Multivalency of the Partitioning Species in Quantitative Affinity Chromatography. Evaluation of the Site-Binding Constant for the Aldolase–Phosphate Interaction from Studies with Cellulose Phosphate as the Affinity Matrix†

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ABSTRACT: Theory is presented which describes the competitive interaction of a multivalent solute with a univalent ligand and an affinity matrix. The formulation accounts for cross-linking interactions of the multivalent solute, and of its complexes with ligand, with matrix interaction sites in terms of two site-binding constants pertaining respectively to ligand–solute and solute–matrix interactions. Explicit expressions are derived which permit evaluation of these constants from experimental results obtained in partition equilibrium experiments or in frontal affinity chromatography studies. These relations are explored in partition equilibrium experiments conducted with cellulose phosphate as the matrix, aldolase as the solute, and phosphate as the ligand. At pH 7.4, $I = 0.15$, a value of 350 ± 60 M^{-1} was obtained for the aldolase–phosphate site-binding constant, in close agreement with the corresponding value deduced from competitive inhibition studies. It is concluded that the present approach is particularly suited to the elucidation of weak interactions, which cannot be reliably studied by conventional means.

The use of affinity chromatography for evaluating equilibrium constants (Andrews et al., 1973; Dunn & Chaiken, 1974) has introduced an analytical potential of the technique that requires further development. In a theoretical treatment of frontal affinity chromatography (Nichol et al., 1974), consideration was restricted to situations in which the partitioning solute either possessed only a single site for interactions with the affinity matrix or, if multivalent, was capable of attachment to only one matrix site because of restrictive steric requirements. A similar but less rigorous theory has been developed for the behavior of such systems in zonal affinity chromatography (Dunn & Chaiken, 1974, 1975). The limitations of these treatments have been emphasized recently in a study of the interaction between a bivalent antibody and an immobilized antigen where elution was effected by the small monovalent antigen phosphorylcholine (Eilat & Chaiken, 1979). In that work, the established theory (Dunn & Chaiken, 1974; Nichol et al., 1974) sufficed to describe the results when the concentration of matrix affinity sites was low but proved inadequate when this concentration was sufficiently high to permit cross-linking interactions of the matrix sites with the bivalent antibody. The recognition of the importance of cross-linking interactions when certain systems are subjected to affinity chromatography provides the motivation for the present investigation, which aims to present explicit relations pertinent to the quantitative description of the system and to evaluation of the relevant site-binding constants. As noted previously (Nichol et al., 1974), this requires consideration of equilibrium partition experiments or frontal chromatography since the approximate theory developed for zonal chromatography with this type of system (Chaiken et al., 1979) fails to distinguish between free and total concentrations of reactant species. Use of the present quantitative theory is illustrated in a partition equilibrium study with aldolase as the solute and with phosphate and cellulose phosphate as the small ligand and affinity matrix, respectively.

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Theory

Basic Relations. Consider a partition equilibrium experiment in which a solute, A, possesses f sites for interaction either with univalent ligand, S, or with sites (X) on a q-valent matrix, M, the reactions being competitive. For a tractable presentation, we shall consider initially situations where no gel partitioning occurs, but this will be examined later. Let the solute–matrix interactions be governed by a single site-binding constant, k_{AX}, irrespective of whether the remaining A sites are vacant or occupied by S or X, and the solute–ligand interactions be described by a site-binding constant k_{AS}. Such equivalence and independence of binding was also assumed by Chaiken et al. (1979). In the mobile (liquid) phase, the solute exists as free A and complexes AS_i ($1 \leq i \leq f$), the constituent molar concentration, $\bar{m}_A$, in this phase being related to the molar concentration of free A, m_A, by (Klotz, 1946)

$$\bar{m}_A = m_A(1 + k_{AS}m_S)^f \quad (1)$$

where m_S is the equilibrium concentration of unbound ligand in the mobile phase. Free A possesses f sites capable of interaction with the matrix, and each AS_i species has $(f - i)$ such sites, the fully saturated complex, AS_f, being incapable of binding to the matrix. Thus, when account is taken of all types of interaction, it follows from reacted-site probability theory (Flory, 1941; Singer, 1965; Calvert et al., 1979) that the total concentration of A in the system, $\bar{m}_A$, is given by

$$\bar{m}_A = \frac{m_A}{(1 - P_{AX})^f} + \frac{m_{AS}}{(1 - P_{AX})^{f-1}} + \frac{m_{AS_2}}{(1 - P_{AX})^{f-2}} + \ldots + m_{AS_f} \quad (2)$$

where P_{AX} denotes the probability that an A site has reacted with a matrix site.

The concentrations of the AS_i complexes in the mobile phase may be expressed as (Klotz, 1946)

$$m_{AS_i} = C_i^f k_{AS}^i m_A m_S^i \quad (3)$$

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Combination of eq 2 and 3 yields

$$\bar{m}_A = m_A\{1 + \sum_{i=1}^{i=f} C_i^f[k_{AS}m_S(1 - P_{AX})]^i\}/(1 - P_{AX})^f \quad (4a)$$

and thus, by application of the binomial theorem

$$\bar{m}_A = m_A[1 + k_{AS}m_S(1 - P_{AX})]^f/(1 - P_{AX})^f \quad (4b)$$

It proves helpful to rearrange eq 4b with the use of eq 1 as

$$P_{AX}/(1 - P_{AX}) = (1 + k_{AS}m_S)[(\bar{m}_A/m_A)^{1/f} - 1] \quad (4c)$$

It is now required to interrelate P_{AX} with P_X, the probability that an X site on the matrix element is occupied by an A or AS_i ($i \neq f$) molecule. If $\bar{m}_X$ is the total molar concentration of X sites present (equalling $q\bar{m}_M$, where $\bar{m}_M$ is the total molar concentration of matrix), the condition of conservation of reacted sites requires that the concentration of reacted sites be given by

$$P_X\bar{m}_X = \frac{fP_{AX}m_A}{(1 - P_{AX})^f} + \sum_{i=1}^{i=f-1}\left[\frac{(f - i)P_{AX}m_{AS_i}}{(1 - P_{AX})^{f-i}}\right] \quad (5a)$$

which on substitution of eq 3 for m_{AS_i}, collection by the binomial theorem, and elimination of m_A using eq 1 becomes

$$P_X\bar{m}_X = \frac{fP_{AX}\bar{m}_A[1 + k_{AS}m_S(1 - P_{AX})]^{f-1}}{(1 + k_{AS}m_S)^f(1 - P_{AX})^f} \quad (5b)$$

Combination of eq 5b with eq 4b (with m_A substituted from eq 1) gives

$$P_X\bar{m}_X = fP_{AX}\bar{m}_A/[1 + k_{AS}m_S(1 - P_{AX})] \quad (5c)$$

The concentration of sites on A or AS_i ($i \neq f$) reacted with matrix sites is given by the right-hand side of eq 5c and is seen to be less in the presence of S than in its absence, as required in this competitive situation. This concentration divided by the product of the concentration of unreacted X sites, $(1 - P_X)\bar{m}_X$, and of unreacted A sites, $f(1 - P_{AX})\bar{m}_A/[1 + k_{AS}m_S(1 - P_{AX})]$, defines the site-binding constant, k_{AX}, as

$$k_{AX} = P_{AX}/[(1 - P_{AX})(1 - P_X)\bar{m}_X] \quad (5d)$$

A combination of eq 4c, 5c, and 5d yields, after extensive rearrangement

$$k_{AX} = \frac{(1 + k_{AS}m_S)[1 - (m_A/\bar{m}_A)^{1/f}]}{(m_A/\bar{m}_A)^{1/f}\{\bar{m}_X - f\bar{m}_A[1 - (m_A/\bar{m}_A)^{1/f}]\}} \quad (6)$$

The basic set of relations is completed by consideration of appropriate concentrations of ligand, S. The constituent concentration of ligand in the liquid phase is given by (Nichol et al., 1967)

$$m_S = m_S + \sum_{i=1}^{i=f} im_{AS_i} = m_S + fk_{AS}m_Am_S(1 + k_{AS}m_S)^{f-1} \quad (7)$$

where, as before, m_S is the concentration of unbound ligand in this phase. The total molar concentration of ligand in the system, however, must also account for ligand associated with the stationary phase via interaction of AS_i ($i \neq f$) molecules with matrix sites. This total concentration is given by

$$\bar{m}_S = m_S + \sum_{i=1}^{i=f}\frac{im_{AS_i}}{(1 - P_{AX})^{f-i}} \quad (8a)$$

which may be simplified with the use of eq 3 and 4c to the form

$$\bar{m}_S = m_S + fk_{AS}m_Am_S(1 + k_{AS}m_S)^{(f-1)}(\bar{m}_A/m_A)^{(f-1)/f} \quad (8b)$$

Use of eq 1 provides the following alternative expressions for $\bar{m}_S$ and $\bar{\bar{m}}_S$

$$\bar{m}_S = m_S[1 + [fk_{AS}\bar{m}_A/(1 + k_{AS}m_S)]] \quad (8c)$$

$$\bar{\bar{m}}_S = m_S[1 + [fk_{AS}\bar{m}_A(\bar{m}_A/m_A)^{(f-1)/f}/(1 + k_{AS}m_S)]] \quad (8d)$$

These relations show that in cases of relatively weak solute–ligand interaction (k_{AS} small), where the experimental design will ensure that $\bar{\bar{m}}_S$ (and hence $\bar{m}_S$) are considerably larger than $\bar{\bar{m}}_A$ (and $\bar{m}_A$), both $\bar{m}_S$ and $\bar{\bar{m}}_S$ will approximate m_S. The elucidation of this type of system is particularly suited to study by affinity chromatography rather than by conventional means such as equilibrium dialysis, and it is in this chromatographic situation that the approximation $m_S \simeq \bar{\bar{m}}_S$ is likely to be reasonably fulfilled, as it is in initial velocity studies of enzymatic reactions with low molecular weight substrates.

Applications. In a batch partition experiment, conducted in the absence of S and with an affinity matrix that excludes partitioning of solute A by means other than interaction with matrix sites, eq 6 is directly applicable and reduces to

$$k_{AX} = (1 - \psi)/\psi[\bar{\bar{m}}_X - f\bar{\bar{m}}_A(1 - \psi)] \qquad \psi = (\bar{m}_A/\bar{\bar{m}}_A)^{1/f} \quad (9)$$

A series of such experiments conducted with assigned values of $\bar{\bar{m}}_X$ and $\bar{\bar{m}}_A$ with determination of corresponding values of $\bar{m}_A$ from the liquid supernatant permits evaluation of k_{AX} from eq 9 and of f (if this number of binding sites on the partitioning solute is not already known) on the basis of the required constancy of k_{AX} within the series. Extension of these experiments to include a known concentration of ligand, $\bar{\bar{m}}_S$, then allows the evaluation of k_{AS} from eq 6, utilizing the approximation $m_S \simeq \bar{\bar{m}}_S$, if appropriate, or the numerical solution of the simultaneous eq 6 and 8d.

Since most affinity chromatography systems employ a gel (for example, Sepharose) as the matrix for immobilization of the affinity groups X, it is now required to discuss situations where gel partitioning of solute occurs as well as interaction with X groups. The discussion is restricted to commonly encountered systems where S is of sufficiently small size to render the partition coefficients of all AS_i complexes indistinguishable from that of unbound A. Moreover, in the same context, it is useful to consider the way in which the basic eq 6 must be modified for application to chromatographic experiments in which the primary experimental parameter is an elution volume or a partition coefficient. The first point to note in relation to eq 6 is the existence of the parameter $\bar{\bar{m}}_A$, not only in the ratio $\bar{m}_A/\bar{\bar{m}}_A$ but also in the coefficient of one of the denominator terms. Consequently, we reinforce our earlier assertion (Nichol et al., 1974) that rigorous quantitative interpretation of affinity chromatography experiments requires the collection of data by the frontal chromatographic technique (Winzor & Scheraga, 1963) to allow specification of the total solute concentration to which the measured elution volume refers. In zonal chromatography, $\bar{m}_A$ and hence $\bar{\bar{m}}_A$ are undefined and varying quantities.

In a frontal experiment with plateau concentration $\bar{m}_A$, the corresponding total concentration, $\bar{\bar{m}}_A$, is given by (Nichol et al., 1974)

$$\bar{\bar{m}}_A = \bar{m}_A(1 + K_{av}) \quad (10a)$$

the partition coefficient of the A constituent, K_{av}, being defined (Laurent & Killander, 1964) as

$$K_{av} = (\bar{V}_A - V_0)/(V_t - V_0) \quad (10b)$$

where $\bar{V}_A$ is the measured elution volume of the A constituent for a column with void volume V_0 and total volume V_t. The product $K_{av}\bar{m}_A$ denotes the concentration of all forms of A immobilized, of which a quantity $K_{av}{}^*\bar{m}_A$ is in the stationary

phase due to gel partitioning (Nichol et al., 1974), where K_{av}* is the partition coefficient of A (and, by assumption, of all AS_i) in the absence of any interaction with matrix affinity sites. As noted previously (Nichol et al., 1974), this quantity may be obtained from experiments with saturating concentrations of competing ligand or estimated on the basis of the molecular size of A. It is now noted that the right-hand side of eq 2 does not express the total concentration of the A constituent in the system for cases where gel partitioning operates. Nevertheless, eq 2 may be modified to retain validity by subtracting from the left-hand side the contribution due to gel partitioning. Thus

$$\bar{m}_A - K_{av}^*\bar{m}_A = \bar{m}_A(1 + K_{av} - K_{av}^*) = \frac{m_A}{(1 - P_{AX})^f} + \sum_{i=1}^{i=f}\frac{m_{AS_i}}{(1 - P_{AX})^{f-i}} \quad (11)$$

Allowance for gel partitioning in equations which follow eq 2 may therefore be made by replacing $\bar{m}_A$ with $\bar{m}_A(1 + K_{av} - K_{av}^*)$, whereupon the basic eq 6 becomes

$$k_{AX} = \frac{(1 + k_{AS}m_S)\,[(1 + K_{av} - K_{av}^*)^{1/f} - 1]}{\bar{m}_X - f\bar{m}_A(1 + K_{av} - K_{av}^*)^{(f-1)/f}[(1 + K_{av} - K_{av}^*)^{1/f} - 1]} \quad (12)$$

Equation 12 may be employed to determine k_{AX}, k_{AS}, and f, as described previously. In this connection, the gel partitioning of S is accounted for by calculating $\bar{m}_S$ for use in eq 8d on the basis of the total volume accessible to the ligand.

Experimental Procedures

Materials. Rabbit muscle aldolase was obtained as an ammonium sulfate suspension from Boehringer Mannheim GmbH and used without further purification. A crystalline suspension of a mixture of α-glycerophosphate dehydrogenase and triosephosphate isomerase was supplied by Sigma Chemical Co., who also provided NADH, fructose 1,6-bisphosphate, and cellulose phosphate. The enzymes were first dialyzed for 16 h at 4 °C against the appropriate buffer to bring them into dialysis equilibrium with the medium to be used for the particular enzyme kinetic or partition study. Experiments were performed at pH 7.4 in buffers with a nominal ionic strength of 0.15. Buffers routinely contained 0.010 M imidazole and 0.0023 M HCl, the remainder of the ionic strength being provided by sodium phosphates and/or sodium chloride: Na_2HPO_4 and NaH_2PO_4 were added in a 3.99:1 molar ratio to ensure identity of the pH of phosphate-containing buffers with that (pH 7.4) of the imidazole–chloride medium. Concentrations of aldolase were determined spectrophotometrically at 280 nm by using an extinction coefficient ($A_{1cm}^{1\%}$) of 9.1 (Baranowski & Niederland, 1949) and converted to a molar scale on the basis of a molecular weight of 160000 (Kawahara & Tanford, 1966).

Kinetic Studies. In kinetic studies of the inhibition of aldolase by phosphate, aldolase activity was estimated by a coupled assay system in which the triosephosphates formed from fructose 1,6-bisphosphate were converted to α-glycerophosphate (Richards & Rutter, 1961). The rate of the aldolase-catalyzed hydrolysis of fructose 1,6-bisphosphate was thus estimated spectrophotometrically at 340 nm by observing the disappearance of NADH from mixtures of NADH and substrate. In these experiments, conducted at 25 °C in a Cary 14 recording spectrophotometer, the aldolase concentration was 0.82 μg/mL, the α-glycerophosphate dehydrogenase–triosephosphate isomerase mixture concentration was 8.6 μg/mL, the NADH concentration was 0.145 mM, and the fructose 1,6-bisphosphate concentration was varied between 25.0 μM and 0.98 mM.

Table I: Partition Equilibrium Data on the Binding of Aldolase to Cellulose Phosphate in Imidazole–Chloride Buffer (pH 7.4, I = 0.15)

$\bar{m}_A$ (μM)	m_A (μM)	V (mL)	$\bar{m}_X$[a] (μM)	k_{AX}[b] (10^{-4} M^{-1})
1.07	0.16	14.30	11.68	6.04
1.33	0.25	14.40	11.60	5.32
1.58	0.37	14.50	11.52	4.55
2.08	0.46	14.70	11.36	5.24
2.57	0.64	14.90	11.21	5.08
3.15	0.89	15.15	11.02	4.89
3.72	1.18	15.40	10.84	4.68
4.27	1.44	15.65	10.67	4.72
4.81	1.71	15.90	10.50	4.83
5.83	2.28	16.40	10.18	4.98
6.78	2.80	16.90	9.88	5.48

[a] Based on a value of 167 nmol for the amount of accessible matrix sites (see Figure 1). [b] Calculated from eq 9 with f = 4.

Affinity Chromatography Experiments. The effect of phosphate on the affinity of cellulose phosphate for aldolase was studied by a recycling partition equilibrium procedure in which the liquid phase of a stirred slurry of ion exchanger and enzyme was analyzed spectrophotometrically at 280 nm by means of a flow cell placed in the line returning the liquid phase to the partitioning system. Before use, the cellulose phosphate was subjected to the washing procedure recommended (Rutter et al., 1966) as a precursor to the purification of aldolase by affinity chromatography on this medium. A slurry containing 0.3–0.8 g of pretreated cellulose phosphate (in water) was then placed in a preweighed column (2.3 × 5 cm) fitted with a sintered-glass disk, and equilibration with the appropriate buffer (pH 7.4, I = 0.15) was effected by passage of the buffer through the ion exchanger. After removal of surplus buffer by suction, the column was reweighed and a further 10 mL of buffer added. The outlet from the column was then connected to the recycling system containing the flow cell and a peristaltic pump to maintain a flow rate of 1.6 mL/min; at this stage, the column was placed in a water bath maintained at 25 °C. After temperature equilibration had been realized, an aliquot (0.10 mL) of stock enzyme solution (6.03 mg/mL) in the appropriate buffer was added to the stirred slurry, approximately 15 min being allowed to elapse before the effluent absorbance (at 280 nm) was recorded and a further aliquot of enzyme solution added. At the end of the experiment, the slurry was washed with buffer and water to remove all protein and buffer components and finally with ethanol. Excess ethanol was then removed by suction and the remainder by heating the column in an oven at 40 °C for 16 h, after which the column was weighed again to determine the weight of dry cellulose phosphate used in the experiment.

Results

Partition Equilibrium Studies in the Absence of Ligand. It is first noted that the recycling procedure employed is entirely equivalent to a series of batch partition equilibrium experiments in which $\bar{m}_A$ is varied and the corresponding m_A determined; such values are reported in the first two columns of Table I. Column 3 presents the corresponding volumes, V, of the liquid phase. The advantage of this recycling procedure is that the amount of matrix is held fixed within the series of experiments; but a comment is required on the evaluation of $\bar{m}_X$, the concentration of accessible matrix affinity sites, appropriate to eq 6. At an infinite concentration of A ($1/\bar{m}_A \rightarrow 0$), it is evident that all accessible matrix sites would

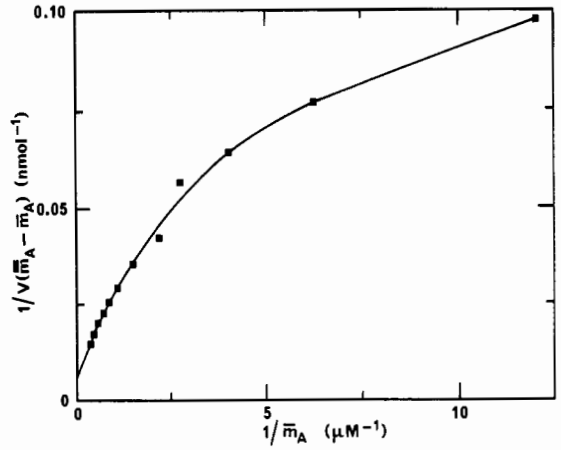

FIGURE 1: Evaluation of the molar amount ($V\bar{m}_X$) of matrix binding sites for aldolase in a recycling partition equilibrium experiment with 0.783 g of cellulose phosphate in imidazole–chloride buffer (pH 7.4, I = 0.15).

be saturated (Calvert et al., 1979), and thus a plot of the reciprocal of the amount of aldolase bound $[1/V(\bar{m}_A - m_A)]$ vs. $1/m_A$ may be extrapolated to the ordinate intercept to determine the amount of accessible affinity sites in the recycling system ($1/V\bar{m}_X$). Figure 1 utilizes the results shown in Table I to present a plot in this format. Although the indicated extrapolation is somewhat uncertain, a value for $V\bar{m}_X$ of 167 nmol was indicated and used in subsequent calculations. It is indeed possible to refine this value, if required, on the basis of iterative use of eq 9 in determining constant values of the parameters f and k_{AX}, a procedure that is clearly facilitated when f is known. Column 4 of Table I summarizes the values of $\bar{m}_X$ on the basis of the extrapolation shown in Figure 1 and column 5 the corresponding values of k_{AX} calculated from eq 9 with f = 4; the latter value is consistent with the involvement of the active sites in the aldolase–cellulose phosphate interaction (Rutter et al., 1966; Masters et al., 1969) and the observation that phosphate is a competitive inhibitor of aldolase, which possesses four active sites (Lai & Horecker, 1972). Analysis of the results shown in column 5 of Table I yields a value of (5.1 ± 0.3) × 10^4 M^{-1}, the indicated error being twice the standard deviation of the mean. This variation in the mean could not be improved by minor changes in the extrapolated value of $V\bar{m}_X$ obtained from Figure 1 and, indeed, is a reasonable error in the determination of a site-binding constant of this magnitude.

Partition Experiments in the Presence of Ligand. Table II presents results obtained in three sets of experiments, each conducted with a different phosphate concentration and with $\bar{m}_A$ varied within each set. Comment is only required on the value of m_S used in eq 6 to calculate k_{AS} from these results. For reasons already given in the theoretical section, it was judged reasonable to make the approximation that $\bar{m}_S \simeq \bar{m}_S \simeq m_S$ for this system since there is more than a 500-fold molar excess of ligand to enzyme in each experiment. The values of k_{AS}, calculated as the sole remaining unknown in eq 6, are shown in column 4 of Table II and yield a site-binding constant of 350 ± 60 M^{-1} ($\pm 2s_m$) for the aldolase–phosphate interaction.

The system was selected to permit comparison of the value of k_{AS} determined by affinity chromatography with the competitive inhibition constant estimated from kinetic studies. The results of these inhibition studies are summarized in Table III, which reports, for the same environment as employed in the affinity chromatographic studies, values of the apparent Michaelis constant, K_m^{app}, and the reciprocal of the conventional inhibition constant K_I for a series of inhibitor (phosphate) concentrations. The mean value of the latter quantity is 350 ± 50 M^{-1}, which clearly agrees with k_{AS} deduced from affinity chromatography.

Table II: Evaluation of the Site-Binding Constant for the Aldolase–Phosphate Interaction by Affinity Chromatography

$\bar{m}_A$ (μM)	m_A (μM)	$\bar{m}_X$[a] (μM)	k_{AS}[b] (M^{-1})
	m_S = 0.30 mM[c]		
0.153	0.088	3.07	100
0.305	0.186	3.06	440
0.455	0.277	3.05	320
0.604	0.366	3.04	170
0.752	0.458	3.02	110
	m_S = 1.00 mM[c]		
0.113	0.069	3.09	180
0.224	0.143	3.07	280
0.335	0.221	3.06	360
0.445	0.299	3.05	410
0.554	0.374	3.04	400
0.662	0.443	3.02	340
0.769	0.509	3.01	270
0.875	0.593	3.00	330
	m_S = 5.00 mM[c]		
0.080	0.055	3.58	170
0.160	0.125	3.57	370
0.239	0.191	3.57	420
0.319	0.261	3.56	490
0.397	0.322	3.55	460
0.476	0.392	3.54	510
0.554	0.456	3.54	500
0.632	0.520	3.53	500
0.709	0.595	3.52	570

[a] Based on the weight of cellulose phosphate and Figure 1. [b] Calculated from eq 6. [c] Established by preequilibration of the cellulose phosphate and the enzyme with imidazole–chloride–phosphate buffer (pH 7.4, I = 0.15).

Table III: Evaluation of the Aldolase–Phosphate Interaction by Competitive Inhibition Studies

phosphate concn (10^3 M)	K_m^{app}[a] (10^5 M)	$1/K_I$[b] (M^{-1})
0	3.11	
0	3.45	
0.5	3.97	420
5.0	8.39	310
10.0	14.1	330
20.0	25.8	340

[a] $K_m^{app} = K_m[1 + (m_I/K_I)]$, values being obtained from standard double-reciprocal analysis. [b] Value obtained by using the mean value of 3.28 × 10^{-5} M for the Michaelis constant K_m.

Discussion

In relation to the particular aldolase–phosphate system selected for study, three points merit comment. It is noted that a major assumption of the theoretical formulation, namely, that a single site-binding constant sufficies to describe all solute–matrix interactions, appears to be vindicated by the constancy of the k_{AX} values shown in Table I. Nevertheless, the possibility cannot be discounted that some steric limitations in relation to the cross-linking interactions may have been operative, which would necessitate consideration of additional site-binding constants. The present analysis does not indicate such complexity, which may, however, have been obscured by experimental error, especially in relation to the extrapolation of Figure 1. Even if k_{AX} is regarded as an approximate average measure of cellulose phosphate–aldolase interactions, the interesting point emerges that it is some 150-fold greater than k_{AS}, the site-binding constant for the interaction between

aldolase and free phosphate. One possible explanation of this difference is that a cluster of immobilized phosphate residues form the matrix affinity site for interaction with each binding site on the aldolase molecule. From inhibition studies with a series of analogues of fructose 1,6-bisphosphate, Hartman & Barker (1965) consider a distance of 1.0–1.2 nm to be the optimal spacing between phosphate groups for their interaction with aldolase.

Of greater general importance than these observations at the microscopic level is the finding that a ligand-acceptor site-binding constant of small magnitude may be reliably assessed by the use of the affinity chromatography procedure and theory presented herein. In the present context, this conclusion could be drawn on the basis of a favorable comparison between k_{AS} and $1/K_I$, but it should be stressed that this reassurance is even more relevant to studies of nonenzymatic systems that cannot be studied kinetically. It may appear at first sight that weak binding to nonenzymatic acceptors could be elucidated by a more conventional procedure such as equilibrium dialysis. In this connection, phosphate binding to aldolase has been examined by this method (Ginsburg & Mehler, 1966), but only at low ionic strength ($I = 0.017$), where the site-binding constant was increased to a value of 2.8×10^4 M^{-1}, some 800-fold greater than the corresponding constant in the present environment ($I = 0.15$). In general terms, the assessment of a site-binding constant from equilibrium dialysis results requires accurate determination of the difference between the concentrations of total and free ligand, which in the case of weak binding involves subtraction of quantities of similar magnitude with consequent large error. Affinity chromatography does not explicitly require the determination of this difference but rather is based on estimations of total and free constituent acceptor concentrations.

With presently available theory, the application of this sensitive method is restricted either to systems in which no gel partitioning of solute arises (where eq 6 is applicable) or to systems where the ligand is sufficiently small to permit the approximation that K_{av}^* is identical for A and all AS_i complexes (where eq 12 is applicable). It may be possible to ensure the former condition in situations where both solute and ligand are macromolecular by the suitable choice of an affinity matrix that excludes the larger reactant; but it should be noted tht with the commonly employed Sepharose-based systems some extension of the present theory would be required to allow for any differential partitioning of the A and AS_i species. Nevertheless, there are many systems of biological interest for which the present treatment is already adequate, and thus it is hoped that this work will assist in the elucidation of their interaction parameters, especially where weak interactions are encountered.

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