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Investigation of liquid phase cooperative binding interactions on the capacity of insoluble affinity adsorbents

A. G. MAYES
Biochemistry Department, University of Bath, Claverton Down, Bath BA2 7AY (U.K.)
J. HUBBLE*
School of Chemical Engineering, University of Bath, Claverton Down, Bath BA2 7AY (U.K.)
and
R. EISENTHAL
Biochemistry Department, University of Bath, Claverton Down, Bath BA2 7AY (U.K.)

ABSTRACT

Experimental data is presented to support the theoretical prediction of an enhancement of adsorption arising from a positively cooperative liquid phase interaction between a multivalent adsorbate and free ligand. The results obtained with glyceraldehyde 3-phosphate dehydrogenase show a 4-fold increase in adsorption to 5′-adenosine monophosphate cellulose in the presence of 3 μM nicotinamide adenine dinucleotide compared with that obtained in the absence of cofactor. Although the magnitude of the effect, and the optimal free ligand concentration do not correspond to those predicted in the original model, the discrepancies may at least in part be accounted for by a maldistribution of immobilised ligand, leading to multiple cooperative interactions between adsorbate and affinity matrix. This can be qualitatively predicted by an extension to the original model.

INTRODUCTION

Separation techniques based on affinity adsorption have great potential for the production of high value, high purity bioproducts. This results from the high degree of selectivity and the consequently large purification factors which can be achieved. However, as affinity adsorption is adopted as a process scale operation the optimisation of empirically developed laboratory-scale methodology becomes important for maximising yield and quality of the end product.

Although much effort has been directed towards optimisation of the solid phase, *i.e.*, investigation of ligand immobilisation chemistry and the effect of both the length and nature of spacer arms, there appears to have been little consideration given to the possible contribution of the liquid phase to the overall performance of a system, for example the possible gains achievable by using free ligand in the liquid phase to enhance the binding of a positively cooperative enzyme to the solid phase.

The property of positive cooperativity, whereby proteins show sigmoid ligand binding isotherms is a fairly common feature of multimeric proteins in free solution.

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The performance and capacity of affinity adsorbents is strongly influenced by the affinity of the protein for the immobilised ligand. It therefore seems likely that adding free ligand to positively cooperative protein in the presence of immobilised ligand will increase the affinity of the protein for all available ligand and hence lead to tighter binding of protein to matrix ligand. This hypothesis was examined quantitatively in computer simulations by Hubble [1], which suggested that significant gains could be achieved in an appropriate system. In this paper we report attempts to obtain experimental evidence for this behaviour and compare the results with the theoretical predictions obtained from computer simulation.

MATERIALS

N^6-(6-aminohexyl)AMP, N^6-(6-aminohexyl)NAD^+, long fibrous cellulose powder, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glyceraldehyde 3-phosphate diethyl acetal and NAD^+ were obtained from Sigma (Poole, Dorset, U.K.).

Free glyceraldehyde 3-phosphate was liberated from the diethyl acetal (Sigma) by heating an aqueous solution in the presence of Dowex 50 H^+ resin, and was assayed using GAPDH and NAD^+ to check for complete hydrolysis of the acetal. This was stored in aliquots at $-20°C$ until required.

Sepharose 4B came from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade.

METHODS

Preparation of nucleotide matrix derivatives

Sepharose 4B or long fibrous cellulose was activated with cyanogen bromide using the method of March *et al.* [2]. The final wash was with 0.1 *M* sodium bicarbonate pH 8.9 (buffer A). Matrix was resuspended in an equal volume of buffer A and sufficient N^6-(6-aminohexyl)AMP or N^6-(aminohexyl)NAD^+ added to give the desired concentration of matrix ligand. This was agitated gently for 16 h at 4°C. The matrix was washed with buffer A and the washings retained for spectrophotometric determination of unbound ligand. The washed matrix was added to 1 *M* ethanolamine and left at room temperature for 2 h to block any remaining reactive groups, then washed with water, 1 *M* sodium chloride and water again before storage at 4°C as a moist cake. Ethanolamine–Sepharose was prepared by activation followed by immediate blocking with ethanolamine.

Ligand bound to the matrices was estimated by calculation of the difference between ligand added and ligand remaining in the washings. Absorbance was measured at 267 nm and a molar absorptivity of 17.7 $mM^{-1}cm^{-1}$ used for calculations [3]. Ligand bound to Sepharose was also estimated by direct spectroscopy of a 10% suspension of matrix in glycerol–water (50:50, w/w) using a similar suspension of underivatised Sepharose as a blank. The wavelength maximum and molar absorptivity of the coupled ligand were assumed to be the same as for the free ligand. The two methods showed close agreement.

Coupling was consistently found to be 94–96% for Sepharose and 50–60% for cellulose.

Nucleotide removal from GAPDH

Nucleotides were removed by charcoal treatment using the method of Gennis [4]. Treated GAPDH had an A 280 nm/A 260 nm ratio of about 2 and was stored as an ammonium sulphate suspension at 4°C until required.

Assay of GAPDH

GAPDH was assayed at pH 8.5 and 25°C in 1 ml of a buffer containing (final concentrations) 50 m*M* sodium pyrophosphate, 5 m*M* EDTA, 10 m*M* sodium dihydrogen orthophosphate, 0.1 *M* potassium chloride, 10 m*M* cysteine, 1 m*M* NAD^+, and 1 m*M* D-glyceraldehyde 3-phosphate (2 m*M* DL racemate) which was added last to initiate the reaction. Enzyme (about 0.005 I.U.) was preincubated for 8 min in the assay buffer to ensure complete reduction of active site thiol groups. Increase in absorbance at 340 nm was followed using a Cecil 272 UV spectrophotometer fitted with a jacketed cuvette holder. Temperature was maintained using a recirculating water bath and cuvettes were left for 15 min to equilibrate prior to use.

Equilibrium batch adsorption

A quantity of matrix equal to 4 ml settled volume was added to 20 ml 50 m*M* sodium pyrophosphate buffer pH 8.5 containing 1 m*M* 2-mercaptoethanol and 1 m*M* EDTA. The total volume was measured and the suspension added to a water jacketed vessel maintained at 25 ± 0.3°C, stirred with an overhead stirrer to minimise physical matrix degradation. A known amount of GAPDH was added and allowed to equilibrate for about 10 min.

A 100-μl sample was withdrawn, spun briefly to sediment the matrix and 20-μl samples of supernatant removed for triplicate enzyme assays. The total added ligand concentration in the vessel was increased by adding a small volume of NAD^+ solution and the system allowed to re-equilibrate for 8 min before repeating the cycle. This was continued until the desired range of added ligand had been covered.

The concentration of free enzyme is found from the assays so, knowing the total amount of enzyme initially added and the system volume (recalculated after each cycle), bound enzyme can be calculated from the enzyme mass balance for each concentration of added ligand.

RESULTS AND DISCUSSION

Fig. 1 shows the simulated effect of free ligand concentration on GAPDH adsorption using theory developed by Hubble [1] and literature values for the binding constants [4]. In contrast Fig. 2a–c shows plots of bound enzyme/total enzyme against log added NAD^+ from batch adsorption experiments using AMP–Sepharose matrices with different ligand densities. In order to keep the bulk average matrix ligand concentration comparable the volume of the highest ligand density AMP–Sepharose used was lower than that used in the other two experiments. As might be expected the fractional binding in the absence of free NAD^+ (arbitrarily plotted at −6.5) increased with increasing ligand density but the predicted enhancement of binding on adding free NAD^+ was not apparent under the experimental conditions adopted for any of the matrix ligand densities tested. Similar results were obtained for NAD^+–Sepharose matrices (data not shown).

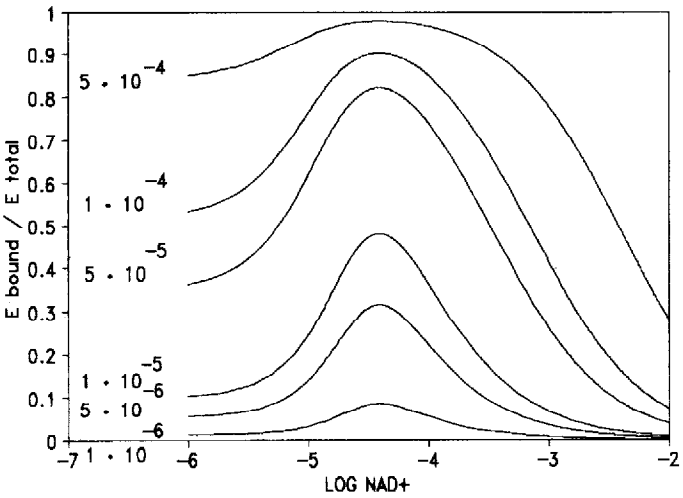

Fig. 1. The predicted effect of matrix ligand concentration on the relationship between free ligand concentration and the fraction of enzyme bound for glyceraldehyde 3-phosphate dehydrogenase. The affinity constants used were derived from the data of Gennis [4]. $K_1 = 2.6 \cdot 10^3$, $K_2 = 1.5 \cdot 10^4$, $K_3 = 1.9 \cdot 10^5$, $K_4 = 5.7 \cdot 10^3\ M^{-1}$. The matrix ligand concentrations were: $1 \cdot 10^{-6}$, $5 \cdot 10^{-6}$, $1 \cdot 10^{-5}$, $5 \cdot 10^{-5}$, $1 \cdot 10^{-4}$, $5 \cdot 10^{-4}\ M$.

The data obtained using AMP cellulose is shown in Fig. 3. Due to the low levels of binding observed in this system the scatter of points in individual experiments was greater than for the Sepharose experiments since the magnitudes of the changes in bound enzyme were similar to the magnitudes of the errors. All the individual experiments suggested that some enhancement of binding was occurring, with control experiments using blank cellulose and ethanolamine-derivatised cellulose showing no

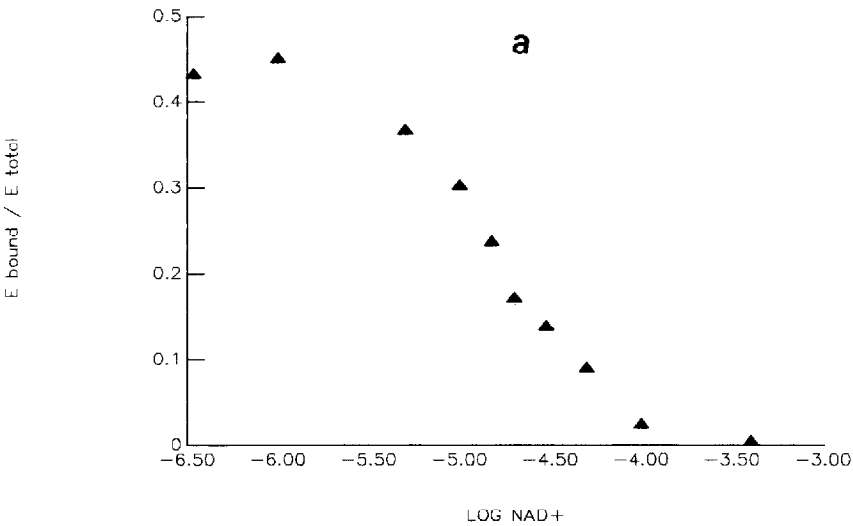

Fig. 2.

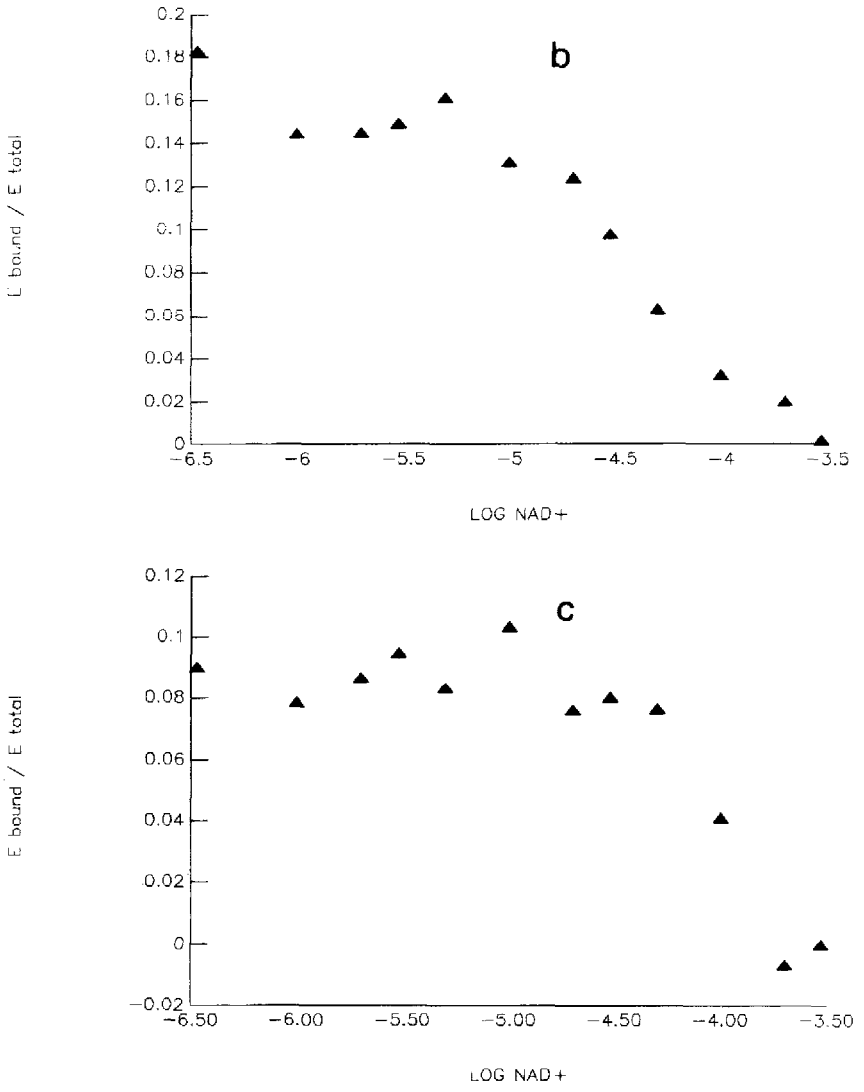

Fig. 2. Effect of added NAD^+ on the fraction of enzyme (GAPDH) bound to AMP–Sepharose. (a) High density: bulk average ligand concentration $1 \cdot 10^{-4}$ *M*. Matrix volume 6.5% of total. Matrix ligand concentration $1.5 \cdot 10^{-3}$ *M*. (b) Medium density: bulk average ligand concentration $9.3 \cdot 10^{-5}$ *M*. Matrix volume 18% of total. Matrix ligand concentration $5.2 \cdot 10^{-4}$ *M*. (c) Low density: bulk average ligand concentration $2.5 \cdot 10^{-5}$ *M*. Matrix volume 18% of total. Matrix ligand concentration $1.4 \cdot 10^{-4}$ *M*. Values for no added NAD^+ are arbitarily plotted at −6.5.

effect. On combining the data from several runs with AMP cellulose the enhancement is quite clearly seen (Fig. 3). An approximately 4-fold increase in binding was apparent on adding free ligand and this compared well with that predicted for the bulk average matrix ligand concentration used. In two other respects, however, the data were not comparable with the predicted behaviour. The fractional binding of enzyme to matrix

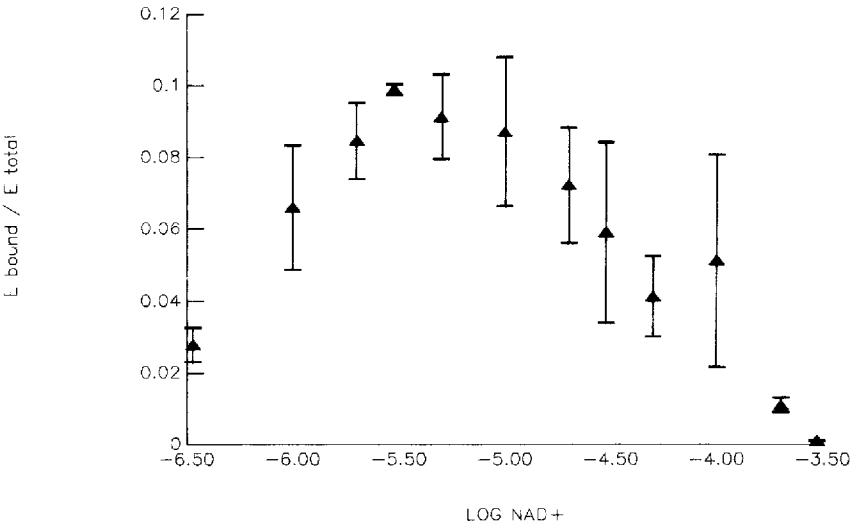

Fig. 3. Effect of added NAD^+ on the fraction of enzyme bound for GAPDH binding to AMP cellulose. The bulk average ligand concentration was $7.8 \cdot 10^{-5}$ M and the matrix volume = 20% of total (matrix ligand concentration $3.9 \cdot 10^{-4}$ M). Values plotted are means ± standard errors (n = 3). Values for no added NAD^+ are arbitarily plotted at −6.5.

ligand was about 10-fold lower than predicted. This observation probably results from restrictions imposed on ligand accessibility by the support matrix, and/or changes in binding constants arising from the chemical effects of immobilisation. The second deviation from the theoretical prediction is that the maximum observed binding enhancement occurs at a lower free ligand concentration. This can in part be explained by the effects of immobilised ligand maldistribution.

The difference in the behaviour of the cellulose and Sepharose matrices probably arises as a result of their different physical structures and consequent differences in the pattern of derivatisation. Sepharose beads are approximately spherical with a size range of 60–140 μm. Fibrous cellulose approximates to cylinders of length 100–250 μm and diameter about 25 μm. Sepharose 4B beads are known to be porous with pore sizes 80–230 nm, averaging about 170 nm [5]. The porosity of the cellulose used is unknown due to batch to batch variation of this natural product. It is generally composed of porous "amorphous" regions, with greater porosity than cross-linked polysaccharides like Sepharose 4B interspersed with compact "microcrystalline" regions [6]. Thus both matrices used should be freely permeable to both small molecules and GAPDH. Hence it does not appear that differences in accessibility could be a major contributor to the different behaviour of cellulose and Sepharose matrices.

The diffusional path length to the centre of a particle is up to 6 times longer for Sepharose assuming similar degrees of contortion in both matrices. Since both activating chemicals and coupling ligands must diffuse into the particle from the liquid phase it is likely that the end product will not be a uniformly derivatised particle but one with a "shell" of high ligand density at the surface and a concentration gradient running to a minimum at the centre of the particle. The shorter the path length the less

pronounced this would be expected to be. The result of this will be that the cellulose matrix will have a more uniform ligand distribution, more closely approximating the bulk average concentration than will the Sepharose matrix where the majority of the ligand will be concentrated towards the external surface of the beads. This would have two possible repercussions.

(1) The Sepharose matrix will behave as one with a higher ligand density thus tending to swamp out the cooperative effect.

(2) The higher ligand density in the Sepharose matrix will lead to an increased probability of multivalent interactions between protein and immobilised ligand.

This behaviour has been observed for a number of proteins with multiple binding sites [7–9]. A bivalent interaction will have a higher affinity constant than a monovalent one and if this is greater than or equal to the enhanced affinity of enzyme molecules partially saturated with free ligand then this will also swamp out any enhancement which might have been achieved due to the cooperative effect.

The effect of localised high concentrations of matrix ligand together with the consequences of multivalant interaction between macromolecule and resin can be qualitatively considered using an extension to the model described by Hubble [1]. In the original theoretical assessment of liquid phase cooperativity the assumption was made that with low matrix ligand densities only monovalent interactions would be possible between adsorbate and support. However, if localised high concentrations of immobilised ligand occur the possibility of multivalent interactions between enzyme and support cannot be discounted. The original model can easily be extended to consider all theoretically possible interactions between a tetravalent enzyme and both free and immobilised ligand. The broader model can be used to give a qualitative indication of the effects that these multivalent surface interactions might be expected to have on observed binding enhancements.

Development of the revised model leads to the formulation of fourteen equilibria describing interactions between individual complex species. For interaction with soluble ligand:

$$[E][L] \overset{K_1}{\rightleftharpoons} [EL]$$

$$[EL][L] \overset{K_2}{\rightleftharpoons} [EL_2]$$

$$[EL_2][L] \overset{K_3}{\rightleftharpoons} [EL_3]$$

$$[EL_3][L] \overset{K_4}{\rightleftharpoons} [EL_4]$$

A similar set of equilibria can be formulated for the interactions of E with immobilised ligand (M) using association constants $K_{m1} - K_{m4}$. Binding between enzyme and both soluble and immobilised ligand can be described in terms of six further association constants:

$$[EM][L] \overset{K_{l1}}{\rightleftharpoons} [EML]$$

$$[EML][L] \overset{K_{l2}}{\rightleftharpoons} [EML_2]$$

$$[EML_2][L] \overset{K_{l3}}{\rightleftharpoons} [EML_3]$$

$$[EM_2][L] \overset{K_{l4}}{\rightleftharpoons} [EM_2L]$$

$$[EM_2L][L] \overset{K_{l5}}{\rightleftharpoons} [EM_2L_2]$$

$$[EM_3][L] \overset{K_{l6}}{\rightleftharpoons} [EM_3L]$$

Fractional binding of enzyme to the affinity support can be described in terms of:

$$Y_m = \frac{\Sigma[EM_i] + \Sigma[EM_iL_j]}{[E] + \Sigma[EL_j] + \Sigma[EM_i] + \Sigma[EM_iL_j]}$$

where i denotes number of sites bound to immobilised ligand and j denotes number of sites bound to free ligand (for a tetramer $1 \leqslant (i + j) \leqslant 4$).

This equation can be expressed in terms of the equilibrium concentrations of L and M together with appropriate products of the individual association constants. Where the association constants are known this relationship can be used to predict the effect of free ligand concentrations on fractional binding as previously described. However, the more general form allows the effect of permitting progressively more complex multiple interactions between enzyme and adsorbate to be predicted. An example of a prediction of this type is given in Fig. 4 using the same association

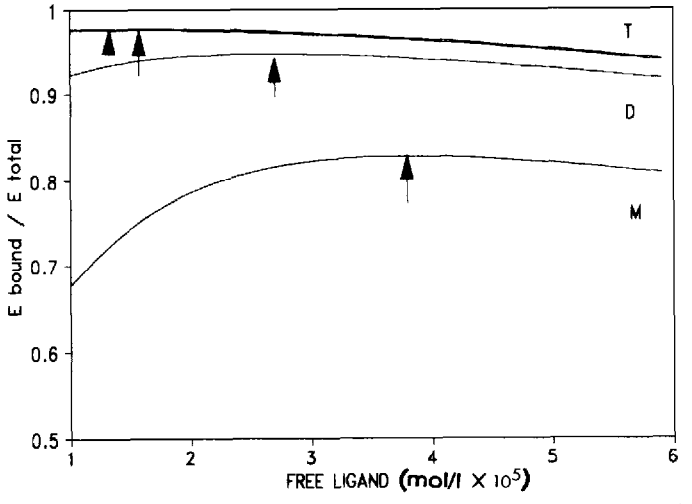

Fig. 4. The predicted effect of multivalent interactions between enzyme and matrix ligand on the relationship between free ligand concentration and fractional binding (association constants as for Fig. 1, bulk average ligand concentration $5 \cdot 10^{-5}$ M). M denotes monovalent interaction, D divalent and T tri- and tetravalent. Arrows denote the free ligand concentration giving maximal fractional binding.

constants for GAPDH as detailed for Fig. 1. The curves obtained as the number of permitted interactions with immobilised ligand is increased from 1 to 4 clearly show a shift towards a lower optimal free ligand concentration together with a masking of the enhancement effect. This was clearly observed in the Sepharose experiments where masking of enhanced binding prevented the identification of an optimum free ligand concentration.

The optimum free ligand concentration in the cellulose experiment was lower than that predicted, indicating perhaps, that even in this case some multivalent interactions were occurring.

No quantitative observations can be made at this stage concerning the levels of binding (which should increase if multivalent interactions are occurring), since the levels observed are much lower than those predicted. This may be due to changes in the intrinsic affinity constants for the interaction of protein with immobilised ligand relative to free ligand, as a result of the immobilisation process. Analysis is further complicated as onlyl a proportion of the immobilised ligand molecules will be in sufficiently close proximity to allow multiple simultaneous interactions with the enzyme. It does, however, clearly demonstrate the trends produced by multiple interactions.

CONCLUSIONS

The results presented demonstrate the occurrence of a liquid phase modification of adsorption capacities arising from the cooperative interaction of free ligand with a multivalent macromolecule. The effect observed is unlikely to be of significance in currently available affinity supports given that it is only observed under conditions of low immobilised ligand density and sub-optimal pH for binding (GAPDH shows optimal binding at about pH 7, but shows no cooperativity in the liquid phase at this pH). However, as both theoretical predictions, and the experimental results presented here suggest that cooperative effects are masked by high localised concentrations of immobilised ligand, it is possible that more significant capacity enhancements might be observed with the soluble supports used in aqueous two-phase partition [10].

In formulating the theoretical relationships described we have only considered the intrinsic cooperative properties of the adsorbate molecule. A more rigourous modelling approach would be needed to take account of the surface cooperative effects as described by Yon [11]. These arise from an increased valency of interaction even where there is no intrinsic molecular cooperativity. The concept of cooperative surface interactions resulting from immobilised ligand "clusters" in orientations suitable for multivalent interactions with a multimeric enzyme has been used to describe the binding of aldolase to phosphocellulose [11,12]. The increased apparent affinity arising from multiple surface interactions is an additional factor which will tend to mask the consequences of liquid phase cooperativity and lead to deviations from the response predicted by simple models. In generating the predictions used in this work we have assumed that the association constants between enzyme and insoluble ligands are similar to those describing the interactions with soluble ligands. Clearly this is a gross simplification even in the case of monovalent interactions with the support. When polyvalent interactions are possible determination of appropriate surface association constants becomes extremely complicated and requires much further work if accurate predictive models are to be developed.

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