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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

LXVI*. INVESTIGATIONS ON THE EFFECTS OF CHROMATOGRAPHIC DWELL IN THE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF PROTEINS

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SUMMARY

Polypeptide and protein retention on chemically modified hydrocarbonaceous surfaces such as *n*-alkylsilicas is generally assumed to be mediated by weak solvophobic effects with relatively rapid kinetics of adsorption and desorption. Recent studies have, however, indicated that multi-zoning effects can be generated for many proteins under certain chromatographic conditions. These effects can lead to asymmetric or multiple peaks for an apparently homogeneous polypeptide or protein. The present study examines retention models for the reversed-phase liquid chromatographic separation of polypeptides and proteins undergoing conformational changes in the mobile phase and at the stationary phase surface. In addition the influence of the chromatographic dwell or residence time on resolution and recovery is examined. Analytical expressions for the change in retention and peak shape have been used to evaluate the role of secondary dynamic effects which may lead to broad asymmetric peaks or multiple peaks for an apparently homogeneous protein chromatographed on *n*-alkylsilicas under defined chromatographic conditions. Methods of classifying protein retention behaviour in reversed-phase systems according to the characteristics of these multiphasic transitions are suggested.

INTRODUCTION

Of all the complex secondary equilibria associated with the reversed-phase high-performance liquid chromatographic (RP-HPLC) separation of polypeptides and proteins, the dynamic effects of conformational equilibria, established by these macromolecules in the bulk mobile phase and at the stationary phase surface remain the least well understood. In common with all solutes undergoing slow dynamic interconversion under specified chromatographic conditions, broad asymmetric peaks and even multiple peaks corresponding to native and unfolded or denaturated

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forms of an apparently homogeneous protein can in principle be induced in either the isocratic or the gradient elution mode in reversed-phase systems. The presence of the same protein or polypeptide in different chromatographic zones, which can be assigned to functionally active and inactive forms, has been observed experimentally with a variety of globular and non-globular proteins, including ribosomal proteins[1,2], papain[3], soyabean trypsin inhibitor[4], collagen chains[5], lysozyme[6,7], ribonuclease[7] and multisubunit protein hormones[8]. Because of the relevance of these phenomena to the optimisation of resolution and recovery in preparative reversed-phase separations of proteins, the composite effects of different ionic and non-ionic modifiers and buffer components, the influence of the type and concentration of the organic solvent modifier and the role of pH and temperature in secondary equilibrium and non-equilibrium processes associated with protein retention to hydrocarbonaceous stationary phases has attracted much interest recently in this and other laboratories. In recent studies[9,10], we have argued that these dynamic effects become particularly evident in reversed-phase chromatographic systems (as well as in the other interactive chromatographic modes) when the overall time constants for the processes of equilibration between native and unfolded forms of a particular protein are of comparable magnitudes to the time of separation. In such circumstances the solute is subjected to differential migration processes due to a variety of competing secondary effects. Both the apparent retention factor and the peak variance will be affected as a consequence. The appearance of the chromatographic profile will thus be very condition-dependent and solute-dependent when the kinetics of competing equilibria due, for example, to aggregation, dissociation or denaturation are slow. Similar behaviour, attributed to slow kinetics of secondary equilibria, has been documented[11–13] in electrophoretic and ultracentrifugal analysis of proteins.

The present study examines an important factor now implicated in this complex retention behaviour, namely the influence of chromatographic dwell (or residence time at the stationary phase surface) on retention, peak shape and recovery for several small globular proteins chromatographed on macroporous *n*-alkylsilicas. Furthermore, different models for protein retention with *n*-alkylsilicas are discussed.

MATERIALS AND METHODS

Equipment

All chromatographic experiments were performed with a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatographic system consisting of two M6000A pumps, a U6K injector and a M660 gradient programmer. The detector used was a M450 variable-wavelength UV monitor operating at 215 nm. Chromatographic measurements were carried out at various temperatures using columns of different dimensions, namely 10–25 cm × 4.6 mm I.D. stainless steel. The stationary phases used were (1) octadecylsilica with mean particle diameter of *ca.* 6 μm and average pore size (from mercury intrusion experiments) of 13 nm corresponding to a specific surface area of 145 m^2/g, and, based on carbon analysis, to a ligand density of 2.0 μmoles dimethyloctadecylsilyl groups per square meter; (2) *n*-butylsilica with a mean particle diameter of 10 μm and average pore size 30 nm, corresponding to a specific surface area of 45 m^2/g and a ligand density of 2.9 μmol dimethylbutylsilyl groups per square meter; (3) μBondapak C_{18}; and (4) μBondapak alkylphenyl. Sample in-

jections were made with SGE Model 50A syringes (SGE, Melbourne, Australia) whilst pH measurements were performed with a Radiometer PHM64 meter, equipped with a combination glass electrode.

Chemicals and reagents

Acetonitrile and 1-propanol (HPLC grade) were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was quartz-distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, U.S.A.). The proteins used in this study were obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) and purified by affinity and/or ion-exchange chromatography. Trifluoroacetic acid was obtained from Pierce Chemical Co., (Rockford, IL, U.S.A.). The phosphate buffers were made from analytical reagent-grade orthophosphoric acid and monobasic sodium phosphate obtained from BDH chemicals (Poole, U.K.). Acrylamide, bisacrylamide, sodium dodecylsulphate, glycine and Tris–HCl were purchased from Sigma Chem. Co., (St. Louis, MO, U.S.A.).

Chromatographic procedures

Bulk solvents and appropriate mobile phases were degassed by sonication. Sample sizes of the various proteins varied between 5–100 μg injected in 10–40 μl. Protein solutions were prepared by dissolving the protein in the initial eluent of a gradient elution system at a concentration of 1–5 mg/ml depending on the particular protein and experimental conditions. Gradient times were varied between t_G = 15 min and t_G = 120 at flow-rates between 0.5 and 2.0 ml/min as indicated in the text. The influence of the chromatographic dwell time at the stationary phase surface under static conditions was measured by interrupting the flow for times varying between 10 min and 16 h immediately following the injection. Analysis of the peak shape and retention data was carried out by previously described methods[9]. The programmes for calculating the various chromatographic parameters and the probability distribution functions were written in Basic language for a Hewlett-Packard Model 86B computer, Model 7470A X–Y plotter and Model 82905B printer. The void volumes of the various chromatographic columns were determined with sodium nitrate. The gradient elapse time, t_e, required for Solvent B to reach the column inlet was determined in the usual manner from the onset of mobile phase absorbance increases with the volume fraction of Solvent B. Experimental mass profiles were compared and quantified in terms of absorbance values of the various proteins at different concentrations with a Cary model 15 UV–VIS spectrophotometer. Mass recoveries were determined by a modified form of the Bradford assay[14]. Because of the inherent errors in the quantitation of components which are eluted as asymmetric peaks the standard error of the peak area determination was *ca.* ± 15% when compared to the absolute recoveries over the same mass range. Enzymatic activity measurements for bovine trypsin were carried out by a procedure based on the method of Anderson *et al.*[15], whilst trypsin inhibitor activity was determined by the method of Kassel *et al.*[16]. Sodium dodecylsulphate polyacrylamide gel electrophoresis was carried out by the Laemmli procedure[17] with 15% slab gels.

RESULTS AND DISCUSSION

The separation of peptides and polypeptides by RP-HPLC is now a widely employed technique. Application of similar chromatographic methods to the purification of globular, membrane and fibrous proteins is a logical extention and an area currently of intense interest. Numerous studies over the past several years (for recent reviews see refs. 10 and 18) have revealed that a wide variety of proteins and protein fragments, in terms of both polarity and size, can be eluted from reversed phase *n*-alkylsilicas with excellent resolution and almost quantitative mass recovery. However, related studies have also demonstrated that low mass recovery, greatly reduced separation peak capacities and generally unsatisfactory resolution can also be observed with the same polypeptides and proteins under slightly different chromatographic conditions. For example, an increase in ionic strength from $\mu = 0.006$ to 0.205 (all other conditions being held constant) was sufficient to result in the complete retention of *a*-chymotrypsinogen to a *n*-butyl LiChrospher Si 500 column whilst under the initial low ionic strength conditions ($\mu = 0.006$) excellent resolution, narrow bandwidth and high recovery were obtained[6]. This dichotomous behaviour is typical of many globular proteins chromatographed on *n*-alkylsilicas where a small change in eluent or stationary phase conditions will be manifested as a major change in chromatographic performance. Often these changes in chromatographic performance are also associated with dramatic loss of recovered mass and biological function, *i.e.* the test substances are recovered in denatured form and the biological activity is abolished due to unfolding phenomena. Such effects are obviously not unique to reversed-phase systems but rather represent general phenomena associated with protein adsorption on polar and non-polar surfaces or interfaces.

For a given protein the intrinsic capacity factor, k', is determined by the overall equilibrium constant, K_d, for its distribution between the stationary phase and the mobile phase. In RP-HPLC as well as in other interactive chromatographic modes it is often assumed that the chromatographic retention time is very much greater than the half-life of protein unfolding, protein re-orientation at the stationary phase surface, protein–buffer ion interaction or protein-solvation phenomena. When such conditions apply, *i.e.* when $t_R \gg t(x)$ and no detectable change in the normalised concentration profile occurs throughout the duration of the experimental observations then the system is considered to be at apparent equilibrium. However, it is well known that the mass distribution coefficient of the observed chromatographic capacity factor is a time-averaged function of the different forms of the solute as it traverses the column. Often secondary phenomena impinge upon the dominant chromatographic distribution process established between the solute and the two phases. Some of these effects can be advantageously employed to enhance selectivity in a predictable way. For example, equilibrium changes associated with pH-controlled ionisation, ion-pair interaction, or the formation of substrate-stabilised protein complexes are widely employed to modulate chromatographic selectivity in reversed-phase and ion-exchange HPLC. Collectively these secondary equilibria can be considered as weak thermodynamically reversible phenomena. However, other secondary processes such as conformational interconversion, protein–protein aggregation, multimer–monomer dissociation, metastable adsorption or sol–gel thermal transitions can also occur with polypeptides and proteins in chromatographic systems with generally undesirable consequences.

The participation of all these different secondary phenomena will affect the apparent capacity factor (given by the sum of the capacity factors for each form weighted by its mole fraction) and also the peak variance (σ_v^2) and peak asymmetry (a_s) when the kinetics of the various processes involved are not rapid compared to the time scale of the chromatographic separation. Clearly in many chromatographic systems the assumption that peak shape and relative retention are independent of the reaction rates for secondary processes is untenable, *i.e.* the near-equilibrium assumption does not apply. In such circumstances the appearance of asymmetrical or multiple peaks for an apparently homogeneous solute will thus be very dependent on the nature of the solute used as well as the choice of the chromatographic conditions. In contrast, however, to the small-molecule situation, it is quite likely that anomalous peak profiles will more readily occur when random or empirical selection of chromatographic conditions is made. The appearance of multiple chromatographic peaks corresponding to biologically active and denatured forms of a particular protein or dissociated subunits of a multimeric protein, *e.g.* ribosomal proteins[1,2], papain[3] or glycoprotein hormones[8], and changes in the specific activity of a biologically active protein across an asymmetric peak of apparently constant amino acid composition illustrate practical problems encountered in protein purification by RP-HPLC. The ability to detect and resolve intermediate states of a multistep conformational transition typified by the above examples will thus depend on the sensitivity of the measurement, the relaxation times associated with the different phenomena, the magnitude of the differences in retention times for the different species and the peak variance of each species.

Most current models of protein retention in reversed-phase systems are based on the concept of reversible binding between the protein and a heterogeneous hydrocarbonaceous surface. Previous studies (*e.g.* refs. 9, 10, 18–21) have concluded that the adsorption involves multisite interactions between hydrophobic residues (or regions) on the protein surface and the chemically bonded non-polar ligand. Under regular reversed-phase conditions, the overall process of protein retention can be represented[10,20] by three discrete events, namely protein solvation in the bulk mobile phase, hydrocarbonaceous ligand solvation by the mobile phase components and interaction of the solvated protein with the solvated stationary phase surface. According to this approach, the chromatographic distribution can be represented by the relationship

$$\mathrm{P} \cdot (\mathrm{Solv})_a + n\ \mathrm{L}(\mathrm{Solv})_b \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{P}(\mathrm{Solv})_{(a-f)} \cdot n\ \mathrm{L} + (nb + f)\mathrm{Solv} \qquad (1)$$

Hence, the dependency of protein retention on the number, n, of hydrocarbonaceous ligands (L) associated with the protein (P) at the stationary phase surface and the solvent concentration required to affect desorption can be given by

$$k' = \Phi \cdot K_d\,[\mathrm{L}(\mathrm{Solv})]^n\,[\mathrm{Solv}]^{-(nb+f)} \qquad (2)$$

where k' ($= \Phi k_1/k_{-1}$) is the capacity factor; K_d is the equilibrium distribution constant; Φ is the phase ratio of the chromatographic system; the variables a and b are the number of solvent molecules associated with the protein and each non-polar

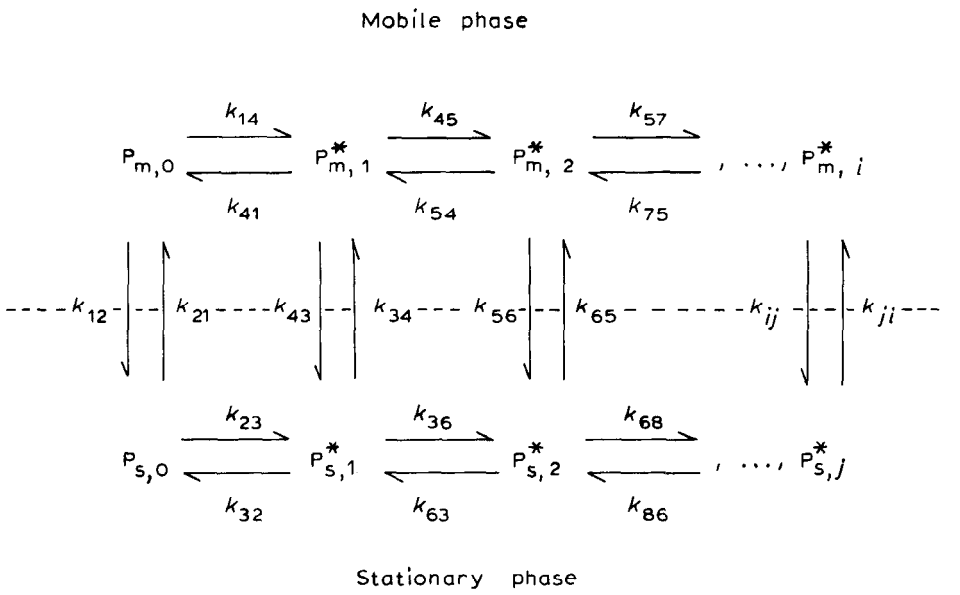
ligand respectively and the variable f is the numberof solvent molecules which occupy a surface area on the stationary phase equivalent to the interfacial hydrophobic contact area, ΔA, established between the protein and the non-polar surface. In reversed-phase isocratic elution, these retention dependencies can also be evaluated[10,21,22] through the relationship of capacity factor to the mole fraction of organic solvent modifier, φ, namely

$$\log k' = \log k'_w - S\varphi \tag{3}$$

where k'_w is the extrapolated capacity factor at $\varphi = 0$ and S is the slope (or tangent) of the plot log k' *versus* φ over a defined k' range. Although both eqns. 2 and 3 provide a reasonable approximation to the retention behaviour of small proteins eluted from *n*-alkylsilicas under isocratic and gradient elution conditions, they assume that an equilibrium mixture of the various structural forms has been established by the time elution commences and that no further change in three-dimensional structure occurs during elution. In reality, protein retention on hydrocarbonaceous surfaces is much more complex and can be considered to arise initially from the hydrophobic expulsion of the solute from the polar aqueous mobile phase, the primary nucleation step being involved with protein binding at the stationary phase surface, reinforced by subsequent conformational changes or re-orientations due to Brownian motion. In both cases an increase in entropy due to water or solvent extrusion from the surface will result, accompanying the increase in the number of hydrophobic binding sites involved and the increase in binding affinity. At high protein concentrations and with eluents of high ionic strength it is also possible that aggregated networks of proteins may arise on the stationary phase surface. These processes may account for anomalous temperature-dependent adsorption breakthrough and column pressure drop effects (due to gelation of the protein in the stationary phase pores) seen in some preparative separations. However, the studies of Jennissen and co-workers[23,24] related to the adsorption of phosphorylase *b* on *n*-butylSepharose suggest that such capillary gelation phenomena are not generally a dominant mechanism for protein retention on non-polar surfaces.

Besides these various surface-induced dynamic effects, other mobile phase-mediated processes can lead to a change in protein three-dimensional structure. For example, the addition of many water miscible organic solvents to an aqueous solution of a protein in its biological conformation leads to regional disruption of hydrophobic interactions between non-polar side chains in the protein with concomitant change in the hydrated three-dimensional structure, resulting in reversible or alternatively irreversible unfolding. Due to the greater accessibility of the exposed hydrophobic regions in the unfolded protein, enhanced retention on the *n*-alkylsilica will ensue, *i.e.* the number of stationary phase ligands involved will be increased and the affinity constant for binding also will be greater. Because of these composite stationary phase and mobile phase dynamic effects, relatively high concentrations of organic solvents are usually required to affect desorption of most proteins. This behaviour is directly manifested as the pronounced dependency of log k' on the mole fraction, φ, of the organic solvent modifier and is reflected in the magnitude of the retention parameters S and log k'_w[10,21,22,25,26]. Since the elution conditions may be above the denaturation midpoint[27,28] of a particular protein the situation can readily arise in

which the resolution and mass recovery may be high but the recovery of biological activity is low and very dependent on elution conditions and column history, *i.e.* the recovery may fall off very rapidly after several repetitive injections of the same protein mixture onto the same column. In order to discriminate adequately each of these secondary effects sufficient case histories for different proteins are required together with phenomenological as well as more fundamental classifications of their behaviour. When this knowledge is available, more systematic selection of chromatographic conditions, germane to optimal resolution and recovery, should prove feasible. Because of their structural variety and complexity the precise molecular mechanisms of adsorption onto and desorption from non-polar surfaces for most proteins remain largely unknown. The present paper examines the simplest case, namely the first order binding of monomeric proteins to hydrocarbonaceous surfaces. However, models similar to those outlined below would appear also pertinent to multimeric proteins. Thus if a first order reversible binding of a native protein, P_0 and various unfolded forms, P_1^*, P_2^*, ..., P_i^*, to a non-polar stationary phase occurs concomitantly with interconversion between various structural forms then the apparent equilibrium process can be schematically represented by



where the subscripts m and s refer to the mobile phase and the stationary phase respectively and k_{ij} represents the first order rate constant for the conversion of state i to state j. The above scheme thus illustrates regular chromatographic behaviour associated with the first order distribution of the native species $P_{m,0} \rightleftharpoons P_{s,0}$ (retention for the native form being defined by $k' = [P_{s,0}]/[P_{m,0}]$ in the absence of secondary time-dependent processes) as well as the unfolding of the protein in the mobile phase or at the stationary phase surface to various conformational intermediates on the denaturation pathways.

The kinetics of reversible unfolding and refolding of proteins, *e.g.* ribonuclease

$A^{29,30}$, in solution have largely been analysed in terms of a two-state model, *i.e.* $P_{m,0} \rightleftharpoons P^*_{m,i}$ and $P_{s,0} \rightleftharpoons P^*_{s,j}$ with the assumption that all intermediates are present in negligible amounts. If, however, unfolding in the mobile phase and at the stationary phase is represented by an initial nucleation step followed by a series of propagation steps then detectable intermediate species can in principle occur as part of the overall separation process. Recent studies on the mechanism of unfolding of ribonuclease A^{29-34}, parvalbumins[35], and *a*-chymotrypsinogen[33,34] have suggested that denaturation is associated with a kinetically slow process between a partially unfolded and a denatured form and a rapid process between the native and the partially unfolded form. The slow kinetic step has been attributed[36,37] to *cis–trans* isomerism around the peptide bonds that contain the pyrrolidine nitrogen of proline residues. In earlier investigations on the origin of bandbroadening of polypeptides and proteins separated by RP-HPLC methods we have proposed[8] that the concentration of the native protein in the mobile phase in a three state model changes biphasically due to unfolding with similar biphasic changes occurring at the stationary phase surface. This situation is represented by the forms $P_{m,0}$, $P_{s,0}$, $P^*_{m,1}$, $P^*_{s,1}$, $P^*_{m,2}$ and $P^*_{s,2}$ in the above scheme. The ability to detect the intermediate state $P^*_{m,1}$ as a discrete chromatographic peak will depend on its concentration and overall half-life. In many situations it can be anticipated that the observed chromatographic profile will approximate the direct process $P_{m,0}/P_{s,0} \rightarrow P^*_{m,2}/P^*_{s,2}$. For example if the rate constants k_{14}, k_{41} and k_{23}, k_{32} of the above scheme are considered to represent the respective fast processes and the rate constants k_{45}, k_{54} and k_{36}, k_{63} are considered to represent the respective slow processes of unfolding in the mobile phase and the stationary phase then the relaxation times for the fast (τ_1) and slow (τ_2) processes in either chromatographic phase can be expressed, at the limiting condition $\tau_{2,m} \gg \tau_{1,m}$ or $\tau_{2,s} \gg \tau_{1,s}$ and $k_{14}, k_{41} \gg k_{45}, k_{54}$ or $k_{23}, k_{32} \gg k_{36}, k_{63}$, by the relationships

$$\frac{1}{\tau_{2,m}} = k_{54} + k_{45}\,(1 + k_{41}/k_{14}) \tag{4}$$

$$\frac{1}{\tau_{2,s}} = k_{63} + k_{36}\,(1 + k_{32}/k_{23}) \tag{5}$$

$$\frac{1}{\tau_{1,m}} = k_{14} + k_{41} \tag{6}$$

$$\frac{1}{\tau_{1,s}} = k_{23} + k_{32} \tag{7}$$

Since the effects of the bulk mobile phase and the heterogeneous stationary phase on protein unfolding will usually be different, then the special situation will rarely arise where $1/\tau_{1,m} = 1/\tau_{1,s}$ or $1/\tau_{2,m} = 1/\tau_{2,s}$. More frequently it can be anticipated that $\tau_{1,m}, \tau_{2,m} \ll \tau_{1,s}, \tau_{2,s}$, *i.e.* the stationary phase component to denaturation dominates.

Superimposed upon these unfolding processes will be the chromatographic retention phenomenon characterised by the distribution processes $P_{m,0} \rightleftharpoons P_{s,0}$,

$P^*_{m,1} \rightleftharpoons P^*_{s,1}$, etc. As a consequence the overall chromatographic relaxation time will be the sum of the individual relaxation times for each process in the mobile phase and the stationary phase weighted by the fraction of the time each form is found in the stationary phase. When the ratio [denoted by the Damkohler number (D_a)[38]] of the time the protein spends in the mobile phase to the overall relaxation time of the chromatographic system is small, and in the limit approaches zero, then the chromatogram for the simplest case of a four-component cycle ($P_{m,0}$, $P_{s,0}$, $P^*_{m,1}$ and $P^*_{s,1}$ or $P_{m,0}$, $P_{s,0}$, $P^*_{m,2}$, $P^*_{s,2}$ when the intermediate state $[P^*_1]$ is essentially zero) will reflect the average macroscopic behaviour of the two forms and consist of two peaks separated by a time interval of $t_m\,(k_{12}/k_{21} - k_{43}/k_{34})$ or by a time interval of approximately $t_m\,(k_{12}/k_{21} - k_{56}/k_{65})$. Furthermore, the plate height component due to the kinetics of the secondary processes will be essentially independent of flow-rate[38]. Since the partially or completely unfolded form of the protein will behave as a more hydrophobic substance it will generally be eluted as the second peak under these conditions when $D_a \approx 0$. Conversely, when D_a is very large, kinetic effects associated with conformational interconversion essentially vanish. Under these conditions the concentrations of the various species approach their apparent equilibrium values. Melander *et al.*[38] have demonstrated that in the simplest case of a four-component cycle with first order or pseudo first order rate constants the resolution for the system can be written as

$$R_s = \frac{t_0\,\Phi(k_{12}/k_{21} - k_{43}/k_{34})\,(1 - e^{-D_a})}{4D_a\,(\sigma_0 + \sigma_1)} \tag{8}$$

where

$$D_a = \frac{L\,(\Phi k_{12}\,k_{23} + k_{14}\,k_{21})\,(k_{14} + k_{41})}{(k_{14}\,k_{21})u_0} \tag{9}$$

Hence, if bandwidths can be fixed such that σ^2_v for the early and later eluted peaks are similar, resolution will depend on the maximum difference in time between the eluted zones, Δt_R, the chromatographic void time, t_0, the relaxation time for mobile phase induced processes $(k_{14} + k_{41})$, the linear flow velocity, u_0, of an inert substance, the phase ratio, Φ, of the system, and the attendant rate constants for distribution (k_{12}, k_{21}) and interconversion (k_{14}, k_{23}), respectively. Similar resolution expressions for the conversion $P_{m,0}/P_{s,0} \rightarrow P^*_{m,1}/P^*_{s,1} \rightarrow P^*_{m,2}/P^*_{s,2}$ can be written in terms of the constants k_{45}/k_{54}, k_{36}/k_{63} and k_{56}/k_{65}. If it is assumed that the rate constants for conversion are finite but small the mass balance in the chromatographic column can be formulated in terms of the following two differential equations:

$$(1 + \Phi k_{12}/k_{21})\frac{\partial[P_{m,0}]}{\partial t} + u_0\frac{\partial[P_{m,0}]}{\partial z} =$$

$$(\Phi k_{32}k_{43} + k_{34}k_{41})\,k_{34}^{-1}\,[P^*_{m,1}] - (\Phi k_{12}k_{23} + k_{21}k_{14})\,k_{21}^{-1}\,[P_{m,0}] \tag{10}$$

and

$$(1 + \Phi k_{43}/k_{34})\frac{\partial[P^*_{m,1}]}{\partial t} + u_0\frac{\partial[P^*_{m,1}]}{\partial z} =$$

$$(\Phi k_{12}k_{23} + k_{14}k_{21})\, k_{34}^{-1}\,[P_{m,0}] - (\Phi k_{32}k_{43} + k_{34}k_{41})\, k_{34}^{-1}\,[P^*_{m,1}] \qquad (11)$$

where t and z denote the position of the solute in terms of time and distance and $[P_{m,0}]$ and $[P^*_{m,1}]$ are the number of moles of $P_{m,0}$ or $P^*_{m,1}$ per unit volume of the accessible mobile phase. Using computative methods similar to those developed by Melander *et al.*[38] for evaluating kinetic effects associated with *cis–trans* isomerism of proline dipeptides[39], numerical solutions to these two differential equations can be obtained when the rate constants and composition of the forms at apparent equilibrium in the mobile phase are known. Investigations currently underway indicate that such data can be readily acquired from size exclusion HPLC and used to supplement other results from spectroscopic measurements on the influence of mobile phase composition on protein unfolding.

In the limiting circumstance when the apparent rate constants of the overall forward (*i.e.* unfolding) and reverse (*i.e.* refolding) reactions approach zero, that is when

$$(\Phi k_{12}k_{23} + k_{14}k_{21})/k_{21} = 0 \qquad (12)$$

and

$$(\Phi k_{32}k_{43} + k_{34}k_{41})/k_{34} = 0 \qquad (13)$$

then each species will migrate independently, and the extent of zone broadening for each species will be defined by its effective diffusion coefficient. If the interconversions $P \rightarrow P^*$ occur with finite reaction rates then for first order reactions the concentration profiles of the zones due to P and P* will both change exponentially with the emergence of a new intermediate zone, its precise position depending on the average time the protein spends in the P_0, P^*_1, P^*_2, ..., P^*_i forms. For sufficiently large separation times, nearly all molecules of P_0 will have statistically undergone several transitions through the interconversion cycle and ended up as $P_{m,0}$, $P_{s,0}$, $P^*_{m,1}$, $P^*_{s,1}$, $P^*_{m,2}$, $P^*_{s,2}$, etc. Quantitatively, the resultant concentration profile can be estimated from the probability distribution of the relative time P spends in the various forms. Almost 25 years ago Keller and Giddings[40] derived a stochastic probability model to account for multiple zones due to interconverting species in paper and other forms of polar chromatography. This model appears equally pertinent today for evaluating multiple peaks associated with conformational equilibria or other secondary equilibria in the reversed-phase and ion-exchange HPLC of polypeptides and proteins. Thus, if the fraction of time the protein, P, spends in one form is represented by t, then the probability that this fraction is in the range $t + \Delta t$ is given by $P_i(t)$ where $i = 1, 2, 3, 4, \ldots$, representing each of the forms. If the interconversion cycle is represented by only four forms, the probability that P starts as $P_{m,0}$ and after one cycle ends as $P_{m,0}$ can be given by

$$P_1^1(t)dt = \frac{r_1 r_2(1 - t)}{t} \exp[-r_1(1 - t) - r_2 t]\, I[4r_1 r_2 t(1 - t)]^{\frac{1}{2}} dt \quad (14)$$

where

$$r_1 = (\Phi k_{12} k_{23} + k_{14} k_{21}) t^*/k_{21} \quad (15)$$

$$r_2 = (\Phi k_{32} k_{43} + k_{34} k_{41}) t^*/k_{34} \quad (16)$$

where t^* = separation time
and

$$I = \frac{\exp 2[r_1 r_2 t(1 - t)]^{\frac{1}{2}}}{4\pi^{\frac{1}{2}}[r_1 r_2 t(1 - t)]^{\frac{1}{2}}} \quad (17)$$

Similarly, if the molecule starts out as $P_{m,0}$ but ends up as $P^*_{m,1}$ the probability distribution can be given by

$$P_1^4(t)dt = r_1 \exp[-r_1(1 - t) - r_2 t]\, I[4r_1 r_2 t(1 - t)]^{\frac{1}{2}} dt \quad (18)$$

Finally, if the molecule starts out as $P_{m,0}$ but after one cycle no reaction has occurred, *i.e.* $t = 0$, the probability distribution is given by

$$P_1^0(t)\, dt = \exp(-r_1)dt \quad (19)$$

At separation time $t^* = 0$ a mixture of $P_{m,0}$ and $P^*_{m,1}$, in apparent equilibrium will be introduced at the column inlet at a position $z = 0$ compared to the overall column length L. Hence, the probability that $P^*_{m,1}$ ends up as $P^*_{m,1}$ after one cycle will be given by

$$P_4^4(t)\, dt = \frac{r_1 r_2 t}{(1 - t)} \exp[-r_1(1 - t) - r_2 t]\, I[4r_1 r_2 t(1 - t)]^{\frac{1}{2}} dt \quad (20)$$

and

$$P_4^0(t)dt = \exp(-r_2)dt \quad (21)$$

whilst the probability that P begins as $P^*_{m,1}$ but ends as $P_{m,0}$ is given by

$$P_4^1(t)dt = r_2 \exp[-r_1(1 - t) - r_2 t]\, I[4r_1 r_2 t(1 - t)]^{\frac{1}{2}} dt \quad (22)$$

When the overall rate constants for the forward and reverse processes of interconversion (*i.e.* r_1/t^* and r_2/t^*) are of the same order of magnitude as the rate constants for adsorption and desorption from the stationary phase, *i.e.* when the condition $1 < D_a < 10$ applies, then two additional aspects of the probability distribution will assume significance and need to be considered, namely $P_{m,0} \rightleftharpoons P_{s,0}$ and $P^*_{m,1} \rightleftharpoons P^*_{s,1}$. Furthermore, when the native and unfolded forms are retained indefi-

nitely as represented by large values of $P_1^2(t)$ and $P_4^3(t)$ respectively then these possibilities represent the time-dependent exponential loss of the solute to the stationary phase. Consequently the overall pathways for a four-stage cycle after some finite separation time, t^*, can be represented by the following scheme:

$P_1^1(t)$, $P_1^2(t)$, $P_1^3(t)$, $P_1^4(t)$ and $P_1^0(t)$

$P_2^1(t)$, $P_2^2(t)$, $P_2^3(t)$, $P_2^4(t)$ and $P_2^0(t)$

$P_3^1(t)$, $P_3^2(t)$, $P_3^3(t)$, $P_3^4(t)$ and $P_3^0(t)$

$P_4^1(t)$, $P_4^2(t)$, $P_4^3(t)$, $P_4^4(t)$ and $P_4^0(t)$

Several of these processes are non-contiguous interconversions, whilst in circumstances of high mass recovery, *i.e.* in the absence of peak ghosting phenomena, the contribution of the type $P_1^2(t)/P_4^3(t)$ to the overall concentration profile will be negligible. Consequently, under conditions of high mass recovery and relatively rapid adsorption–desorption kinetics the overall concentration profile will be proportional to the summated probability distributions, in each case properly weighted by the fractions, a and b, of molecules of P in the initial $P_{m,0}$ and $P_{m,1}^*$ forms injected onto the column at $t^* = 0$, *i.e.*

$$P(t) = a[P_1^1(t) + P_1^4(t)] + b[P_4^1(t) + P_4^4(t)] \tag{23}$$

$$P(t = 0) = a \exp(-r_1) \tag{24}$$

$$P(t = 1) = b \exp(-r_2) \tag{25}$$

Equivalent expressions can be written for cooperative and non-cooperative transitions involving multistep conformational perturbations with several intermediate states, $P_0 \rightarrow P_1^* \rightarrow P_2^*$, etc. By assuming Gaussian distributions for the concentration profiles of $P_{m,0}$ and $P_{m,1}^*$ centred around $t = 0$ and $t = 1$ respectively and by allowing a single diffusion coefficient to describe the combined effects of the chromatographic process for each conformational form, then in the cases summarised above, both $P(t = 0)$ and $P(t = 1)$ can be made discrete functions and the calculated probability distributions of the interconverting species, separated by retention time differences of $\Delta t_{R,1}$, $\Delta t_{R,2}$ can be computed for different values of the variables a, b, c, ..., and r_1, r_2, r_3, ..., etc. Non-Gaussian concentration profiles for $P_{m,0}$ and $P_{m,1}^*$ at $t = 0$ and $t = 1$ respectively due to heterogeneous interactions at the surface can also be accommodated by considering the appropriate peak variance equal to $(\sigma_G^2 + \tau_e^2)$ where σ_G is the standard deviation of the Gaussian constituent and τ_e is a time constant of an exponential modifier attributable to other kinetic events at the heterogeneous stationary phase surface not directly associated with conformational perturbations.

Because detailed experimental data on the rate constants associated with adsorption–desorption kinetics or conformational interconversion of different forms of a protein chromatographed on *n*-alkylsilicas are currently very sparse, the graphical representations derived from quantitative numerical solutions of the above prob-

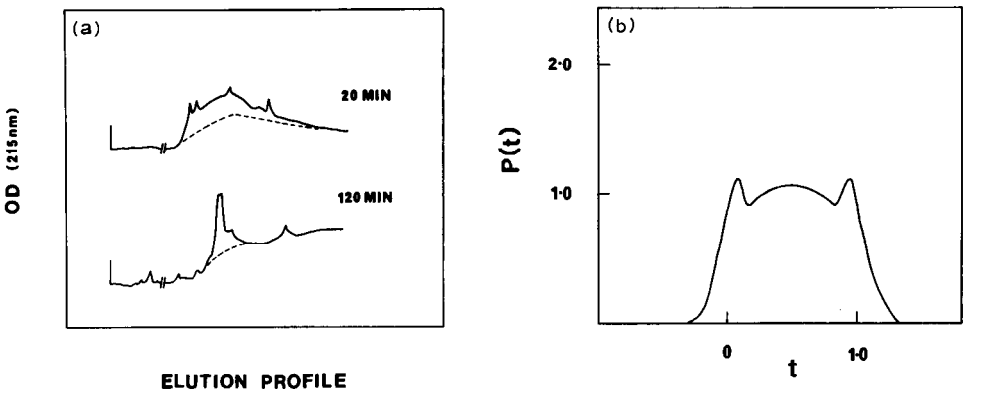


Fig. 1. (a) Elution profile of bovine trypsin chromatographed on a 30 nm *n*-butylsilica stationary phase at a flow-rate of 1.0 ml/min. The primary eluent (A) was 15 m*M* orthophosphoric acid–50 m*M* monobasic sodium phosphate, pH 2.3, and the secondary eluent (B) containing acetonitrile–15 m*M* orthophosphoric acid–50 mM monobasic sodium phosphate (1:1, v/v). A linear gradient elution of 0–100% (B) over 20 min or 120 min was carried out. The dashed lines show the optical density profile for a subsequent blank gradient. (b) The calculated concentration profile for bovine trypsin undergoing dynamic interconversion whilst chromatographed on a reversed-phase column. It is assumed that the interconversion processes occur by a two-state reversible transition with overall forward conversion and reverse conversion rates $r_1/t^* \approx r_2/t^* \approx 1.25 \cdot 10^{-3}$ sec^{-1} and that at $t^* = 0$ an equilibrium mixture of two interconverting components of equivalent mole fractions is initially loaded onto the column. It is further assumed that the migrating zone for each component generates a Gaussian distribution profile with $\sigma_t = 0.1$ and that the effective diffusion coefficients of both forms are the same.

ability distributions with various combinations of selected values for the rate constants, adsorption and desorption equilibrium constants, and relative retention differences can be used to simulate elution profiles and where feasible to provide visual comparison with experimentally determined chromatograms. Fig. 1 shows one such comparison, in which the separation of L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK)-treated bovine trypsin on a 30 nm *n*-butylsilica with a low-pH water–acetonitrile eluent is used as a test case. This protein can be successfully chromatographed on this same stationary phase and other silica-based reversed phases, such as μBondapak C_{18}, Synchropak RP-P[41] or μBondapak CN under low-pH water–acetonitrile or water–2-propanol elution conditions, as a peak of narrow bandwidth with excellent resolution from other proteins and high recovery of biological activity. However, by deliberately scouting out different elution conditions, the far from optimal chromatograms shown in Fig. 1 could be obtained. Simply by further manipulation of changes in the gradient steepness parameter, *b*, including the range of solvent composition, $\Delta\varphi$, and with the addition of 2–10 m*M* calcium chloride to the mobile phase the bandshape could be restored to an essentially symmetrical peak. In essence such changes in elution conditions are equivalent to variation in the chromatographic separation time, or elution time, t_g, plus any additional time due to chromatographic dwell, *i.e.* incubation time at the stationary phase surface and residence time in the mobile phase. Variations in t_g will also be associated with changes in elution volume, V_g, if the flow-rate is also not appropriately adjusted. Figs. 2–3 show further examples of the influence of competing equilibria on the multizoning behaviour of proteins separated on *n*-alkylsilicas as the chromatographic elution time

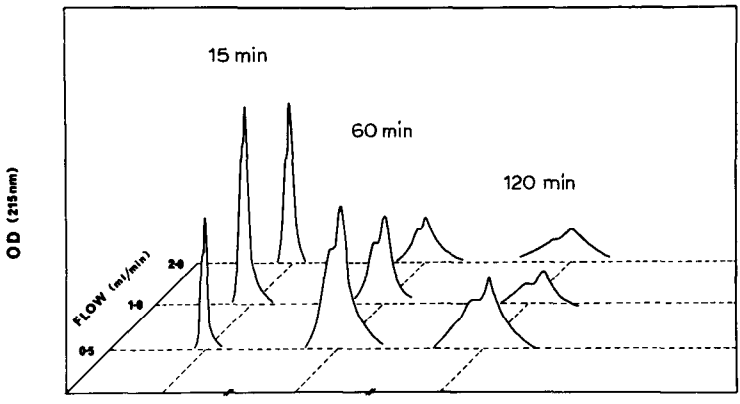


Fig. 2. Changes in bandshape of bovine trypsin chromatographed on a 13 nm octadecylsilica stationary phase as a function of flow-rate and residence time at the stationary phase surface. A linear gradient from 0.1% TFA–water to 0.1% TFA–water–acetonitrile (1:1) was used in all experiments. The bandwidth of the trypsin sample in the 15 min, 0.5 ml/min experiment was equivalent to $4\sigma_v \approx 1050$ µl. In all experiments the trypsin zone was eluted approximately at the same acetonitrile concentration. BAEE assays of the recovered zones revealed that the trypsin-like activity of all zones was also similar.

and/or the dwell time at the stationary phase surface were independently adjusted. The data shown in the composite Fig. 2 summarise a variety of observations pertinent to bandshape changes of TPCK-treated bovine trypsin eluted under different conditions of gradient time and flow-rate following a static incubation step on a 13 nm nominal pore diameter octadecylsilica. Further structural investigations currently in progress will clarify whether the double chain form, *a*-trypsin[42], is present in this preparation and participates in these band broadening effects. Firstly, the composite peaks, following recovery and concentration, corresponded in each case within the limit of experimental errors (*ca.* ± 15%) to zones of equivalent biological activity

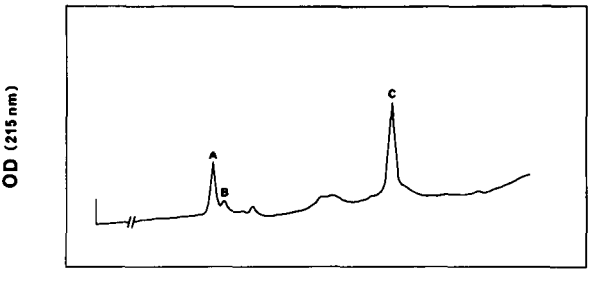


Fig. 3. Representative elution profile of soyabean trypsin inhibitor undergoing dynamic interconversion during chromatographic separation on a 30 × 0.4 cm column packed with 10 µm *n*-alkylphenylsilica; flow-rate, 2 ml/min; linear 30 min gradient from 15 m*M* orthophosphoric acid to acetonitrile–15 mM orthophosphoric acid–water (1:1, v/v). Reinjection of component A or component B demonstrated a time-dependent conversion to component C. Reinjection of component C resulted in the emergence of a single peak with the same elution time and peak shape.

as assessed by the BAEE assay[15]. Furthermore, each component had the same apparent molecular weight as assessed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis performed under non-reducing conditions*. Secondly, for a particular gradient time, the peak shape did not appear to be significantly influenced by flow-rate. Thirdly, significant change in band broadening appeared to occur when the elution time approximated the half-time for deactivation of trypsin in 0.1% trifluoroacetic acid (TFA)–water–acetonitrile, *i.e.* when t^* was approximately equal to or greater than 45 min and this was accompanied by a change in mass recoveries. Fourthly, long static incubation times, *i.e. ca.* 16 h had effects on the band shape of the bioactive zone similar to those of shorter incubation times, *i.e.* little change in the peak shape was evident after a dwell time of *ca.* 60 min. Fifthly, a zone corresponding to fully denatured trypsin could not be eluted from the octadecylsilica support under the elution conditions employed. Fig. 3 shows a typical chromatographic profile for soyabean trypsin inhibitor (STI) when subjected to similar on-column incubation experiments. Comparable results with complex elution patterns have been observed previously by Karger and co-workers[3,4] for this protein when chromatographed on *n*-alkylsilicas. Following collection of each fraction it was evident from reinjection experiments with individual components that components A and B were independently converted to component C. Further, all three components A, B, C had the same molecular weights as assessed by SDS-polyacrylamide gel electrophoresis but only the two earlier eluting components exhibited trypsin inhibition activity. The net effect of these interconversions is the formation and recovery in good mass yield of inactive STI when the incubation or separation time is greater than *ca.* 30 min with the low pH water–acetonitrile gradient system. Interestingly, 1-propanol based gradients appeared to stabilise form A to some extent although the general pattern of A/B → C was still evident.

It should be noted that the experimental profiles shown in Figs. 1–3 represent gradient elution data whilst the plot of P(t) *versus* (t) (Fig. 1b) assumes that the distribution processes involve a first order isocratic elution development of only two converting states. The experimental gradient elution results for trypsin suggest that with this protein a more complex multistep pathway occurs which involves at least a three step interconversion. Similar behaviour is also evident with *a*-chymotrypsinogen[33] and pepsinogen[34]. Recent studies have suggested[25,26] that in well defined situations changes in relative retention and bandwidth data derived from gradient elution experiments correlate well with changes in the same parameters derived from isocratic measurements as dependent and independent variables are experimentally adjusted. Clearly, in the case of gradient elution the rate of accumulation (or disappearance) of a particular form, P_i, per unit time will be dependent on the gradient steepness parameter, b. Even in this more complex elution case the change in gradient slope can be viewed as inducing a series of microscopic steps each of which results in first order, pseudo first order, or higher order kinetics along a non-equilibrium trajectory. Because of the nature of gradient elution, once different forms of a slowly interconverting solute have been desorbed from the column surface they will migrate down the column and be subjected to band compression due to the change in eluent composition across the migrating zone. The propensity of the chromatographic sys-

* See "Note added in proof" on p. 64.

tem with gradient elution to produce discrete peaks or zones of sufficient peak height to allow high-sensitivity detection either on-line spectrophotometrically or off-line in functional assays is thus favoured. It can be appreciated then that gradient systems have great potential for generating non-equilibrium changes associated with phenomena which are in essence thermodynamically irreversible. The work of Katchalsky and co-workers[43,44] suggests that when such non-equilibrium changes lead to long-lived metastable forms, these forms can be analysed in the same way as equilibrium processes with the apparent concentrations of the different forms treated as equilibrium concentrations under that specified condition. Various parameters, such as apparent rate constants and apparent activation energies, can thus be calculated as different experimental variables are changed sequentially. Using this approach the apparent rate constants for the denaturation of lysozyme and soyabean trypsin inhibitor at 20°C and 25°C on the 6 μm octadecylsilica column with a 10 m*M* TFA primary eluent and a 0–40% 1-propanol developing eluent were calculated to be 25, 70, 60 and 115 $\times$ 10^{-4} sec^{-1} respectively. These values are of a magnitude comparable to those previously reported[3] for these two proteins chromatographed on a large-pore-*n*-butylsilica. Since the rates of denaturation of these and other proteins in various eluents can be determined from measurements of changes in effective molecular volume by size-exclusion chromatography and from multidimensional spectroscopic measurements such as UV absorption, fluorescence, light scattering and circular dichroism data, the apparent rates of denaturation of proteins at the stationary phase surface can be thus derived. Investigations currently underway in this laboratory are addressed to differentiate in this way the stationary phase component of these dynamic events which can dramatically affect chromatographic performance.

The importance of the chromatographic retention parameters S and $\log k'_w$, derived from either isocratic or gradient elution data, in the optimisation of resolution of polypeptides and proteins on *n*-alkylsilicas has been previously documented[10,18,22,25,26]. The present study suggests that additional criteria can be added to the growing list of behavioural characteristics which describe protein retention in reversed-phase systems and by analogy in ion-exchange and biospecific affinity systems. Consider first, the following circumstance in which the retention process involves only the transitions $P_{m,0}$, $P_{s,0}$, $P^*_{m,1}$ and $P^*_{s,1}$ and the overall interconversion kinetics in the mobile phase and stationary phase are rapid. Under these constraints a single peak with preservation of biological activity for each protein is to be anticipated for gradient elution even when the separation time is relatively long, *i.e.* $t^* > 30$ min. Proteins with these characteristics will also exhibit considerable resistance to denaturation under static conditions in the chosen mobile phase composition, *e.g.* trypsin under low pH conditions in the presence of low concentrations of calcium chloride. Secondly, when a one-step interconversion occurs which involves slow/slow kinetics with $1/\tau_m$ approximately equal to $1/\tau_s$ two essentially symmetrical peaks differing in relative retention will arise for each protein, the abundance of each form and the free energy of denaturation varying linearly with the concentration of the solvent and the temperature over the whole transition region. The second eluted peak will invariably be associated with the denatured form, as typified by soyabean trypsin inhibitor or ribonuclease A. Thirdly, when a one- or two-step interconversion occurs with comparable kinetics to the separation kinetics, several discrete intermediate

forms may become evident. Depending on the relationship of $1/\tau_2$ and/or $1/\tau_1$ to the separation time a broad asymmetric peak or multiple peaks of biologically active and/or inactive protein with variable mass recovery may arise. The retention behaviour of the follicular protein, inhibin[45], may be an example of this third case. Fourthly, in circumstances when the kinetics associated with renaturation (refolding) in the mobile phase and the stationary phase, *e.g.* k_{75} and k_{86}, are slow (all others being fast) high recovery of the denatured protein as a symmetric peak will ensue in gradient elution experiments as for example seen with bovine fibroblast growth factor[46,47] rabbit muscle phosphorylase kinase[48] or other multimeric proteins[49,50]. A special case can also be identified when desorption rates are very slow, *e.g.* when $k_{ij} \gg k_{ji}$, which would be manifested as a complete inability to elute the denatured protein from the stationary phase irrespective of chosen particle porosity or ligand coverage. The above criteria provide a basis for classifying protein retention behaviour in RP-HPLC systems. Although initially such discriminations will be mainly phenomenological in nature from experimental case histories, this information will allow more precise mechanisms of protein retention to be advanced and evaluated more throroughly in terms of thermodynamic and non-thermodynamic considerations. Besides the relevance of these criteria to the issue of protein recovery in general, they also have implications for the appropriate choice of column configuration and eluent flow-rates. Improvements in column design and stationary phase characteristics, particularly for preparative applications, will certainly result as additional information on the dynamic effects of competing secondary equilibria associated with protein adsorption onto and desorption from non-polar (and also polar) surfaces become more extensively documented.

SYMBOLS

a_s	Peak asymmetry factor
b	Gradient steepness parameter as defined by $b = S\Delta\varphi t_0/t_G = SV_m\theta'/F$
F	Flow-rate of mobile phase
K_d	Equilibrium distribution constant
k_{ij}	First order rate constant for the conversion of state i to state j
k'_i	Capacity factor for solute P_i
$k'_{w,i}$	Capacity factor for solute P_i with a mobile phase where $\varphi = 0$
L	Column length
P^*_{ij}	Conformational form of solute P in state i undergoing interconversion to state j
$P_i^j(t)$	Probability function for solute P undergoing one interconversion cycle from state i to state j, as defined by eqn. 14
r_i	Overall rate constant for the conversion of solute in state i to state j in a chromatographic system of phase ratio, Φ, as defined by eqn. 15
S	Slope (or tangent) of the plot log k' *versus* volume fraction of organic solvent modifier, φ, as defined by eqn. 3
t_d	Chromatographic dwell time as defined by the incubation time at the stationary phase surface

t_g	Gradient elution time of solute P
t_G	Gradient time
t_0	Column dead time
u_0	Mobile phase linear velocity
V_g	Elution volume of solute P
V_m	Column void volume
$[P_{m,0}]$	Concentration of solute P in native state in the mobile phase
$[P^*_{m,i}]$	Concentration of solute P in unfolded state i in the mobile phase
$[P_{s,0}]$	Concentration of solute P in native state on the stationary phase surface
$[P^*_{s,i}]$	Concentration of solute P in unfolded state i in the stationary phase
ΔA	Hydrophobic contact area
$\Delta\varphi$	Change of φ across the gradient
σ_t	Peak bandwidth in time units
σ_v	Peak bandwidth in volume units
σ^2_G	Peak variance for a Gaussian bandshape in units of time or volume
σ^2_v	Peak variance in volume units
$\tau_{i,m}$	Relaxation time for solute P in state i undergoing interconversion in the mobile phase
$\tau_{i,s}$	Relaxation time for solute P in state i undergoing interconversion at the stationary phase surface
μ	Ionic strength
Φ	Phase ratio
θ'	Rate of change of $\Delta\varphi$ with time
φ	Volume fraction of organic solvent modifier

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NOTE ADDED IN PROOF

Further structural characterisation has confirmed that the bovine trypsin used in these chromatographic studies contained *ca.* 30% α-trypsin resulting from an internal cleavage between lysine-125 and serine-126. The calculated concentration profile for two interconverting states for the trypsin species based on the relationship P(t) *versus* (t) will thus generate at least four maxima when r_1 (native trypsin), r_1 (native α-trypsin), r_2 (unfolded trypsin) and r_2 (unfolded α-trypsin) are different and the rate of migration of the various trypsin species do not coincide.

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