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pH effects on non-ideal protein size-exclusion chromatography on Superose 6

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ABSTRACT

The retention behavior of five globular proteins (ribonuclease, lysozyme, β-lactoglobulin, serum albumin, γ-globulin) was determined on Superose 6 in low ionic strength (0.02–0.04 *M*) mobile phases of high, intermediate and low pH (10.0, 7.0, 4.3). Quantitative assessments of attractive or repulsive solute-packing interactions were made by comparing the chromatographic partition coefficient to the value obtained for non-interacting spherical solutes on the same column. The dependence of this "non-ideal" adsorption or exclusion on net protein charge is complex, but not very sensitive to protein type. The results suggests that on size-exclusion chromatography packings that behave as weak cation-exchange resins the electrostatic solute-packing interaction is not governed by a highly localized set of a few charged sites.

INTRODUCTION

Ion-exchange chromatography (IEC) is a key tool in the analysis and preparative separation of peptides and proteins (see, for example, ref. 1). There is a considerable divergence of viewpoints about the fundamental nature of protein-substrate interactions in IEC. At one extreme, it has been proposed that a well-defined, localized set of charged amino acid groups on a protein react in a stoichiometric manner with a complementary set of fixed charges on the packing, releasing an identical number Z (*e.g.* three or four) of small ions [2]. This classical ion-exchange model attributes the effect of ionic strength (which always diminishes the capacity factor k') to a mass-action equilibrium in which the small ions with charge opposite to the fixed charges on the packing play the role of "displacer ions" [3]. Applying to this concept the treatment of Boardman and Partridge [4], Drager and Regnier [5] obtained a linear dependence of ln k' on ln M (where M, the molarity of the displacer ion, is the ionic strength for univalent electrolyte mobile phases) with a slope of Z. Linear plots in support of this model were reported for β-lactoglobulin on Mono-Q, a strong anion-exchange resin [2].

A completely different point of view results if the attractive force between protein and packing is considered to be the result of relatively long-range, spatially averaged interactions between two surface of opposite charge [6]. The role of the small ions is then via Debye–Hückel screening, and the dependence of k' on ionic strength (I) is quite different [7]. In support of this viewpoint, we note the result of Haggerty and Lenhoff [8] who found excellent correlation between k' and the protein mean surface potential, calculated using the molecular modeling software program Delphi [9]. From this perspective, a quasi-global protein charge, not a local one, controls interaction, and no complementary ion-pairing is envisioned. The treatment of Ståhlberg [6] follows similar lines, and explicitly states

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that the charge distribution of packing and protein are likely to be asymmetric, and thus inimical to the stoichiometry central to the Regnier model. Given this disparity of viewpoints, it would seem prudent to understand the phrase "ion-exchange chromatography", as applied to proteins, as meaning "chromatography of proteins on a column packed with a support that can operate as an ion-exchange resin", thus recognizing that the mechanism for protein retardation on such a column may or may not partake of the same stoichiometric features as controls the separation of small ions.

Since the preceding treatments predict different dependences of k' on I, one would expect that experiment should cast a decisive vote. However, the practical range of I in ion-exchange chromatography is quite limited because the strength of the interactions under typical conditions precludes the use of low ionic strength mobile phase. Data obtained in a narrow range of I cannot reveal departures from proposed expressions for $k'(I)$, especially for semi-logarithmic or double-logarithmic relations. (It should be noted that in one study where the ionic strength was varied over several orders of magnitude [10] the log–log dependence of k' on I predicted by Regnier was observed only within limited ranges of I.) Weak ion-exchange resins which can easily be operated over a wide range of I are not in common use. However, virtually all aqueous size-exclusion chromatography (SEC) packings, based on silica or on cross-linked hydrophilic polymers, carry some negative charge at neutral or basic pH [11], and are in this sense weak ion-exchange resins. The dependence of retention time on I can thus be examined over several decades of I.

One difficulty that arises in the use of SEC data to test theories of IEC is the choice of the proper reference compound for the determination of relative peak retention. All theories will lead to the fraction of protein in the bound state, and hence its migration velocity v relative to an unretained solute, *i.e.* $k' = (v_0/v)^{-1} = t/t_0$. But in SEC one conventionally measures the elution volume relative to a solute too big to enter the pores, *i.e.*

$$K_{SEC} = (V_e - V_0)/(V_t - V_0) \quad (1)$$

where V_e is the observed elution volume, V_0 that of the aforementioned large solute, and V_t the elution volume of a compound similar in size to the solvent. However, V_0 does not correspond to the unretained solute embodied in k', and the dependence of K_{SEC} on I would mix together size and interaction effects. The unretained solute with retention time t_0 must be a non-interacting solute of the same size as the analyte in question. Recently, we have established [12] that the dependence of K_{SEC} on solute radius R is coincident for pullulans (non-ionic flexible chain polymers) [13], ficolls (non-ionic, densely branched, quasi-spheres) [14] and carboxylated starburst dendrimers [15] (compact dendritic species with negative surface charge), on Superose 6 in neutral pH, $I > 0.2$ mobile phase. That the combined K_{SEC} *vs.* R plot for these solutes represents the behavior of non-interacting spheres is supported by the observation that data for several proteins obtained at $I > 0.2$ also fall on this curve, particularly when the mobile phase pH is close to the protein pI. Thus, comparisons of protein retention to the value predicted from this "ideal" curve facilitates the analysis of coulombic interaction effects on protein chromatography.

In the current work we present limited results for a number of globular proteins under conditions of rather low ionic strength ($0.02 < I < 0.04$) and over a range of pH (4.3–10). Under these conditions, the protein pI values may be above or below the pH, and the charge on the packing, due to sulfate and/or carboxylic acid groups [16], also may vary with pH. In contradistinction to the IEC studies mentioned above, the results are obtained both in the repulsive (pH > pI) as well as the attractive (pH < pI) regimes. The deviation in K_{SEC} from that for the "ideal" sphere of identical R is observed as a function of I and pH. The results constitute a "range-finding" study in that the number of ionic strengths examined are too small to provide a test of the theories mentioned above. However, the insights obtained set the stage for more detailed examinations.

TABLE I

CHARACTERISTICS OF PROTEINS USED IN THIS STUDY

Protein[a]	Source	MW	pI	R_S (nm)[b]	R_η (nm)[b]
Ribonuclease (R-5503)	Bovine pancreas	13 700	9.0	1.75	1.90
Lysozyme (L-6876)	Egg white	14 000	11.0	1.85	2.0
β-Lactoglobulin (L-2506)	Bovine milk	35 000	5.2	2.7	2.65
Albumin (A-7906)	Bovine serum	66 000	4.9	3.5	3.4
γ-Globulin (G-5009)	Bovine	150 000	7.0	5.6	–

[a] All from Sigma, except γ-globulin also supplied by CalBiochem.
[b] Literature sources for R_S and R_η as cited in ref. 12.

EXPERIMENTAL

Materials

Table I lists the proteins employed in this study, along with their MW, isoelectric point, and Stokes radius and viscosity radius, where available. In the subsequent analyses we use the average of the Stokes radius R_S and the viscosity radius R_η, although it should be noted that the differences between these are too small to significantly affect our interpretation of the results. All buffers and salts were reagent grade, from Sigma, Mallinckrodt, Fisher or Aldrich.

Methods

Size-exclusion chromatography. SEC was carried out on a prepacked Superose 6 HR 10/30 column, which had a column efficiency of 3800–4600 plates/m throughout most of these studies. The HPLC instrument was a Beckmann System Gold, equipped with a Beckman Model 156 refractive index detector or a Waters R401 differential refractive index detector, along with the UV detector supplied with the instrument. Solvent was delivered with a Beckmann 110 B pump and an Altex 210A valve with either a 20-, 50- or 100-μl loop. A Rheodyne 0.2-μm precolumn filter was placed in-line to protect the column. Flow-rates were measured and found to be constant within ±0.5% by weighing of collected eluent. Sample preparation was accomplished by shaking or tumbling for 1–2 h. The concentrations of all polymers and proteins were in the range 2–5 mg/ml, except for concentration effect studies. Samples were filtered through 0.45-μm Gelman filters before injection. K_{SEC} was determined according to eqn. 1 with V_0 determined from the retention of either $2 \cdot 10^6$ MW dextran or $4 \cdot 10^6$ MW poly(ethyleneoxide) as 6.50 ml, and V_t determined from the retention of dextrose as 19.98 ml.

RESULTS AND DISCUSSION

In order to examine effects of both ionic strength and pH, we obtained protein retention data at four sets of conditions: pH 7.0, $I = 0.02$ M; pH 7.0, $I = 0.04$ M; pH 4.3, $I = 0.03$ M, and pH 10.0, $I = 0.02$ M. The results are presented in Fig. 1A for pH 7.0, and Fig. 1B for pH 4.3 and

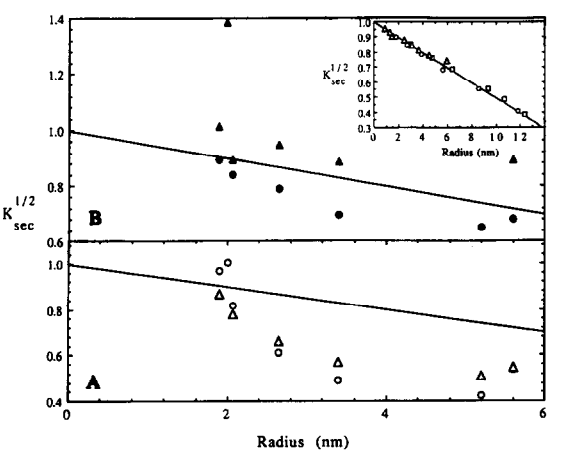

Fig. 1. Dependence of $K_{SEC}^{1/2}$ on protein radius, on Superose 6, in various buffers. A: (△) pH 7.0, $I = 0.04$ M; (○) pH 7.0, $I = 0.02$ M. B: (▲) pH 4.3, $I = 0.02$ M; (●) pH 10.0, $I = 0.03$ M. Insert shows the "ideal curve" generated from data for pullulan (○), ficolls (□) and dendrimers (△) (see ref. 12) corresponding to the solid lines in A and B.

10.0. The data are presented as $K_{SEC}^{1/2}$ *vs.* R, since this linearizes the "ideal curve" obtained for the non-interacting spherical solutes, which is shown as the solid line in Fig. 1A and B, and as the insert. In this treatment, we consider R as a static parameter, unaffected by pH, for the following reasons: (a) limited quasi-elastic light scattering studies for bovine serum albumin (BSA), RNAse and lysozyme, reported elsewhere [17] show no measurable dimensional change for these proteins over the pH range 4–10; (b) the magnitude of the changes in retention are large compared with the expected dimensional change from partial unfolding. It is evident that all the proteins of this study exhibit adsorption (positive deviations from the "ideal" line) for pH 4.3, and nearly all show repulsion at pH 10.0. (We will comment on the pH 7.0 results later). The variation in the extent of deviation from the "ideal" line clearly depends on the individual pI values. Potschka has described these deviations in terms of the horizontal displacements, *i.e.* ΔR, and for SEC in the repulsive range attributes the negative values of ΔR to the electrical double layer around the protein [18]. We follow the same procedure, although we note that (a) there is no compelling reason to assign all of the measured "ΔR" to the protein, as opposed to the electrical double layer near the substrate (ionic strength effects on the repulsive term may be viewed as a diminution in effective pore volume [18,19] with as much justification as an increase in effective solute size [20]); and (b) ΔR has no physical meaning in the case of attraction. Nevertheless, the horizontal displacements of the protein data from the "ideal" curve in Fig. 1A and B provide a good quantitative measure of the magnitude of the non-ideal interaction.

It is not *a priori* evident that the non-ideal contribution is purely coulombic. In this case we should expect considerable variation among the different proteins when ΔR is analyzed on the basis of net charge. Fig. 2 shows the dependence of ΔR on the net protein charge, obtained from published titration data [21–23]. Note that $\Delta R < 0$ refers to repulsion, and $\Delta R > 0$ to attraction. The following conclusions may be reached. (i) As can be seen from pH 7.0 data, higher ionic

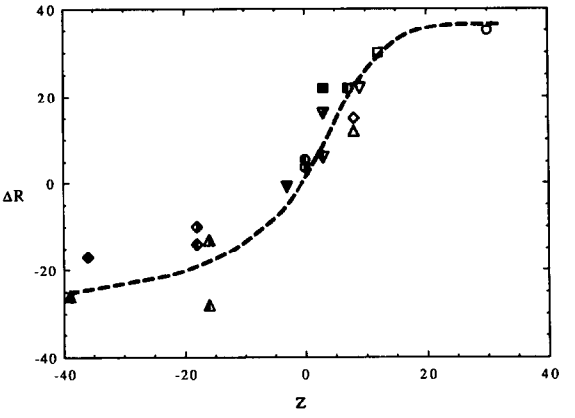

Fig. 2. Data from Fig. 1 plotted as ΔR *vs.* Z, where ΔR is the horizontal separation between the "ideal curve" and the datum (such that positive ΔR corresponds to late elution, or attraction, and negative ΔR to early elution or repulsion) and Z is the net protein charge. In pH 4.3, $I = 0.03$ M eluent: □ = lysozyme; ▽ = RNAse; △ = BSA; ◇ = β-lactoglobulin; ○ = γ-globulin. In pH 7.0, $I = 0.02$ M eluent; ◧ = lysozyme; ▼ = RNAse; ▲ = BSA; ◆ = β-lactoglobulin; ◐ = γ-globulin. In pH 7.0, $I = 0.04$ M eluent: ▼ = RNAse; ▲ = BSA; ◆ = β-lactoglobulin; ◑ = γ-globulin. In pH 10, $I = 0.03$ M eluent: ■ = lysozyme; ▼ = RNAse; ▲ = BSA; ◆ = β-lactoglobulin.

strengths lead to more nearly ideal behavior, but the effect is more pronounced in the repulsion regime; this finding is supported by results obtained for catalase, apoferritin and thyroglobulin at pH 7.0 [24] (not included in the present paper because titration curves needed for net charge values are not available). (ii) With the exception of a single result for BSA at pH 7.0, data obtained for the five different proteins do not deviate excessively from a common curve (broken line). (iii) The common curve is strongly asymmetric with respect to Z: small positive increments in Z at pH < pI produce large changes in ΔR; comparable changes in ΔR in the repulsive regime require much larger absolute increments in $|-Z|$. It could be argued, correctly, that Fig. 2 fails to represent the packing charge, which is changing along with Z, as pH falls. However, the effect of diminishing pH as one progresses in the positive direction from $Z = 0$ should be a decrease in packing charge; nevertheless, ΔR rises rapidly in the range $0 < Z < 12$.

The observation of a common curve, even with some strong individual departures, suggests that both repulsive and attractive forces are

primarily coulombic, and —to a first approximation— more strongly influenced by net protein charge than local charge. It is indisputable that retention on strongly charged columns is often governed by "charge patches", *i.e.* proteins may show strong retention on the "wrong side" of their p*I* values [2], but this appears to be less the case for electrostatic attraction on weakly charged SEC columns. A reasonable hypothesis is that the lower charge density of the SEC column results in a less steep minimum in the orientational energy of "bound" protein, and that the greater orientational mobility of the "bound" protein means that more charged groups may make some contribution to interaction with the packing.

Last, we discuss the asymmetry of Fig. 2, *i.e.* the apparently stronger effect of change in Z on ΔR in the attractive regime. Two possibilities may be considered. The first involves fundamental differences between attractive and repulsive forces, the latter being inherently more long-range. Repulsive interactions may sample the total population of charges on a protein to a greater extent that attractive ones. The effect of a single positive charge on attractive interaction with the anionic packing may thus be greater than the effect of one additional negative charge on the repulsion, because the orientation of the protein at the packing surface is more likely to favor the proximity of the former, and diminish the proximity of the latter. The second consideration is packing charge, which changes along with pH to the extent that the Superose ionophores are carboxylic acid. For acidic proteins, such as BSA, diminution in the pH below p*I* should produce compensating effects, inasmuch as the protein charge (positive) increases while the packing charge diminishes. For highly basic proteins, for which p*I* is well above pK_a of any weak acid packing ionophores, such effects are expected to be negligible in the vicinity of $Z = 0$. A prerequisite to further analysis along these lines is the titration curve of Superose 6, which is one of the objectives of ongoing work.

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