A quantitative study of the biospecific desorption of rat liver (M₄) lactate dehydrogenase from 10-carboxydecylamino-Sepharose

Determination of the number of ligand-binding sites blocked on adsorption

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1. The theory of Nichol, Ogston, Winzor & Sawyer [(1974) *Biochem. J.* **143**, 435–443] for quantitative affinity chromatography, when adapted for use with a non-specific column from which a multi-site protein can be specifically desorbed by its free ligand, permits determination of the concentration of adsorption sites on the column, their adsorptive affinity (as an association constant) and either the intrinsic (site) constant for ligand-binding to the protein or an 'occlusion coefficient' (defined as the number of ligand-binding sites blocked on adsorption), one of which must be known. 2. The theory has been applied to the NADH-specific desorption of rat liver M₄ lactate dehydrogenase from 10-carboxydecylamino-Sepharose. It suggests that most of the enzyme molecules are adsorbed with at least two NADH-binding sites blocked, indicating an extensive adsorption interface in relation to the protein surface. Other chromatographic parameters were also determined for the system. 3. Among topics discussed are (a) factors affecting the experimentally determined value for the number of blocked sites, (b) the nature of the adsorption sites on the column and (c) the similarity of the analysis to that for determining Hill coefficients, and other possible applications.

10-Carboxydecylamino-Sepharose (10-CD-Sepharose, I) has been used as column packing for the purification of a number of enzymes by biospecific-elution chromatography, including wheat-germ aspartate carbamoyltransferase (Yon, 1981a) and rat liver lactate dehydrogenase (Kyprianou & Yon, 1982a). Some qualitative aspects of the specific desorption of these and other enzymes by their ligands have been studied (Yon, 1979, 1981a,b; Kyprianou & Yon, 1982b). The adsorbent shares some properties with the immobilized triazinyl-dye packings that are currently attracting much attention as tools for protein purification (Atkinson *et al.*, 1982; Lowe *et al.*, 1982), in that it incorporates constellations of hydrophobic and ionic groups capable of inter-

acting with complementary constellations on the surfaces of proteins, such interactions being remarkably prone to perturbation by soluble protein ligands. In a recent study the NADH-specific desorptions of rat liver lactate dehydrogenase from 10-CD-Sepharose and from Cibacron Blue-Sepharose were compared, and were found to have several points of similarity (Kyprianou & Yon, 1982b).

Quantitative studies of protein elution by biospecific ligands have been, to a large extent, confined to cases of affinity chromatography, which assumes that biologically significant ligand-binding sites on the protein are exclusively or predominantly involved in the enzyme-adsorbent interaction [see reviews by Turkova (1978) and Chaiken (1979a,b)]. This assumption is untenable when a material such as 10-CD-Sepharose is used. Consequently, in studying the interactions of proteins with 10-CD-Sepharose, information about the nature and extent of the adsorption interface would be useful. The present paper describes an attempt to obtain such information by quantitative analysis. General equations described by Nichol *et al.* (1974) for use

Abbreviation used: 10-CD-Sepharose, 10-carboxydecylamino-Sepharose.

with affinity systems have been adapted for the case of a multi-site protein, such as lactate dehydrogenase, interacting with its ligand and with a non-specific adsorbent, such as 10-CD-Sepharose. By using appropriate plotting forms and data from frontal-elution and zonal-elution experiments, we have determined several parameters of the interaction between rat liver M_4 lactate dehydrogenase and 10-CD-Sepharose, including evidence that more than one NADH-binding site is occluded on adsorption of an enzyme molecule.

Theory

A number of equations have been used to analyse the chromatography of equilibrating protein-ligand complexes on 'affinity' columns when the protein has a single ligand-binding site (reviewed by Turkova, 1978), and in principle these could be applied to non-specific columns. However, special problems arise when one is investigating a multi-site protein such as lactate dehydrogenase. In affinity chromatography one assumes that adsorption involves these same sites, usually with the simplifying further assumption that immobilized ligand groups on the column are widely spaced so that only one ligand-binding site on each protein molecule is involved in adsorption (Masters *et al.*, 1969; Nichol *et al.*, 1974; Veronese *et al.*, 1979), although the case of two-site adsorption has been considered (Eilat & Chaiken, 1979; Chaiken, 1979a). In the present case, which involves a non-specific adsorbent (in the sense that biologically significant binding sites are not uniquely involved in the adsorption of the protein), no such assumption can be made *a priori* about the number of ligand-binding sites blocked by adsorption, since the extent of the adsorption site on the protein is not known. The following treatment permits determination of the number of blocked sites. It is an extension by one of us (R. J. Y.) of the comprehensive theory of quantitative affinity chromatography described by Nichol *et al.* (1974). Although the case of a four-site protein is discussed for purposes of illustration, the essential equation [eqn. (4) below] remains valid for any number (greater than 1) of identical independent sites.

The relevant equilibria to be discussed, and the association constants for the formation of each complex, are listed below, where E represents lactate dehydrogenase, L represents NADH and X represents an adsorption site on the column:

The group of equilibria (A) represents the stepwise binding of NADH to the four non-co-operative binding sites on the enzyme to form free solution complexes, each stepwise association constant being the product of the intrinsic (site) association constant K_L and a statistical factor $(4-i+1)/i$ ($i = 1, 2, 3$ and 4 for successive binding) (Klotz, 1946). The equilibrium (B) represents adsorption of the free enzyme on the column. Equilibria representing adsorption of soluble enzyme-ligand complexes, e.g. $EL + X \rightleftharpoons EXL$, are redundant in this formulation, since, if they can occur, they are simply alternative routes to the complexes in (C). The group of equilibria (C) represents the binding of NADH to accessible sites on the adsorbed enzyme, and must take into account the number of such sites. In the example shown, three accessible sites are assumed. If the intrinsic constant K_L for these sites is unaffected by adsorption, then the stepwise association constants are $(3-i+1)K_L/i$ ($i = 1, 2$ and 3). More generally, if n is the number of blocked sites, then the stepwise association constants for the $4-n$ accessible sites is $(5-n-i)K_L/i$ ($i = 1, 2, \dots, 4-n$). It is important to note that in (B) the nature of X is undefined beyond the property of 1:1 binding to the enzyme, all adsorption sites X having the same affinity, K_X , for the enzyme. In particular, no attempt is made to describe X in terms of the substituted 10-carboxydecylamino groups on the column. Moreover, ligand-binding to non-occluded sites on the adsorbed enzyme is assumed to have no effect on K_X . This assumption, and the assumption of the same value of K_L for ligand-binding to the free and adsorbed enzyme, are made so as to limit the constants in the model to a number determinable by simple analysis. The assumptions are unlikely to be valid if the protein is known to be subject to strong site-site interactions, as may be the case in an allosteric enzyme.

From these equilibria and association constants, expressions for the total concentration of enzyme in the starting solution, $[E_T]$, the total concentration of adsorbed enzyme, $[E]_{ads.}$, and the total concentration of adsorption sites, $[X_T]$, are derived in terms of the free (uncomplexed) concentrations $[E]$, $[X]$ and $[L]$ as follows:

$$[E_T] = [E](1 + K_L[L])^4 \quad (1)$$

$$[E]_{ads.} = K_X[E][X](1 + K_L[L])^{4-n} \quad (2)$$

where n is the number of blocked NADH-binding sites on the enzyme;

$$[X_T] = [X]\{1 + K_X[E](1 + K_L[L])^{4-n}\} \quad (3)$$

[These may be compared with eqns. (15), (18) and (20) respectively of Nichol *et al.* (1974).] For frontal-elution chromatography, these expressions can now be used in a theoretical derivation strictly analogous to that of Nichol *et al.* (1974), eqns. (2)–(8), in which the combined retardation effects of adsorption and gel-permeation (liquid–liquid partitioning) are considered. This treatment yields the following equation, in which V is the variable enzyme elution volume, V_0 is the enzyme elution volume in the absence of adsorption and V_s is the volume of the stationary liquid phase [cf. case 4, Table 1, in Nichol *et al.* (1974)]:

$$\frac{1}{V - V_0} = \frac{[E_T]}{V_s[X_T]} + \frac{(1 + K_L[L])^n}{V_s K_X[X_T]} \quad (4)$$

On setting $[L] = 0$, eqn. (4) reduces to eqn. (5) below, which is equivalent to eqn. (9) of Nichol *et al.* (1974):

$$\frac{1}{V - V_0} = \frac{[E_T]}{V_s[X_T]} + \frac{1}{V_s K_X[X_T]} \quad (5)$$

By measuring V for various values of $[E_T]$ in the absence of NADH and plotting $1/(V - V_0)$ versus $[E_T]$, the values of $[X_T]$ and K_X are obtained from the linear gradient and the vertical intercept respectively. V_s is usually known or can be readily measured.

With $[X_T]$ and K_X known, eqn. (4) is applied to values of V obtained at constant $[E_T]$ and various values of $[L]$. The data can be used to determine one, but not both, of n and K_L , and hence the other must be known or its value assumed. In this context, most quantitative affinity-chromatographic studies on multiple-site enzymes are designed to obtain values of K_L by using equations equivalent to eqn. (4) with $n = 1$, i.e. it is assumed that only one site is blocked to soluble ligand by adsorption (see, e.g., Masters *et al.* 1969; Nichol *et al.*, 1974; Veronese *et al.*, 1979). If it is necessary to assume that $n = 2, 3$ or 4, then appropriate linear forms of eqn. (4) may be derived in which the square, cube or fourth root of $\{1/(V - V_0) - [E_T]/(V_s[X_T])\}$ is plotted versus $[L]$. These analyses all permit estimation of the value of K_L . In the present paper, however, we have taken published values for K_L from kinetic or binding studies, thereby permitting us to obtain a value for n , and hence an assessment of the extent of the adsorption interface in relation to the four NADH-binding sites. Rearranging eqn. (4) and taking logarithms to base 10, we obtain:

$$\log \left(\frac{1}{V - V_0} - \frac{[E_T]}{V_s[X_T]} \right) = n \cdot \log(1 + K_L[L]) - \log(V_s K_X[X_T]) \quad (6)$$

Hence n is conveniently obtained from the gradient

of a double-logarithmic plot. In the use of eqns. (4) and (6) it is assumed that $[E_T]$ is very much smaller than $[L]$, so that initial values can be used for $[L]$. If $[E_T]$ is also much smaller than $[X_T]$, the term $[E_T]/V_s[X_T]$ may be omitted, as is usual in zonal-elution analysis (Dunn & Chaiken, 1974).

Experimental

Materials

Sephacrose 4B was obtained from Pharmacia, London W.5, U.K. 11-Aminoundecanoic acid was from Aldrich Chemical Co., Gillingham, Dorset, U.K. NADH was from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other reagents were from Sigma or from BDH Chemicals, Poole, Dorset, U.K., and were of analytical-reagent grade whenever possible. 10-CD-Sepharose was prepared by coupling 11-aminoundecanoic acid to CNBr-activated Sepharose 4B by the procedure of Cuatrecasas (1970), and extensively washed with alternating large volumes of 1% NaCl and 0.1% Brij 35, and finally with distilled water, to remove unchanged 11-aminoundecanoate. The gel was found to contain $17.6 \mu\text{mol}$ of 10-CD groups/ml of settled gel, by titration of carboxy groups. Lactate dehydrogenase (M_4 isoenzyme) was purified to homogeneity from rat liver by the two-step procedure described previously (Kyprianou & Yon, 1982a), in which the major purification was by NADH-specific desorption from 10-CD-Sepharose. The purified enzyme was concentrated by ultrafiltration (including several washes with buffer on the ultrafilter) and passed through a column of Sephadex G-25 before use. Spectrophotometric measurements on the resulting preparation showed that the molar concentration of residual NADH was at most 4% of the molar enzyme concentration, i.e. at most 1% of the concentration of binding sites. All chromatographic experiments in the present work used fresh preparations taken from a single stock solution of enzyme and a single batch of 10-CD-Sepharose. The stock enzyme concentration was found to be 1.92 mg/ml ($14.2 \mu\text{M}$ on the basis of $M_r = 135000$) by amino acid analysis of a sample hydrolysed in 6M-HCl at 110°C for 24 h.

Assays of enzyme activity

Lactate dehydrogenase was assayed by catalytic activity by the method of Bergmeyer & Bernt (1974).

Frontal-elution chromatography

To conserve the enzyme supply, frontal-elution chromatography was performed in small (0.10 ml) columns formed from adapted narrow-bore glass pipettes. The column length was 5.0 cm. Columns were equilibrated with the appropriate buffer, con-

taining NADH as required, at 4°C. A portion of the stock enzyme was equilibrated with the same buffer by passage through a column (10 ml) of Sephadex G-25, and further diluted with the buffer as appropriate. NADH from a concentrated stock solution was added to the required final concentration. Columns were then fed with enzyme solution under gravity, the flow rate being adjusted to deliver approx. 5 column volumes/h. Elution was continued until the activity in the effluent equalled that in the starting solution. Fractions of size approx. 0.2 ml were collected, the volume of each fraction being individually determined by drawing it up into a calibrated syringe. In each experiment, a separate column was used for each value of the variable under study, the whole set being run simultaneously under otherwise identical conditions. The temperature was 4°C in all cases.

Zonal-elution chromatography

Replicate columns (1.00 ml volume) were set up in 1 ml calibrated plastic syringes of 5 mm internal diameter. Equilibration and preparation of the enzyme sample was as described for frontal-elution experiments. Each column was loaded with 0.1 ml of diluted (80-fold) enzyme sample, so that, on further dilution (20-fold at least) due to passage through the column, the enzyme concentration was low enough to be neglected (see the Theory section). The flow rate was 1.5 column volumes/h. Other conditions were as for frontal-elution chromatography.

Results

Effect of buffer concentration on elution volume

A low-ionic-strength buffer (0.02 M-Tris/HCl, pH 8.5) was used in the purification of the enzyme (Kyprianou & Yon, 1982a). Our original aim was to perform quantitative experiments under the same conditions; however, this aim was abandoned when it was found that low buffer concentrations gave rise to excessively large elution volumes, even when the enzyme was at the relatively high concentration of 0.21 μ M (the effect of enzyme concentration is discussed below). Fig. 1 shows the effect of varying the buffer concentration on the frontal-elution volume at this enzyme concentration. A marked dependence on buffer concentration is seen, indicating that adsorption of the enzyme is due, to a large extent, to ionic forces. At a buffer concentration of 0.02 M the elution volume, V_e , is 23 ml, i.e. 230 times the column volume. Since quantitative analysis required a much lower enzyme concentration than 0.21 μ M, the elution volume would be expected to be even larger. Since large elution volumes would consume relatively large amounts of the enzyme in the frontal-elution approach, we

Fig. 1. Effect of buffer concentration on the enzyme elution volume

Columns (0.1 ml) were frontally eluted at 4°C with a solution of the enzyme (0.21 μ M) in Tris/HCl buffer, pH 8.5, containing 1 mM-2-mercaptoethanol. The buffer concentrations were 0.02 M (○), 0.05 M (●), 0.10 M (□) and 0.20 M (■). Other details are given in the Experimental section.

increased the buffer concentration to 0.20 M for all subsequent experiments.

Determination of V_s and V_0

The stationary-phase volume, V_s , of a gel column is normally found as the difference between the void volume (the elution volume of a large, totally excluded and non-interactive macromolecule) and the elution volume of a very small molecule such as water or ammonia. For an interactive gel such as 10-CD-Sepharose, based on the large-pore Sepharose gels, we could not find a sufficiently large macromolecule that was totally free of interaction with the immobilized column groups. Experience has shown that there is negligible shrinkage or expansion of Sepharose on modification to form 10-CD-Sepharose. Further, the inserted 10-carboxydecyl-amino groups are negligible in size compared with the average pore diameter. For these reasons we have assumed that the stationary-phase volume for 10-CD-Sepharose is the same as that of unmodified Sepharose 4B, the parent gel. Measurements on a large column made with DNA and ammonia as markers showed that V_s for the batch of Sepharose used was 66% of the total bed volume.

The unretarded elution volume, V_0 , for rat liver lactate dehydrogenase was obtained by zonal elution

of the enzyme in the presence of a very large excess (2 mM) of NADH, and was found to be 86% of the total bed volume. This is very close to the value of 82% obtained for the elution of the enzyme from a column of unmodified Sepharose 4B, and confirms that (a) the stationary-phase volumes accessible to the enzyme are almost identical in 10-CD-Sepharose and in unmodified Sepharose, lending support to our assumption that V_s is the same in both, and (b) the fully liganded enzyme has negligible affinity for 10-CD-Sepharose, i.e. the 'biospecific sensitivity' of this system, as defined by Yon (1980), is close to 1.0.

Determination of K_x and $[X_T]$

Elution profiles were determined for various concentrations of the enzyme in the absence of NADH. The elution volumes, V , were measured at the midpoint of the enzyme front in each case. Fig. 2 shows these data plotted in accordance with eqn. (5); the best straight line was fitted by regression analysis. The concentration of adsorption sites on the column, $[X_T]$, was found from the slope of this line to be $22 \pm 6 \mu\text{M}$. The adsorption association constant, K_x , was found from the ordinate intercept to be $(2.8 \pm 1.2) \times 10^6 \text{ M}^{-1}$ corresponding to a mean dissociation constant of $0.36 \mu\text{M}$.

The adsorption-site concentration is lower than the density of substituted 10-carboxydecylamino groups (17.6 mM) by nearly 800-fold. Part of the

Fig. 2. Effect of enzyme concentration on elution volume. Columns (0.1 ml) were frontally eluted at 4°C with solutions containing various concentrations of the enzyme in 0.2 M-Tris/HCl buffer, pH 8.5, as described in the legend of Fig. 1. Elution volumes (V) were determined at the midpoint of the emerging enzyme front in each case. The data are plotted in accordance with eqn. (5) and the straight line was fitted by regression.

difference must be due to steric inaccessibility of some of the 10-carboxydecylamino groups; however, it seems unlikely that this would account for more than a fraction of the difference. A more plausible reason could be that a single adsorption site must contain several 10-carboxydecylamino groups oriented in a precise three-dimensional array, a condition that would be satisfied by a very small proportion of these groups.

Frontal-elution analysis of desorption by NADH

Quantitative analysis required that the concentration of NADH, $[L]$, be much greater than the enzyme concentration, $[E_T]$, so that initial values may be used for $[L]$. Fig. 3(a) shows the elution profiles obtained for an enzyme concentration of 9.0 nM and initial NADH concentrations in the range 2–6 μM . The elution volumes from Fig. 3(a) are plotted in accordance with eqn. (6) in Fig. 3(b); for this purpose the intrinsic (site) constant, K_L , for NADH binding to rat liver lactate dehydrogenase was assigned the value $4.2 \times 10^5 \text{ M}^{-1}$, corresponding to the dissociation constant of 2.4 μM reported by Brodelius & Mosbach (1976) (see the Discussion section). The slope of the double-logarithmic plot was 1.85 ± 0.07 by regression analysis, indicating that significantly more than one NADH-binding site on the enzyme is blocked by adsorption on the column. We take this to indicate an extensive adsorption site involving more than one column (10-carboxydecylamino) group.

Zonal-elution analysis of desorption by NADH

Zonal-elution measurements are much more sparing of the purified enzyme than are frontal-elution measurements, and consequently we explored the feasibility of this approach to using eqn. (6). Since the enzyme concentration at peak maxima varies in this approach, it is only used when the term $[E_T]/V_s[X_T]$ can be ignored. The highest enzyme peak in the experiment (plotted in Fig. 4a) corresponds to an enzyme concentration of 15 nM, and hence the error involved in ignoring the second term on the left of eqn. (6) is less than 0.4%. We have therefore plotted the elution (peak) volumes and NADH concentrations in the simplified form in Fig. 4(b), using the same value of K_L as in the previous paragraph. The slope of the double-logarithmic plot was found to be 1.61 ± 0.06 , in fair agreement with the value obtained by frontal-elution analysis.

Enzyme activity in the presence of 11-amino-undecanoate

To elucidate further the effect of NADH on the adsorption of the enzyme to 10-CD-Sepharose, we examined the effects of free 11-aminoundecanoate, which provides the immobilized groups of 10-

CD-Sepharose [see structure (I)] on the activity of the enzyme. The compound was tested at concentrations up to 4.9 mM (its solubility limit in the experimental buffer system, 0.2 M-Tris/HCl, pH 8.5), although its actual monomeric concentration may be considerably less owing to micelle formation. Two sets of substrate concentration were tested, 75 μ M-NADH/1.5 mM-pyruvate and 6 μ M-NADH/6 μ M-pyruvate. 11-Aminoundecanoate neither activated nor inhibited the enzyme under these conditions. If

Fig. 3. Effect of NADH concentration on frontal-elution volume

Columns (0.1 ml) were frontally eluted with a solution of the enzyme (9.0 nM) and various concentrations of NADH. Other details are given in the Experimental section or the legend to Fig. 2. (a) Elution profiles for NADH concentrations (μ M) of 0 (○), 2 (●), 3 (□), 4 (■), 5 (Δ) and 6 (▲). (b) Data plotted in accordance with eqn. (6). The line was fitted by regression.

the ability of NADH to desorb the enzyme from 10-CD-Sepharose is due to simple competition between NADH and 10-carboxydecylamino groups, one would have expected considerable inhibition by 11-aminoundecanoate in view of the value of K_X , $2.8 \times 10^6 \text{ M}^{-1}$, even if the monomeric concentration of 11-aminoundecanoate were as low as 10 μ M. We conclude that the mechanism of NADH-specific desorption from 10-CD-Sepharose is not due to the binding of single 10-carboxydecylamino groups at, or overlapping, the NADH-binding site(s) (see the discussion of possible modes of ligand-specific desorption in Yon, 1981b).

Fig. 4. Effect of NADH concentration on zonal-elution volume

Columns (1.0 ml) were loaded with 0.1 ml of solution containing the enzyme (0.18 μ M) and various concentrations of NADH, then eluted with the same solution from which the enzyme was omitted. Other details are given in the Experimental section or the legends to preceding Figures. (a) Zonal-elution profiles for NADH concentrations (μ M) of 8 (○), 10 (●), 12 (□), 15 (■) and 18 (Δ). (b) Data plotted in accordance with eqn. (6). The line was fitted by regression.

Discussion

Significance of the 'occlusion coefficient', n

We present in this paper an analytical approach for assessing the extent of the adsorption interface between protein and adsorbent by determining the number of blocked ligand-binding sites on an adsorbed multi-site protein. The analysis is similar in form to that used in the determination of the Hill interaction coefficient for a co-operative protein, which in its simplest form is the number of co-operative ligand-binding sites (Hill, 1910). Since a single ligand-binding site is normally a small portion of the total protein surface, and since replicate sites are usually distributed with spherical symmetry (i.e. maximum separation) over this surface, adsorption that blocks one ligand-binding site can involve a relatively small adsorption interface, whereas adsorption that blocks two or more sites must necessarily be quite extensive (see Fig. 5).

In view of this significance of n , it is worth considering which parameter values, if in error, are most likely to affect the value of n obtained by experiment; in particular we should ask whether, given the likely error-structure of our experiments, our value of approx. 1.7 is significantly greater than 1. Since n is the slope of a double-logarithmic plot, it is relatively insensitive to small errors in the variables and calculated constants contributing to both the ordinate and abscissa terms of eqn. (6). Analysis of likely worst-case errors in the variables/constants that were determined or calculated by us (i.e. all excepting K_L) suggests that their cumulative effect is unlikely to change the value of n by more than about 10%. Hence, if we assume no error in measuring the concentration of NADH, then the value of n is only seriously overestimated if we have used a value for K_L that is about an order of magnitude different from the true value. The majority of literature values of K_L for the binding of

NADH to mammalian M_4 isoenzymes of lactate dehydrogenase are in the range 1×10^5 – $5 \times 10^5 \text{ M}^{-1}$ (Stinson & Holbrook, 1973; Brinkworth *et al.*, 1975; Brodelius & Mosbach, 1976), and include $2 \times 10^5 \text{ M}^{-1}$ for the mouse M_4 isoenzyme (Brinkworth *et al.*, 1975) as well as $1.3 \times 10^5 \text{ M}^{-1}$ (Vestling, 1962) and $4.2 \times 10^5 \text{ M}^{-1}$ (Brodelius & Mosbach, 1976) for the rat liver (predominantly M_4) enzyme. Since in general the value of n decreases with increase in K_L , we chose the highest value for the rat liver enzyme, $4.2 \times 10^5 \text{ M}^{-1}$, which gave n values of 1.85 (frontal-elution analysis) and 1.61 (zonal-elution analysis). These values are therefore likely to be minimal estimates. We conclude that n is significantly greater than 1.

The non-integral value (about 1.7) for n may merely reflect experimental error in determining a true value of 2. Alternatively it may indicate a more complex mechanism than was used to set up eqns. (1)–(6) (see the Theory section). Arguably deviation from this simple theory is most likely to be due to the following two factors. (1) Heterogeneity of adsorption sites with respect to the number of blocked sites. Thus a value of 1.7 might indicate a majority of adsorptions blocking two sites and a minority blocking one. (2) Partial blockage, i.e. some NADH-binding sites on the adsorbed enzyme have finite but decreased intrinsic binding constants (K'_L) compared with those of fully accessible sites (K_L). Although the effects on n of these modifications to the theory have not, as yet, been explored in full, a preliminary study (R. J. Yon, unpublished work) suggests that a value of n significantly greater than 1 will always indicate that at least some enzyme molecules are adsorbed with two or more sites fully or partly blocked. In such cases it is still worth measuring n as a general index of occlusion, in much the same way as the Hill coefficient (frequently non-integral when greater than 1) is used as an index of co-operativity between interacting ligand-binding sites.

Fig. 5. Schematic representation of possible adsorption modes blocking two ligand-binding sites

The large object is the enzyme with four ligand-binding sites. Hatching denotes the column matrix containing immobilized 10-CD groups (black circles).

Nature of the adsorption sites on 10-CD-Sepharose

Occlusion of two or more NADH-binding sites can occur in one of two ways illustrated in Fig. 5. (1) The binding sites are directly involved in adsorption to the 10-carboxydecylamino groups (Fig. 5a), i.e. this is a case of affinity chromatography. This appears very unlikely, since the free analogue of the 10-carboxydecylamino group, i.e. 11-amino-undecanoate, failed to inhibit the activity of the enzyme even at low substrate concentrations. (2) The adsorption is non-specific in the sense that the 10-carboxydecylamino groups interact with parts of the protein surface not involved in catalysis, but in a way that hinders access to the ligand-binding sites (Fig. 5b). This seems the most likely mode of adsorption. The number of 10-carboxydecylamino groups that comprise a single enzyme-adsorption site is not known; however, at least two 10-carboxydecylamino groups would seem to be required to tie down a surface area large enough to include two NADH-binding sites. As mentioned above (see the Results section), the involvement of several 10-carboxydecylamino groups in a precise geometry would explain the very small number of adsorption sites relative to the total number of immobilized 10-carboxydecylamino groups. Such multivalent adsorption is well-documented (see Jennissen, 1976; Yon, 1978).

Wider applications

Determination of the 'occlusion coefficient', n is applicable to the chromatography of any multi-site protein on any adsorbent, provided that the adsorbed protein is subject to ligand-specific desorption, that its ligand-binding sites are identical and non-co-operative, and that the number of such sites and their intrinsic binding constant, K_L , are independently known. When, in cases of true affinity chromatography, two or more ligand-binding sites on a single protein molecule are involved in adsorption (represented in Fig. 5a), theory predicts a relationship between the adsorption constant, K_x , the intrinsic constant, K_L , and the number of occluded sites, n , that is subject to experimental test when these values have been determined. Hence this analytical approach may be of value in resolving questions of specificity in adsorption, as for example in the ongoing debate on the mechanism of adsorption of dehydrogenases and kinases to triazinyl-dye columns (Stellwagen, 1979).

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