaviour at low protein concentrations. A necessary consequence of using the concerted cluster theory is the very large difference in affinities between adsorption to monovalent sites and clusters which it predicts. The reasons for this have been discussed in detail [1]. Effectively, for a monovalent binding constant (K_1) of the order $10^6\ M^{-1}$, binding to a cluster is predicted to be irreversible (hence the K_2 value of the order $10^{18}\ M^{-1}$, see Table I, depicted by the essentially vertical left-hand asymptote in Fig. 1A). Practical limits to the sensitivity of the assay have prevented us obtaining more data points along this upwards curve of the Scatchard plot, hence the approach to irreversibility remains to be demonstrated conclusively.

The magnitude of its binding constant suggests that the immobilized ligand group may not simply be a phosphate group. The estimate of $1.13 \cdot 10^6\ M^{-1}$ for the microscopic association constant for binding to the immobilized ligand is nearly 3000-fold greater than the constant for phosphate measured in free solution. Since both free ligands (phosphate and hexitol bisphosphate) compete with the immobilized ligand on these experiments, the immobilized ligand could resemble either. The microscopic binding constant for the immobilized ligand is similar to that of hexitol bisphosphate ($1.24 \cdot 10^6\ M^{-1}$). Moreover, immobilized ligands occur infrequently in the matrix, since they comprise about 0.3% of the total phosphate immobilized. For these reasons, we speculate that the effective immobilized ligand is a bisphosphate group in phosphocellulose.

The cluster theory treats aldolase as a sphere of radius 4 nm, and assumes that whenever two or more ligands are clustered within the bounds of an equivalent sphere, and are encountered by an enzyme molecule, all the bounded ligands will bind concertedly, up to a maximum of four. The model postulates an immovable, rigid, matrix to fix the clusters in space; however we also envisage considerable local movement of a non-translatory nature (rotations about single bonds, for example) to assist matrix ligands to meet the spatial requirement for specific binding. It is possible that the microcrystalline structure of cellulose approximates to the rigid, inflexible matrix required to hold the component ligands of a cluster permanently in juxtaposition. Interestingly, Yon and Easton [16] were unable to show strong evidence of clusters in Blue Sepharose binding to lactate dehydrogenase. Sepharose is a much "softer" matrix, with much more flexibility and local movement in its polymer chains; ligand-clusters would have only a fleeting existence, if any, in such a structure. It will be necessary to examine other affinity matrices of both types to confirm whether, as we suspect, the clustering phenomenon will be observed only when a "hard" matrix such as cellulose is used.

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APPENDIX

Derivation of a Langmuir-type equation for concerted protein adsorption that blocks i sites on a N-site protein

We consider a protein molecule P with N identical sites for binding soluble ligand S. This protein is adsorbed reversibly at a matrix site X, such that i of the S-binding sites are blocked to S. The remaining $N - i$ sites are able to bind S with the same intrinsic affinity as sites in the unadsorbed protein. Adsorption is concerted, *i.e.*, the i sites are blocked in an all-or-none manner.

Let $[P_s]$ denote the concentration of all soluble protein forms, *i.e.*, the unadsorbed protein measured by experiment. Then, following Klotz [17],

$$[P_s] = [P] + [PS] + [PS_2] + \ldots + [PS_N]$$
$$= [P](1 + K_S[S])^N \quad (A1)$$

where [P] denotes the concentration of free (unbound) protein, $[PS_j]$ denotes concentrations of soluble complexes and K_S is the microscopic (site) association constant for binding S.

Similarly, the total concentration of adsorbed protein is given by

$$[P_b] = [PX] + [PXS] + [PXS_2] \ldots + [PXS_{N-i}]$$
$$= [PX](1 + K_S[S])^{N-i}$$
$$= K_i[P][X](1 + K_S[S])^{N-i} \quad (A2)$$

where $[PXS_j]$ are the concentrations of adsorbed complexes, K_i is the (stoichiometric) association constant for adsorption of P to X, and [X] is the concentration of unoccupied X. Note that $[P_b]$ is the adsorbed protein obtained by experiment.

The total concentration of adsorption sites $[X_t]$ is $[X_t] = [X] + [P_b]$. Substituting from eqn. A2, this becomes

$$[X_t] = [X]\{1 + K_i[P](1 + K_S[S])^{N-i}\} \quad (A3)$$

Substituting from eqns. A1 and A3 into eqn. A2 to eliminate [P] and [X], we obtain

$$[P_b] = \frac{K_i[X_t][P_s]}{K_i[P_s] + (1 + K_S[S])^i} \quad (A4)$$

which is in the required Langmuir format.

For several independent populations of adsorption sites, the total adsorbed protein is the sum of several terms of the type in eqn. A4. Thus for two X-populations which block 1 and 2 S-sites respectively, the adsorbed protein will be

$$[P_b] = \frac{K_1[X_1][P_s]}{K_1[P_s] + (1 + K_S[S])} + \frac{K_2[X_2][P_s]}{K_2[P_s] + (1 + K_S[S])^2}$$

which is eqn. 1 in the main text.

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