Sensitivity and other Factors Affecting Biospecific Desorption in Chromatography of Proteins

A STUDY BY COMPUTER SIMULATION

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Some theoretical aspects of the desorption of a column-bound protein by elution with its biospecific ligand are considered in cases where, in comparison with the unliganded protein, the protein–ligand complex has a diminished but finite affinity for the adsorbent. A quantity termed the biospecific sensitivity, B , is introduced to facilitate comparison between different systems. Biospecific sensitivity may be defined as the fractional change in standard free energy of adsorption on formation of the protein–ligand complex. The effects of a moderate-to-low biospecific sensitivity on theoretical desorption profiles have been examined by using a computer simulation of the classical multiple-plate column model. Desorption was simulated under various boundary conditions involving protein–adsorbent and protein–ligand affinities and the initial concentrations of adsorption sites, protein and ligand. These simulations suggest that, when the biospecific sensitivity is low, desorption is optimized if (a) the unliganded protein is adsorbed as weakly as possible, (b) the column is loaded to near-saturation with the required protein, (c) the free ligand concentration is many times greater than that giving near-saturation of the protein in free solution, and (d) protein contaminants with high affinity for the adsorbent, and present in large amount, are removed in preliminary purification steps.

The desorption of column-bound proteins by elution with their biospecific ligands is a powerfully selective method of protein purification. It is used in affinity chromatography, where it is usually viewed as a competition between immobilized and free ligands (O'Carra, 1978; Mosbach, 1978), and in biospecific-elution (affinity-elution) chromatography, where it is viewed as the perturbation of a largely non-specific adsorption, i.e. not involving predominantly or exclusively the ligand-binding site on the protein (von der Haar, 1974; Scopes, 1977a,b; Yon, 1977). In both cases desorption is due to the formation of a specific protein–ligand complex whose affinity for the adsorbent is lower than that of the free protein.

In general only some of the weak non-covalent bonds involved in the adsorption of the protein are perturbed by introducing the ligand whereas others are not. This is certainly the case in those instances of affinity chromatography in which non-specific bonds are known to be involved. In an attempt to incorporate this distinction into theoretical discussions of biospecific desorption, and to find out how much non-

specific 'interference' can be tolerated, a parameter termed the biospecific sensitivity was introduced and its effects on computer-simulated desorption profiles were examined. These developments are described in the present paper.

Computer Simulation

The theoretical elution profiles shown in the present paper were produced by a computer program that modelled the column as the classical stack of plates. The progress of solutes down the column was treated as a succession of alternating advances and equilibrations (Martin & Synge, 1941; Morris & Morris, 1976), the relevant equilibria being as follows:

where P, L and A represent protein, ligand and adsorption site respectively, and the K values are

equilibrium association constants. The composition of each multiple-equilibrium mixture was calculated by the method of Storer & Cornish-Bowden (1976).

$$B = \frac{\text{No. of weak bonds cancelled by the ligand}}{\text{Total no. of weak bonds in adsorption of the free protein}} \quad (3)$$

The required elution profile is the output of P + PL from the last plate.

Theory and Results

Biospecific sensitivity

The difference in the chromatographic behaviour of a protein in the absence and presence of its biospecific ligand reflects the difference in the standard free energies of interaction of the adsorbent with the free protein (ΔG_{PA}^0) and with the protein–ligand complex (ΔG_{PLA}^0). It is assumed that the introduction of the ligand makes negligible changes in non-specific factors that are likely to affect the interaction such as pH, ionic strength or polarity. It is also assumed that the ligand itself is not adsorbed. Ligand-induced changes in chromatographic behaviour are then determined by ($\Delta G_{PA}^0 - \Delta G_{PLA}^0$). However, whether this difference is large enough to be experimentally discernible will depend on its magnitude relative to ΔG_{PA}^0 . That is, the sensitivity of the protein–adsorbent interaction to perturbation by the ligand is given by a quantity B defined as follows:

$$B = \frac{\Delta G_{PA}^0 - \Delta G_{PLA}^0}{\Delta G_{PA}^0} = 1 - \frac{\Delta G_{PLA}^0}{\Delta G_{PA}^0} \quad (1)$$

The quantity B is here termed the biospecific sensitivity of the protein–adsorbent interaction. From eqn. (1) it may be defined as the fractional change in the standard free energy of the interaction on formation of the protein–ligand complex. For the purpose of simulating column behaviour, equilibrium association constants rather than standard free energies are required. Assuming ideal conditions the two are related by $\Delta G^0 = RT \cdot \ln K$. Hence at constant temperature eqn. (1) may be written as:

$$B = 1 - \frac{\ln K_{PLA}}{\ln K_{PA}}$$

or

$$K_{PLA} = K_{PA}^{(1-B)} \quad (2)$$

Only two of the three quantities K_{PA} , K_{PLA} and B are independently variable by eqn. (2). In the remainder of the present paper I have chosen to discuss biospecific desorption in terms of K_{PA} and B . In practice, having decided on values for these independent variables, eqn. (2) was used to calculate K_{PLA} for use in the computer simulations described above.

If one assumes that the protein–adsorbent interaction is multivalent, that the individual weak bonds are equivalent (i.e. make equal contributions to the

free energy of the interaction) and that the effect of ligand-binding is to cancel out some of these weak bonds, then it follows from eqn. (1) that:

This simplified treatment will probably be strictly applicable to rather few cases; however, it will be a rough approximation in many more cases, since most actual weak bonds (hydrogen bonds, ionic bonds and hydrophobic ‘bonds’), though not equivalent, in fact vary by not much more than one order of magnitude (Barker, 1971). It is a simplification that permits a more mechanistic interpretation of B than does eqn. (1). Eqn. (3) limits B values to between 0 and 1, whereas eqn. (1) permits values greater than 1 if ΔG_{PLA} is positive, i.e. if the adsorbent repels the protein–ligand complex.

Factors affecting biospecific desorption

Purification by biospecific desorption usually involves adsorption of the unliganded protein, washing of the column to remove weakly adsorbed contaminants and application of a front of the ligand. In ideal cases the required protein is eluted in a sharp high-concentration peak and in 100% yield. By using the computer-simulation approach, a number of factors that affect the size, shape and yield of the desorbed protein peak were examined, with the emphasis on cases of moderate-to-low B values, which are thought to represent most actual cases. The model column (5 or 50 plates) is loaded with 1 column-volume of the sample protein, washed with 9 or more column-volumes of solvent, and the ligand front is then introduced. The column contains a single population of adsorption sites at a concentration of $50 \mu\text{M}$; however, the elution profiles will remain valid if all concentrations are multiplied by a factor x and all (bimolecular) association constants are multiplied by $1/x$.

(a) *Magnitude of K_{PA}* . The required protein must be adsorbed strongly enough to resist desorption by the prolonged wash necessary to remove unadsorbed or weakly adsorbed contaminants; this sets a minimum to the value of K_{PA} . At high values of K_{PA} the idealized desorption profile [as defined above; see also Fig. 2 and (b) below] will always be obtained when $B = 1$, since eqn. (2) then gives $K_{PLA} = 1$ whatever the value of K_{PA} . When B is considerably less than 1 the protein is desorbed poorly or negligibly at very high values of K_{PA} , and hence permissible values of K_{PA} are confined to a relatively narrow band. This is shown in Fig. 1, where (under the boundary conditions chosen) the minimum value of K_{PA} is about 10^5 M^{-1} . With B set at 0.5, an excellent desorption profile is obtained when $K_{PA} = 10^6 \text{ M}^{-1}$. At $K_{PA} = 10^7 \text{ M}^{-1}$ the desorbed peak

Fig. 1. Effect of K_{PA} on adsorption and subsequent biospecific desorption

Computer-simulated elution profiles are labelled with the appropriate value of K_{PA} in M^{-1} . Sample: 1 column-volume of $10\mu M$ -protein solution. Column parameters: plate number = 5; concentration of adsorption sites = $50\mu M$. Desorption conditions: $B = 0.5$; $K_{PL} = 10^4 M^{-1}$; ligand concentration = $10mM$. The arrow indicates the introduction of the ligand front.

is more extended and dilute, whereas at $K_{PL} = 10^8 M^{-1}$ the width and dilution are unacceptable. Thus for our model column K_{PA} is restricted to about two orders of magnitude, 10^6 – $10^7 M^{-1}$. This restricted range of permissible values of K_{PA} is perhaps the single most important consideration in choosing conditions for biospecific desorption.

(b) *Magnitude of B* . The effect of varying the biospecific sensitivity is shown in Fig. 2, for two values of K_{PA} and of sample concentration. The sample concentrations were such as to nearly saturate the column, as shown by the incipient leakage of protein. Desorption conditions were the same as in Fig. 1. As expected, when $K_{PA} = 10^6 M^{-1}$ and B approaches 1 the protein is desorbed in a high-concentration peak of less than 1 column-volume and in 100% yield. A considerable amount of non-specific adsorption can be tolerated without affecting this mode of desorption, as shown by the fact that the desorption profiles at $B = 0.6, 0.8$ and 1.0 are practically indistinguishable. As B is decreased below 0.5 the desorbed peak becomes increasingly diluted and extended; however, even when B is as low as 0.2 , about 90% of the desorbed peak is spread over only 2 column-volumes of the effluent, a result that would be acceptable by the standards of many present-day protein purifications (see Fig. 2a). In general, the same comments may be made when $K_{PA} = 10^7 M^{-1}$, except that the concentration of the desorbed peak is lower in every case and there is considerably more 'tailing'. Provided that K_{PA} is kept as low as possible, therefore, biospecific desorption will be a viable purification step even when as much as 80% of the

Fig. 2. Effect of B on biospecific desorption when (a) $K_{PA} = 10^6 M^{-1}$ and (b) $K_{PA} = 10^7 M^{-1}$

Computer-simulated elution profiles are labelled with the appropriate value of B . Sample concentration was $20\mu M$ in (a) and $50\mu M$ in (b). The column plate number was 50. Other details are as given in the legend to Fig. 1.

Fig. 3. Effect of loading on biospecific desorption

Computer-simulated elution profiles are labelled with the appropriate value of sample concentration (M); 1 column-volume of sample was loaded in each case. Note that the protein concentration (ordinate) values are expressed relative to the concentration of the sample. Column plate number = 50, $K_{PA} = 10^7 M^{-1}$ and $B = 0.34$. Other details are as given in the legend to Fig. 1.

adsorption free energy is unaffected by the presence of the ligand.

(c) *Sample loading*. The effect of varying the concentration of protein in the loaded sample (1 column-volume) is shown in Fig. 3, under relatively unfavourable adsorption conditions ($K_{PA} = 10^7 M^{-1}$

and $B = 0.34$). Desorption conditions are as for Figs. 1 and 2. At the sample concentration of $50\mu\text{M}$, when the loaded protein is stoichiometrically equivalent to the available adsorption sites, there is some leakage in the early wash fractions. However, most of the protein is eluted immediately after the ligand front in an initially sharp but highly asymmetrical peak. More than 80% of the adsorbed protein is released within 2 column-volumes of the ligand front. As the sample loading is decreased there is an increasing delay before the emergence of the main protein peak, whose asymmetry is concomitantly diminished. When the concentration is less than about $1\mu\text{M}$, the desorbed peak becomes symmetrical and its elution position is independent of further decrease in sample concentration. The column is then being operated over the linear part of the adsorption isotherm for the formation of the PLA complex, as in normal high-resolution chromatography. For preparative work, however, this represents a rather inefficient use of the column, since there are advantages in loading to near-saturation, both in the sharp immediate response to the ligand front and in more efficient use of the available adsorption sites. Moreover, the purification mechanism does not rely on separating sample constituents by continuous or gradient elution, which requires high-resolution conditions, namely a narrow starting zone, a high plate number and operation in the linear range of the adsorption isotherm.

(d) *Ligand concentration and the magnitude of K_{PL} .* Figs. 4(a) and 4(b) show the effect on the desorption profile of varying the concentration of the ligand front at two values of the protein-ligand affinity (K_{PL}). Relatively unfavourable conditions ($K_{PA} = 10^7\text{M}^{-1}$ and $B = 0.34$) were selected, and the sample concentration is $50\mu\text{M}$, i.e. stoichiometric with the available adsorption sites. Note that gel-filtration effects that might alter the shape of the ligand front are not considered here; in the absence of adsorption, protein and ligand are assumed to migrate at the same rate. In general, desorption is favoured by a high value of K_{PL} . For similar desorption profiles, the required ligand concentration is 10-fold greater when $K_{PL} = 10^4\text{M}^{-1}$ than when $K_{PL} = 10^5\text{M}^{-1}$. The results suggest that the desorption profile is determined approximately by the product $K_{PL} \times (\text{ligand concentration})$; for the conditions chosen, excellent desorption is obtained when this product is greater than 10^2 .

In free solution a ligand concentration of $10/K_{PL}$ (i.e. 10 times the half-saturation concentration $1/K_{PL}$) would convert 91% of the protein into the PL complex. In the presence of the adsorbent, however, this ligand concentration [1 mM in (a), 0.1 mM in (b)] is relatively ineffective in desorbing the protein, which is released in a broad diluted band. To obtain a reasonably narrow and concentrated peak a ligand

Fig. 4. Effect of ligand concentration on biospecific desorption when (a) $K_{PL} = 10^4\text{M}^{-1}$ and (b) $K_{PL} = 10^5\text{M}^{-1}$

Computer-simulated elution profiles are labelled with the appropriate concentration (M) of the ligand front. Column plate number = 50, $K_{PA} = 10^7\text{M}^{-1}$, $B = 0.34$ and sample concentration = $50\mu\text{M}$. Other details are as given in the legend to Fig. 1.

concentration approaching $100/K_{PL}$ is required. Thus ligand concentrations that would nearly saturate the protein in free solution may be inadequate for effective biospecific desorption. This problem is dependent on the biospecific sensitivity; the lower the value of B , the higher is the concentration of an effective ligand front. In practice the use of high ligand concentrations may pose problems, since the ligand may substantially change non-specific bulk properties of the irrigant (ionic strength or polarity, for example) that may desorb the protein together with impurities. This problem may be partly circumvented by including a 'dummy' compound, physicochemically similar to the ligand but having no biospecificity towards the required protein, in all samples and washes preceding the ligand front, and to replace this compound by the ligand when the front is applied. When this approach is used, the upper limit of the ligand concentration is determined by the extent to which the 'dummy' compound decreases K_{PA} , which may become too low for effective adsorption.

(e) *Impurities in the loaded sample.* In real purifications the sample will contain many contaminants in addition to the required protein. Since adsorption is essentially non-specific, these contaminants may be adsorbed but will not be desorbed by the ligand. Their only effect will therefore be to decrease the capacity of the column for the required protein. Fig.

Fig. 5. Effect of a single protein contaminant on bio-specific desorption of the required protein

Elution profiles of the required protein (—) and of the contaminant (---) are shown. Sample concentration of the required protein is 50 μM in each case. The contaminant protein is absent in (a) and present at 50 μM in (b) and 150 μM in (c). K_{PA} for both proteins was $2 \times 10^6 \text{ M}^{-1}$, and for the required protein $B = 0.32$. Column plate number = 5, ligand concentration = 500 μM and $K_{PL} = 2 \times 10^5 \text{ M}^{-1}$.

Discussion

Previous experiments in this laboratory on the effects of ligands on the interactions between enzymes and 'imphilytes' (immobilized amphiphilic ampholytes), some of which have been reported (Yon, 1977; Grayson *et al.*, 1979; Yon & Grayson, 1979), showed considerable variation in the sensitivity of response to the ligand in different cases. The need to express this variation quantitatively led to the definition of biospecific sensitivity given in eqn. (1), and its less rigorous but conceptually very useful extension in eqn. (3). It is instructive to compare the more common types of protein chromatography in current use in terms of probable values of B . Three categories may be recognized. (1) At one extreme are stationary phases where the density of substitution is so high that a large number of non-covalent bonds to a single protein molecule are present. Unless the ligand is very large, the fraction of these perturbed by the ligand will be negligible, i.e. B will tend to zero. This category will probably include most uses of ion-exchange, hydroxyapatite, hydrophobic-interaction and other essentially non-specific types of chromatography of proteins. However, there are relatively few reports on attempts to use biospecific desorption on stationary phases other than ion-exchangers, and its potential (or lack of it) remains largely unexplored. (2) At the other extreme are cases of 'pure' affinity chromatography, in which the non-covalent bonds involved in adsorption of the unliganded protein are strictly confined to the ligand-binding site on the protein and are all cancelled by the binding of the ligand. In such cases the numerator and denominator of eqn. (3) are equal and B is equal to 1 (or possibly greater; see previous comments on the possibility of repulsive interactions). (3) Between these extremes are all cases where B is a substantial fraction less than 1. These include all cases of biospecific desorption from non-specific stationary phases such as ion-exchangers and 'imphilytes'. Also included are the many cases of affinity chromatography where non-specific interactions have been demonstrated or where the possibility clearly exists, as when CNBr-activated matrices, hydrophobic spacer-arms or immobilized dyes are used at a high density of substitution or in low-ionic-strength buffers, or when the strength of adsorption is incompatible with the known (and frequently quite small) association constant for the protein-ligand interaction in free solution (O'Carra, 1978). When used in such cases, biospecific desorption may well be characterized by quite low values of B ; as we have seen, up to 50% of the adsorption free energy may be attributable to non-specific interactions without seriously impairing the excellent desorption profile characteristic of 'pure' ($B = 1$) affinity chromatography.

5 shows the effect of an increasing concentration of a single protein contaminant that has the same K_{PA} value as the required protein. Clearly the problem will be most severe when the contaminants have higher K_{PA} values than the required protein and are present at higher concentrations. It may be necessary to remove such contaminants before applying biospecific desorption.

For the column modelled in the present paper K_{PA} was confined to the range 10^6 – 10^7 M^{-1} . This suggests a relatively small number of non-covalent bonds between adsorption site and unliganded protein, by analogy with known protein–ligand association constants. For example, many dehydrogenase enzymes bind their coenzymes with association constants in the same range; in the case of NAD^+ binding to lactate dehydrogenase there are six non-covalent bonds involved (Adams *et al.*, 1973). For most proteins an adsorption involving such a small number of bonds would be relatively weak, since a typical protein has a much higher potential for interaction with the column on account of its extended size and the large number of active groups on its surface. This potential for excessive interaction between proteins and some types of adsorbent has long been recognized as the reason for the tendency to ‘all-or-nothing’ behaviour, the value of K_{PA} tending either to zero or to an extremely high value (Tiselius, 1954; Giddings, 1967; Morris & Morris, 1976). The requirement for K_{PA} to be in the range 10^6 – 10^7 M^{-1} therefore calls for careful and critical adjustment of the protein–adsorbent interaction. There are several possible ways of achieving this, e.g. decrease in the density of substitution of the stationary phase or manipulation of solvent factors such as pH or ionic strength. An example of such adjustment to optimize K_{PA} was described in experiments on the adsorption of pig heart malate dehydrogenase on an ‘imphilyte’ and its desorption by NADH (Yon, 1977). It is perhaps significant that the most productive use of ion-exchangers in biospecific desorption appears to involve cation-exchangers (phosphocellulose and CM-cellulose) at near-neutral pH conditions, when the adsorption of most proteins (isoelectric points in the range 5–7) must be very weak (see, e.g., Scopes, 1977a,b).

Computer simulation studies have provided some general conclusions on optimizing biospecific desorption in protein chromatography. For convenience these are summarized again here. (1) Adsorption of the unliganded protein should not be excessively strong; the weakest adsorption compatible with efficient washing of the loaded column should be used. (2) The column should be loaded to near-saturation with the required protein. (3) The concentration of the ligand front may need to be much higher than the concentration giving near-saturation of the protein in free solution, and care must be taken to ensure that the introduction of the ligand does not significantly disturb the bulk solution

properties of the irrigant. (4) When the required protein is a very small fraction of the total protein present, it may be necessary to subject it to several purification steps before application of biospecific desorption.

Finally, an important problem that has not been discussed in the present paper is adsorption-site heterogeneity. Such heterogeneity must be considered whenever mixed multivalent adsorption on adsorbents with high densities of substitution is under study, since there will then be a high probability that more than one distribution of active groups on the protein surface can be matched by a complementary adsorbing distribution on the stationary phase.

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References

- Adams, M. J., Buehner, M., Chandrasekhar, K., Ford, G. C., Hackert, M. L., Liljas, A., Rossman, M. G., Smiley, I. E., Allison, W. S., Everse, J., Kaplan, N. O. & Taylor, S. S. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1968–1972
- Barker, R. (1971) *Organic Chemistry of Biological Compounds*, p. 103, Prentice-Hall, Englewood Cliffs
- Giddings, J. C. (1967) *J. Gas Chromatogr.* **5**, 413–419
- Grayson, J. E., Yon, R. J. & Butterworth, P. J. (1979) *Biochem. J.* **183**, 239–245
- Martin, A. J. P. & Synge, R. L. M. (1941) *Biochem. J.* **35**, 1358–1368
- Morris, C. J. O. R. & Morris, P. (1976) *Separation Methods in Biochemistry*, 2nd edn., p. 36 and pp. 80–87, Pitman and Sons, London
- Mosbach, K. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol.* **46**, 205–278
- O’Carra, P. (1978) in *Chromatography of Synthetic and Biological Polymers* (Epton, R., ed.), vol. 2, pp. 131–157, Ellis Horwood, Chichester
- Scopes, R. (1977a) *Biochem. J.* **161**, 253–263
- Scopes, R. (1977b) *Biochem. J.* **161**, 265–277
- Storer, A. C. & Cornish-Bowden, A. (1976) *Biochem. J.* **159**, 1–5
- Tiselius, A. (1954) *Ark. Kemi* **7**, 443–449
- von der Haar, F. (1974) *Methods Enzymol.* **34**, 163–171
- Yon, R. J. (1977) *Biochem. J.* **161**, 233–237
- Yon, R. J. & Grayson, J. E. (1979) *Protides Biol. Fluids Proc. Colloq.* **27**, in the press