A New Method of Quantitative Affinity Chromatography and its Application to the Study of Myosin

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A new method of quantifying the interactions between two or three components of an interacting system, one of which is insoluble, is described. The method differs from those previously applied to affinity chromatography systems in that it does not require that elution volumes be measured, but is instead dependent on measurements of the quantity of affinity-bound material. Theoretical expressions are derived for systems in which the acceptor molecule (usually the enzyme) is in free solution and those in which the acceptor is immobilized. Examples presented to illustrate the validity of the theory are of the latter type and are from studies on the myosin-adenosine nucleotide-PP_i system. With Sepharose-myosin columns (myosin covalently coupled to CNBr-activated Sepharose) a dissociation constant of 1.8 μ M for ATP⁴⁻ was found. Data were also obtained under conditions that closely approximate to those found *in vivo*, i.e. on columns packed with a slurry of Sephadex G-50 and precipitated myosin filaments formed at low ionic strength. The binding of MgATP²⁻, MgADP⁻, ATP⁴⁻ and MgPP_i²⁻ to 'filamentous' myosin in both two- (myosin and nucleotide) and three- (myosin, nucleotide and PP_i) component systems at different temperatures was studied and the dissociation constants obtained agreed well with previously published values. Except for the binding of ATP⁴⁻ to filamentous myosin at 4°C when 85% of the protein was interacting with the nucleotide, much lower values for the number of available sites occupied by the nucleotides were as a routine found in this system. Although this apparent discrepancy is difficult to explain, it is not an anomaly of the theoretical approach and may reflect the present state of understanding of the myosin system.

During the past 5 years the applications of affinity chromatography in biochemical research have expanded and diversified. These applications have included use of the technique in quantitative investigations of protein-protein and protein-ligand interactions. Andrews *et al.* (1973) provided the first example of such a quantitative approach, using immobilized α -lactalbumin to determine the equilibrium constant between the components of lactose synthetase and glucose. Their simplified approach was only applicable to this ligand-retarded elution system. However, Nichol *et al.* (1974) greatly extended the theory of this quantitative approach to encompass many of the possible equilibria and situations. They also considered possible effects of gel/liquid phase partition and presented a series of general equations, which cover a wide range of chromatographic conditions. In applying these equations (Nichol *et al.*, 1974; Brinkworth *et al.*,

1975), they make the necessary volume measurements by partition experiments or by frontal chromatography. Similar, if less rigorous, theories were also used by Dunn & Chaiken (1974, 1975), Lowe *et al.* (1974) and Kasai & Ishii (1975). These methods also involved measurements of elution volumes.

The quantitative approach described here differs from the above methods in that it involves direct measurements of the amount of affinity-retarded material and does not require that measurement of elution volumes be made. This both simplifies and accelerates the rate of acquisition of useful data. The situation where the acceptor molecule is immobilized is used to demonstrate the validity of the theoretical approach, although the method is applicable to any interacting system where one of the components is insoluble. The particular system used is a 'natural' form of the muscle protein myosin. This 'filamentous' myosin (myosin precipitated at low ionic strength) much more closely resembles myosin as it is in the muscle than do the proteolytic fragments of the protein, heavy meromyosin and subfragment-1, from which many of the published binding and kinetic data have been obtained (e.g. Bagshaw *et al.*, 1974).

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Theory

All the following derivations are based on the nomenclature below and apply to the situation where the immobilized component is packed into a column and the soluble components applied to this column.

- A Immobilized component.
- [A_T] Total concentration of A.
- B Soluble component 1.
- [B_f] Concentration of free or soluble B, i.e. the concentration of B not complexed with A. In the situation below, [B_f] = [B] or [B] + [BD].
- [B_b] Concentration of bound (column-retained) B. In both situations described below, [B_b] = [AB].
- C 'Eluting' component (usually high concentrations of B or D, denoted B_∞ or D_∞ respectively; see below).
- D Soluble component 2

The following equilibria could exist (assumed to be rapidly equilibrating):

and the following dissociation constants therefore apply:

$$K_{AB} = \frac{[A][B]}{[AB]} \quad (1a)$$

$$K_{AD} = \frac{[A][D]}{[AD]} \quad (1b)$$

$$K_{BD} = \frac{[B][D]}{[BD]} \quad (1c)$$

where the square brackets denote molar equilibrium concentrations. In the situations considered below, either B or D compete for the same site on A, or A and D compete for the same site on B, and hence the ternary complex ABD is not formed (or considered in the theory). The formation of ternary complexes can, however, be readily handled by the ensuing approaches.

Theory (i): evaluation of the situation where A is the immobilized ligand and B is the protein (acceptor molecule) in free solution

This might apply to subfragment-1 (B) applied to a column of Sepharose-6-aminohexan-1-ol pyrophosphate (A).

A solution of B of known concentration, in the above example subfragment-1, containing a known low concentration of D ('competing' ligand, in this example PP_i or ADP) is applied to the column

of A (Sepharose-6-aminohexan-1-ol pyrophosphate) until equilibrium is reached. Component C (e.g. D_∞) is now added to the running solution, resulting in the elution profile of the type shown in Fig. 1(a). The area under the peak corresponds to B_b at this concentration of D and can be experimentally determined by measurement of protein or enzyme activity. [A similar approach was introduced into gel-filtration studies by Hummel & Dreyer (1962) and used by Fairclough & Fruton (1966) to determine binding constants for the interaction of serum albumins with tryptophan derivatives.] It is necessary to introduce component D into consideration here, since, although it is possible to perform these experiments with components A and B alone (see below), it is difficult to assess the effects of immobilization of the ligand A on K_{AB}. On the other hand determination of K_{BD}, the dissociation constant of subfragment-1 and soluble PP_i (or soluble ADP etc.) in the above experiment, is not open to this criticism.

The relevant equilibria in this situation are those to which dissociation constants K_{AB} and K_{BD} apply, i.e. eqns. (1a) and (1c). Now, [B_f] = [B] + [BD], and substitution for [BD] from eqn. (1c) gives:

$$[B_f] = [B] \left(1 + \frac{[D]}{K_{BD}} \right) \quad (2)$$

In addition, [B_b] = [AB] by definition, and substitution for [AB] from eqn. (1a) gives:

$$[B_b] = \frac{[B][A]}{K_{AB}} \quad (3)$$

Combining eqns. (2) and (3) we have:

$$\frac{[B_f]}{[B_b]} = \frac{\left(1 + \frac{[D]}{K_{BD}} \right) K_{AB}}{[A]} \quad (4)$$

As:

$$[A] = [A_T] - [AB]$$

or

$$[A] = A_T - [B_b] \quad (5)$$

this can be substituted in eqn. (4) leading to the expression:

$$\frac{[B_f]}{[B_b]} = \frac{\left(1 + \frac{[D]}{K_{BD}} \right) K_{AB}}{[A_T] - [B_b]} \quad (6)$$

which rearranges to give:

$$\frac{[B_f]}{[B_b]} = \frac{K_{AB}}{[A_T]} \left(1 + \frac{[D]}{K_{BD}} \right) + \frac{[B_f]}{[A_T]} \quad (7)$$

If [BD] ≪ [D]_{total}, i.e. K_{BD} ≫ [B], then [D] = [D]_{total} and is therefore known. B_f and B_b are both obtained from the elution profile (Fig. 1a), and plotting [B_f]/[B_b] against [B_f] for various concentrations of B

Fig. 1. Experimental design and theoretical data plots used in this study

(a) Elution profile for B applied to columns of immobilized A. This hatched area represents the amount of B bound to A (i.e. B_b) under the conditions used. This is displaced from the column by some competing ligand C, which is usually a high concentration of either D (usually in the three-component system involving A, B and D, and when D does not interfere with the measurement of B) or even B (usually in the two-component system when radioisotopes can be used; see Fig. 2). In the three-component system this run is repeated at several concentrations of B at a fixed concentration of D ($[D] = D_1$) and then repeated at another concentration of D ($[D] = D_2$) etc. In the two-component system, only B is varied, since D is absent. (b) Primary plot for the three-component system. Values of B_f and B_b are obtained from the chromatography experiments in (a) at different concentrations of D (D_1, D_2 etc.). The slopes of all the lines should be the same and equal to $1/A_T$.

at a fixed value of $[D]$ results in a straight line (Fig. 1b). Taking the abscissa intercept for each of a series of such lines (obtained by using different values for $[D]$) and plotting the negative of this against the relevant value of $[D]_{\text{total}}$ results in a straight line (Fig. 1c) with ordinate intercept K_{AB} and abscissa intercept K_{BD} .

The above evaluation does not take into consideration any partitioning of either B or D. However, since B in this situation will invariably be a large (protein or acceptor) molecule, there will always be some partitioning of B on the columns. It is necessary, therefore, to define a partition coefficient, α_B , where:

$$\alpha_B = \frac{\text{Volume of column accessible to molecule of B}}{\text{Volume of column accessible to solvent molecules}} \quad (8)$$

and the true values of $[B_f]$ and $[B_b]$ ($[B_f]_{\text{true}}$ and $[B_b]_{\text{true}}$) are:

$$[B_f]_{\text{true}} = \frac{[B_f]_{\text{measured}}}{\alpha_B} \quad (9)$$

and

$$[B_b]_{\text{true}} = \frac{\text{Total amount of B eluted}}{\text{Volume of column accessible to molecule of B } (V_B)} \quad (10)$$

Since V_B is constant for a given column, it can be eliminated from the calculations (assuming that each experimental series is carried out on the same column), leaving the determined values of B_b (and the derived value of A_T) as amounts, not concentrations. α_B may be determined by adding to a dry sample of the column-support material a solution of B of known concentration and measuring the new concentration of B in the supernatant. Full experimental details of the method for obtaining the partition coefficient are given by Nichol *et al.* (1974).

If D is a small ligand (e.g. ATP), partitioning effects can be ignored, i.e. D molecules will have the same potential access as solvent molecules. However, if D is a large molecule (e.g. a protein) it is necessary to introduce a second partition coefficient, α_D , where:

$$\alpha_D = \frac{[D]_{\text{measured}}}{[D]_{\text{true}}} \quad (11)$$

and this can be determined as described above for α_B .

(c) Secondary plot for the three-component system. The abscissa intercepts from (b) (I_1, I_2 etc.) are plotted against the appropriate $[D]$. (d) Plot for the two-component system. D is absent and thus only K_{AB} (abscissa intercept) and A_T (1/slope) are determined.

Partition effects lead to the following modification of eqn. (2):

$$[B_f] = [B] + \frac{[B][D]}{rK_{BD}} \quad (12)$$

where r is a partition parameter, and $r = 1$ when $\alpha_B \leq \alpha_D$. If $\alpha_D < \alpha_B$, then:

$$r = \frac{\alpha_B}{\alpha_D}$$

Under most conditions (i.e. those in which B is a protein and D a competing ligand) therefore r will be equal to 1. Substitution of eqn. (12) for eqn. (2) in the derivation of eqn. (7) leads to the modified expression:

$$\frac{[B_f]}{[B_b]} = \frac{K_{AB}}{[A_T]} \left(1 + \frac{[D]}{rK_{BD}} \right) + \frac{[B_f]}{[A_T]} \quad (13)$$

For this (and eqn. 7) to be useful, it is necessary, as mentioned above, that $[B] \ll K_{BD}$, i.e. that $K_{BD} \gg 100[B]$. Hence for the concentration of [B] to be manageable, K_{BD} should preferably be $\geq 10 \mu M$.

Such a restriction does not apply to the situation where the acceptor molecule is immobilized.

Theory (ii): evaluation of the situation where A is the immobilized protein (or other acceptor molecule) and B and D are both soluble ligands which bind to the same site on A

This might apply to a column of Sepharose-myosin to which is applied an ATP/ADP mixture.

The practical methodology in this situation is identical with that described in theory (i) above. In this case the dissociation constants of the complexes formed in the relevant equilibria are K_{AB} and K_{AD} , i.e. eqns. (1a) and (1b), and $[B_f] = [B]$. Substituting for [B] from eqn. (1a) we have:

$$[B_f] = \frac{K_{AB}[AB]}{[A]} \quad (14)$$

Again $[B_b] = [AB]$, and in this case can be expressed as:

$$[B_b] = [A_T] - ([A] + [AD]) \quad (15)$$

Substitution in eqn. (15) for [AD] from eqn. (1b) results in:

$$[B_b] = [A_T] - [A] \left(1 + \frac{[D]}{K_{AD}} \right) \quad (16)$$

Rearrangement of eqn. (14) gives:

$$[A] = \frac{K_{AB}[AB]}{[B_f]} = \frac{K_{AB}[B_b]}{[B_f]} \quad (17)$$

and substitution of this for [A] in eqn. (16) leads to:

$$[B_b] = [A_T] - \frac{K_{AB}[B_b]}{[B_f]} \left(1 + \frac{[D]}{K_{AD}} \right) \quad (18)$$

Rearrangement of eqn. (18) gives the general expression:

$$\frac{[B_f]}{[B_b]} = \frac{K_{AB}}{[A_T]} \left(1 + \frac{[D]}{K_{AD}} \right) + \frac{[B_f]}{[A_T]} \quad (19)$$

which is of the same type as eqn. (7) and can be used in the same way (Figs. 1b and 1c). Since the solution of B and D is applied until the column is saturated, i.e. $[B_f] = [B]_{\text{applied}}$ and $[D] = [D]_{\text{applied}}$, both $[B_f]$ and $[D]$ are known and do not have to be measured. However, measurement of $[B_f]$ is useful, as it gives an indication of whether the equilibrium situation has in fact been attained. Eqn. (19) may also be subject to partition effects, as described above for eqn. (7).

However, in most situations (including those presented below) both B and D will be small molecules (e.g. ATP, PP_i) and these partition effects can be ignored. If either B or D should be a large (protein) molecule, a partition coefficient (α_B or α_D) may be introduced and determined as previously described.

Both eqns. (7) and (19) describe three-component systems, which, although providing measurements of three parameters (K_{AB} , A_T and K_{AD} or K_{BD}), also involve the determination of a large number of values of B_b . These latter can be decreased if component D is eliminated. In this case both eqns. (7) and (19) both simplify to:

$$\frac{[B_f]}{[B_b]} = \frac{K_{AB}}{[A_T]} + \frac{[B_f]}{[A_T]} \quad (20)$$

and the simple plot of $[B_f]/[B_b]$ against $[B_f]$ (Fig. 1d) provides values for K_{AB} and A_T .

It should be re-emphasized that, for the situation described by theory (i), K_{AB} , the dissociation constant of the complex formed between the immobilized ligand, A, and the protein (acceptor molecule), B, may be of only limited interest except in a completely academic sense, since the effects of immobilizing ligand A can never be fully assessed (Lowe *et al.*, 1974; O'Carra *et al.*, 1973). Similarly, although it is interesting to know A_T in this situation (indeed this is a very good method of determining the 'capacity' of an affinity column), A_T does not have much relevance to the protein under investigation. Hence eqn. (20) is more useful in the situation described by theory (ii), especially in the later examples given below.

It is also important to note that, although eqns. (7), (19) and (20) derived above contain terms in $[B_b]$ and $[A_T]$, these become amounts provided that each complete experiment is performed on the same column. The area under the eluted peak (hatched area

in Fig. 1*a*) is thus the amount of B bound to A, i.e. B_b not $[B_b]$ ($[B_b] = B_b/V_B$), and will be plotted as such in the Results and Discussion section. The values of A_T derived from these graphs will therefore also be in amounts. This value of A_T will be a measure of the available sites (which bind B) and not necessarily of the total amount of enzyme-acceptor molecule present.

Materials and Methods

Materials

[8-³H]ATP and [8-³H]ADP were from The Radiochemical Centre, Amersham, Bucks., U.K. ATP was from Kyowa Hakko Kogyo Co., Tokyo, Japan, and ADP was from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Sepharose 4B and Sephadex G-50 were from Pharmacia (G.B.), London W.5, U.K. All other chemicals were AnalaR grade and used as supplied.

Methods

Myosin was prepared from the back and leg muscles of New Zealand White rabbits by the method of Trayer & Perry (1966). Subfragment-1 was prepared from this by digestion with Sepharose-papain and the active fraction of this digest was isolated by affinity chromatography on Sepharose-6-amino-hexan-1-ol pyrophosphate (Trayer *et al.*, 1974*a,b*; Bottomley & Trayer, 1975). Protein was determined by the microtannin turbidimetric method of Mejbaum-Katzenellenbogen & Dobryszczyka (1959) as described by Bottomley & Trayer (1975). Ca^{2+} -stimulated ATPase (adenosine triphosphatase) activities were determined by the direct measurement of P_i (Fiske & SubbaRow, 1925). Mg^{2+} -stimulated ATPase activities were similarly determined, but required a 30 min incubation time.

Sepharose-myosin was prepared and characterized, and radioactivity measured as described by Bottomley & Trayer (1975).

All graphs plotted in the Results and Discussion section were subjected to a linear-regression analysis where appropriate.

Results and Discussion

To illustrate the potential and also the limitations of affinity chromatography for the quantitative study of molecular interactions, we have chosen examples from our studies on a myosin-adenosine nucleotide- PP_i system. The permutations possible in this system fully cover the different situations analysed above.

Sepharose-(6-amino-hexan-1-ol pyrophosphate)-subfragment-1- PP_i (theory i)

Early experiments on the practical application of this approach were conducted on the system evaluated in theory (i), i.e. that represented by eqn. (13). In these experiments, subfragment-1 (component B) was passed over columns of Sepharose-6-amino-hexan-1-ol pyrophosphate (component A) in the presence of various concentrations of PP_i (component D). The amount of subfragment-1 bound to the affinity column was found by including 20 mM- PP_i (D_∞) in the developing buffer (which included the fixed applied concentration of subfragment-1). Although these experiments were encouraging and qualitatively successful, the results are not given in detail here since problems were encountered in obtaining accurate values for B_b . The subfragment-1 emerging from the column after including D_∞ in the developing buffer was spread over a large volume, making an accurate determination of B_b very difficult. This is almost certainly due to the fact that the dissociation constant (K_{BD} in our nomenclature) for subfragment-1 and PP_i is about $5 \times 10^{-7} M$ (Nauss *et al.*, 1969), making it very difficult to satisfy the requirement $[B] \ll K_{BD}$ (theory i). This problem was similar to that encountered by Nichol *et al.* (1974) when studying their Sepharose-lectin-galactose system.

Sepharose-myosin-ATP (theory ii)

The restriction encountered above does not apply where the acceptor molecule (in this case myosin) is immobilized (theory ii) and the practical applicability of the general theory can be readily demonstrated. Experiments were conducted by using columns of Sepharose-myosin (myosin covalently coupled to CNBr-activated Sepharose 4B), particularly as the properties of this immobilized myosin had been previously characterized (Bottomley & Trayer, 1975). In order to simplify data collection, a two-component system was used (eqn. 20).

In the experiment whose results are given in Fig. 2, various fixed concentrations (0.5–5.0 μM) of [8-³H]-ATP⁴⁻ were used as component B and passed over a column of Sepharose-myosin (component A). The experiment was conducted as described in Fig. 1*a*), and the results were plotted as shown in Fig. 1*d*). The values of K_{AB} and A_T were obtained from a linear-regression analysis of the data and were 1.8 μM and 19.3 nmol respectively. The value of K_{AB} found agrees reasonably well with published values for the dissociation constant of myosin and ATP⁴⁻ (Schlisfeld & Barany, 1968) and very well for the value found for filamentous myosin (Table 1), thus illustrating the validity of the theory. The relatively low value of A_T (approx. 1 mol of ATP⁴⁻

Fig. 2. Quantitative affinity chromatography on Sepharose-myosin conjugates

The column (6cm×0.4cm) of Sepharose-myosin, containing 16nmol of immobilized myosin, was equilibrated in 75mm-triethanolamine, adjusted to pH 7.8 with acetic acid, containing 1mM-EDTA at room temperature (20–22°C). Appropriate concentrations of [8-³H]ATP (0.5–5.0 μM) were run through until a plateau had been attained, when 0.5mM-[8-³H]ATP was applied. All ATP solutions contained the same amount of radioactivity (5000 d.p.m./ml). Fractions (1ml) were collected directly into scintillation vials and counted for radioactivity (see the Materials and Methods section). Details of obtaining B_f and B₀ values are given in the legend to Fig. 3.

bound/mol of myosin) may well in this case be a reflection of the immobilization process; this is discussed more fully below.

Studies with filamentous myosin

Since Sepharose-myosin does not show all the properties, particularly with respect to its steady-state enzymic activities (Bottomley & Trayer, 1975), of native myosin, it is not an ideal system with which to work. The interpretation of any results obtained, however good, will always be open to doubt when applied to the native molecule. Such an argument could be similarly made for any protein that has been covalently modified in some way in order to cause its immobilization. Many proteins exist only in the soluble form, and must therefore be covalently modified in this manner before this treatment. Myosin, however, exists *in vivo* as an insoluble filament, and can easily be induced to form insoluble filamentous aggregates by simply lowering the ionic strength. We therefore decided to exploit this behaviour to demonstrate further the practical application of eqns. (19) and (20). This filamentous or precipitated myosin is physically similar to myosin in its natural state, and results obtained with it should therefore be applicable to the situation existing in the intact muscle.

To prepare the myosin for column-chromatography experiments, the following procedure was undertaken. A solution of myosin was precipitated by addition

to a large volume of water, the precipitate collected by centrifugation and mixed with a suspension of Sephadex G-50 in 75mm-triethanolamine adjusted to pH 7.8 with acetic acid. The protein concentration of a sample of this mixture was measured by dissolving the myosin in 0.5M-KCl, removing the Sephadex G-50 by centrifugation and subjecting the supernatant to the micro tannin turbidimetric assay (see the Materials and Methods section). An appropriate volume of the mixture was then packed into a small column, between layers of Sephadex G-50, as illustrated in Fig. 3(a). During equilibration of the column (usually with 75mm-triethanolamine/acetic acid, pH 7.8, containing 0.5mM-2-mercaptoethanol and 2mM-magnesium acetate) a careful check was made on the effluent, but there was never any indication of the presence of protein being leached from the column. The filamentous myosin thus represents component A.

In all the experiments described below the same technique as described in the legend to Fig. 2 was used to elute and measure the amount of the myosin-bound nucleotides. The same basic amount of radioactivity was maintained throughout a single experiment, i.e. the specific radioactivity of the applied ATP/ADP solution was varied. Thus a solution of 0.5–7.5 μM-[8-³H]ATP (or ADP) containing 5000 d.p.m./ml (component B) was applied to the column until equilibrium was attained. Elution of the myosin-bound nucleotide was performed by raising the appropriate nucleotide concentration to 0.5 or 0.75mM (B_∞; i.e. this was in effect C in Fig. 1a), but maintaining the amount of radioactivity at 5000 d.p.m./ml (the higher concentration of C was used when [B] was ≥ 7.5 μM). This resulted in a peak of higher radioactivity, representing the amount of nucleotide bound by the myosin, being eluted from the column. Fractions were collected directly into scintillation vials, mixed with scintillation fluid and counted for radioactivity.

At the end of a particular set or series of experiments, normally completed within 5 days, the column used was carefully unpacked and the contents were thoroughly extracted with 0.5M-KCl as described above and subjected to protein and ATPase-activity determinations. In all cases these were approximately the same after each experiment as before. Hence there was no loss in either the amount or activity of the filamentous myosin during the course of these experiments. A fresh column was used for each experiment.

Fig. 3(b) and 3(c) illustrated some typical data from experiments with these Sephadex G-50-filamentous myosin columns. In Fig. 3(b), the amount of radioactivity reached after elution gives a precise measure of the volume of each fraction (when compared with the radioactivity of a 1 ml sample of the appropriate solution) and thus serves as an internal check. The pre-elution value

Fig. 3. Quantitative affinity chromatography with filamentous myosin and ATP (two-component system)

(a) A typical column. All columns were Pasteur-pipette size (0.4cm×6cm), with a volume of approx. 0.6ml. (b) Two typical elution profiles with (i) 2.5 and (ii) 1.25 μM -ATP. The column was packed as shown in (a) and operated in 75 mM-triethanolamine, adjusted to pH 7.8 with acetic acid, containing 2 mM-magnesium acetate and 0.5 mM-2-mercaptoethanol, at room temperature (approx. 22°C) with a flow rate of 20 ml/h. Fractions (1 ml) were collected directly into scintillation vials after 25 ml of the appropriate concentration of [8- ^3H]ATP solution (containing 5000 d.p.m./ml) had been passed over the column. Previous experimentation had showed that, after this amount of ATP, the eluting concentration of ATP (B_r) was equal to that applied. At the time shown by the arrow the concentration of ATP in the eluting buffer was raised to 0.5 mM, but the radioactivity was maintained at 5000 d.p.m./ml. (c) From the series of elution profiles from which those illustrated in (b) were taken, values of B_r and B_b were obtained and were plotted as illustrated. The line was drawn after a linear regression analysis of the data.

Table 1. Quantitative affinity chromatography with filamentous myosin and ATP or ADP (two-component system)

The data were obtained from filamentous myosin columns (component A) packed and operated as described in the legend to Fig. 3. Four or five points (in the range $[B] = 0.5\text{--}5.0 \mu\text{M}$) were obtained as illustrated in Fig. 3(b) and plotted as shown in Fig. 3(c). All chromatographic columns (except those marked *) were analysed after use for protein content and ATPase activity (see the text). Results marked with † refer to two different myosin preparations.

Component B	Temperature (°C)	K_{AB} (μM)	A_T (nmol)	Total myosin (nmol)	A_T (as % of sites available)
MgATP $^{2-}$ †	20–22	2.4	5.85	8*	
MgATP $^{2-}$ †	20–22	2.1	5.41	9.4	29
MgATP $^{2-}$	4–6	1.1	5.6	16.2	17.3
MgADP $^-$	4–6	1.1	5.6	16	17.5
ATP $^{4-}$	20–22	4.3	10	8*	
(+EDTA)					
ATP $^{4-}$	4–6	3.0	46	27	85
(+EDTA)					

indicates the exact concentration of, in this experiment, MgATP $^{2-}$ (B_r), reached [usually it was necessary to apply about 25 ml of solution before switching to C and eluting the bound nucleotide (B_b)]. From these two radioactivity values an average background can be obtained, and this is subtracted from the points on the eluted peak, whose sum then becomes an accurate value of B_b . The results of this experiment, using MgATP $^{2-}$, are plotted in Fig. 3(c) and the values obtained for K_{AB} and A_T are given in the first line of Table 1. The linearity of the plot obtained in Fig. 3(c) and the value of K_{AB} found (Table 1) readily demonstrated the practicality and validity of the theory. Control experiments performed with columns of either Sephadex G-50 alone or Sephadex G-50 and precipitated myosin first denatured by dissolving in 10 M-urea revealed that there was no significant interaction between MgATP $^{2-}$ (0.5–10 μM) and these matrices.

Table 1 summarizes the data obtained from a series of experiments done to investigate the binding of ADP and ATP, under a variety of conditions, to filamentous myosin. All these experiments were performed as described in the legend to Fig. 3 by using the two-component system and analysing the data according to eqn. (20). The binding of ATP $^{4-}$ was performed in the buffer indicated in the legend to Fig. 3(b), except that the magnesium acetate was replaced by 1 mM-EDTA. For comparison, a selection of published values for myosin–ligand dissociation constants etc. obtained by different methods is shown in Table 2.

Table 2. *Published values for myosin-ligand dissociation constants etc.*

All values were obtained at room temperature (20–22°C) unless otherwise indicated. References: ^a, Schlisfeld & Barany (1968); ^b, Lowey & Luck (1969); ^c, Nauss *et al.* (1969); ^d, Malik & Martonosi (1972).

Myosin		Heavy meromyosin	
K_m (MgATP ²⁻)	$4.1 \times 10^{-7} \text{ M}^a$	K_m (MgATP ²⁻)	$6.5 \times 10^{-7} \text{ M}^a$
K_D (MgATP ²⁻)	$8.4 \times 10^{-7} \text{ M}^a$	K_D (MgADP ⁻)	$1.4 \times 10^{-5} \text{ M}^d$
K_D (MgADP ⁻)	$7 \times 10^{-6} \text{ M}^b$	K_D (MgADP ⁻)	$1.4 \times 10^{-6} \text{ M} (6^\circ\text{C})^d$
K_D (MgPP _i ²⁻)	$5 \times 10^{-7} \text{ M} (3^\circ\text{C})^c$	K_D (MgPP _i ²⁻)	$5 \times 10^{-7} \text{ M} (3^\circ\text{C})^c$

For MgADP⁻ and ATP⁴⁻ (which is not readily hydrolysed by myosin when the triethanolammonium ion is the only cation present), the value of K_{AB} is a direct measurement of the dissociation constant of myosin–MgADP or myosin–ATP interaction. For MgATP²⁻, the issue is not as straightforward. If one assumes that the rate of hydrolysis of MgATP by the ‘filamentous’ myosin is roughly the same as that in solution (i.e. at least 10 nmol of ATP hydrolysed/min per mg), then, with a flow rate of 20 ml/h, the myosin on the column is capable of fully hydrolysing more than 100 nmol of ATP per column volume, i.e. a solution of up to 0.15 mM–MgATP²⁻. Thus the main component of the developing column buffer will be MgADP⁻, possibly with some MgATP²⁻ and P_i. The measured value of K_{AB} will therefore be a complex combination of the equilibrium constants of the complexes formed between myosin and MgADP⁻, MgATP²⁻ and of the major hydrolytic intermediate [M**ADPP_i; Bagshaw *et al.* (1974)]. The above should apply to columns operated at both room temperature and 4°C, since Malik & Martonosi (1972) found that the turnover number of the MgATPase activity of heavy meromyosin at 23°C was only 2.4 times greater than that at 6°C.

The reproducibility of the method is demonstrated by the first two lines of Table 1, where essentially the same value for the apparent dissociation constant of myosin for MgATP²⁻ was found with two different myosin preparations.

It was noteworthy that the K_{AB} values of filamentous myosin for MgATP²⁻ and MgADP⁻ were identical at 4°C, underlining the argument that K_{AB} for MgATP²⁻ is effectively the dissociation constant for myosin–MgADP, at least at 4°C. It should be possible with this method to obtain binding data for the initial event of MgATP²⁻ hydrolysis by using ATP analogues that are either not hydrolysed, such as 5'-adenylyl imidodiphosphate, or only hydrolysed very slowly, e.g. adenosine 5'-(3-thiotriphosphate).

There is thus reasonable agreement between the values of K_{AB} for filamentous myosin and ATP/ADP under various conditions when they are compared with published values shown in Table 2. Any discrepancies found between the two Tables could easily be caused by the different conditions used in

the different studies. For example, all the studies on myosin–ligand interactions in Table 2 were performed on myosin solubilized at high ionic strength.

The most remarkable and puzzling feature of the results given in Table 1 is the value of A_T obtained with MgATP²⁻ and MgADP⁻. Under no conditions does this value reach 50% of the available sites, i.e. 1 mol of bound nucleotide/mol of myosin, and at 4°C it falls below 25% of the sites. It is tempting to suggest that these results with filamentous myosin may represent the situation *in vivo*, since under one set of conditions it was possible to occupy virtually all of the sites on the myosin molecule (the binding of ATP⁴⁻ at 4°C; Table 1). The actual cause of the low values for A_T must, however, await further investigation. It has been pointed out (Huxley, 1963) that the fine structure of the filaments formed after precipitation of myosin by lowering the ionic strength is dependent on the actual conditions used to attain this. Myosin was always precipitated in an identical manner throughout this study, but this could still have caused the myosin to be precipitated in such a way as to hinder sterically nucleotide binding. Nevertheless, any modifications to the properties of soluble myosin that result from packing it into the A filament have not been fully assessed. It may be that this causes some of the molecules to be ‘turned-off’ and/or results in a negative co-operativity being expressed between the two heads of the myosin molecule with respect to nucleotide binding. Both could result in low A_T values in the above experiments. It should be possible to study this further by using A filaments isolated whole from muscle (Morimoto & Harrington, 1973) and operating the columns under a wider range of nucleotide concentrations, perhaps in the presence of controlled concentrations of Ca²⁺.

An additional development of this system was to show that it could be used in the three-component system described by eqn. (19). Thus a series of chromatography experiments was done, under the same basic conditions as previously described in Fig. 3, with MgADP as component B and PP_i as component D. Since a preliminary application of eqn. (19) to the Sepharose–myosin system (not shown

Fig. 4. Quantitative affinity chromatography with filamentous myosin, ADP and PP_i (three-component system)

(a) The experiment was conducted as described previously in the legend to Fig. 3, except that the column was operated at 4°C , and each of the series of MgADP concentrations (0.4 , 1.25 , 2.0 and $2.5 \mu\text{M}$) was used at a different fixed concentration of MgPP_i . The data were analysed as described in Fig. 1. $\square$, $0.5 \mu\text{M}$ - PP_i ; $\blacksquare$, $1.0 \mu\text{M}$ - PP_i ; $\circ$, $2.5 \mu\text{M}$ - PP_i ; $\bullet$, $5.0 \mu\text{M}$ - PP_i . (b) The value for each abscissa intercept ($-I$ in Fig. 1b) was then plotted against the PP_i concentration (component D, Fig. 1c). The values of K_{AB} , K_{AD} and A_T obtained are given in the text.

here) had indicated that binding constants for both ADP and PP_i were quite low, the concentrations were varied between 0.4 and $2.5 \mu\text{M}$ (ADP) and between 0.5 and $5.0 \mu\text{M}$ (PP_i). The results obtained (at 4°C) are expressed graphically in Fig. 4. Values for K_{AB} (dissociation constant of myosin and MgADP) and K_{AD} (dissociation constant of myosin and MgPP_i^{2-}) were 0.94 and $4.9 \mu\text{M}$ respectively. This value for K_{AB} from the three-component system is in excellent agreement both with that found in the two-component system operated under similar conditions ($1.1 \mu\text{M}$; Table 1) and with published values for heavy meromyosin (Table 2), again establishing the validity of this method. The value of K_{AD} is rather higher than other published values for the dissociation constant of MgPP_i^{2-} (Table 2). In this system, a low value for A_T was again found: 6nmol , 18% of available sites (average of the four slopes in Fig. 4a), although this was virtually identical with that found with the two-component system at 4°C (Table 1).

This method is the first by which a determination of the dissociation constants and stoichiometry of binding sites for whole myosin have been obtained

under conditions closely resembling those found *in vivo*. Although the apparent discrepancies in the values of A_T are difficult to explain, this is not an anomaly of the theoretical approach and may well reflect the present state of our understanding of the myosin system.

General discussion

Previous investigations (see the introduction) using the potential of affinity chromatography for obtaining equilibrium constants in interacting systems have all depended for their success on the measurement of volumes. In situations, such as those described here, where the interacting components have a very low dissociation constant, the measurement of these volumes by either frontal affinity chromatography (Nichol *et al.*, 1974), or particularly by zonal affinity chromatography (Dunn & Chaiken, 1974, 1975), can be difficult in practice and may be prone to errors. The method described here allows the direct measurement of the affinity-bound material over a wide range of concentrations of component B. A particularly desirable aspect of this approach is that it allows, by using specific elutant ligands, the measurement of only material bound specifically to the column. Volume measurements do not discriminate in this way and would assess both specific and non-specific interactions of the soluble component(s) with the column matrix. Such non-specific interactions in affinity-chromatographic systems are well-documented (e.g. O'Carra *et al.*, 1973; Holroyde *et al.*, 1976). This aspect is greatly extended when radioactive material can be used as in the myosin/adenosine nucleotide system described in Fig. 3.

It must be emphasized that most of the work presented has been performed with precipitated myosin of Sepharose-myosin and the data analysed by eqns. (19) or (20) of theory (ii). It was not possible to validate the equations derived by theory (i) with the system used, i.e. subfragment-1 binding to an immobilized pyrophosphate derivative, since the dissociation constant in this system was too low to satisfy the requirement $[B] \ll K_{BD}$. The two theories have very similar derivations, however, and the proof of theory (ii) by the results presented implies that it should be possible to apply theory (i) to situations where the interactions are weaker than those between subfragment-1 and PP_i . The method is, in fact, well-suited to the study of weak interactions where methods such as equilibrium dialysis have severe limitations.

Finally, this theoretical treatment has been applied to insoluble myosin (as it exists *in vivo*) as well as to myosin covalently immobilized to CNBr-activated Sepharose (a 'classical' affinity-chromatography system). Since the theory is applicable to any biological system comprising two interactions among

three reactants, one of which is insoluble, it is expected that it should find application in a study of components immobilized on membranes.

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