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SURFACTANT-MEDIATED PROTEIN HYDROPHOBIC-INTERACTION CHROMATOGRAPHY

D. B. WETLAUFER* and M. R. KOENIGBAUER
Department of Chemistry, University of Delaware, Newark, DE 19716 (U.S.A.)

SUMMARY

Three proteins have been subjected to hydrophobic-interaction chromatography in the presence of submicellar concentrations of the surfactant (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS). At several concentrations of CHAPS below the critical micelle concentration, CHAPS increased the retention of lysozyme and pancreatic trypsin inhibitor, but decreased that of ribonuclease A. The dependence of retention on CHAPS concentration was substantially different for the three proteins, *i.e.*, the surfactant showed selectivity in its interactions with the proteins.

In the solvents that were used for chromatography the surface tension decreases monotonically with increasing CHAPS concentration. Since different proteins may either be eluted or retained by the addition of CHAPS, our findings are inconsistent with the idea that retention is a simple function of surface tension. It also appears unlikely that the selectivity we have observed can be accounted for by a formulation of retention as a function of surface tension. As an alternative interpretation, we present a scheme of multiple equilibria and their kinetic components as a basis for formulating the dependence of retention on surfactant concentration.

INTRODUCTION

An elementary and recurrent problem in the purification of proteins is solubilizing the proteins. A suitable solvent is usually a buffered aqueous solution, often containing some additive to increase protein solubility. We commonly use urea, ionic detergents, guanidinium chloride, or other "salting-in" electrolytes. Less frequently, co-solvents like dimethyl sulfoxide, N-methylformamide, and non-ionic surfactants are employed. It is generally found that solubilizing agents also tend to be denaturing agents. If preservation of the native protein is an objective, a substantial amount of experimentation is usually required to solubilize and purify a particular protein. While the native structure is not required for certain investigative purposes, it is almost always desirable in preparative work. This is surely the reason why protein chromatography in conventional low-pH reversed-phase chromatography (RPC) systems (strongly denaturing) is being challenged by ion-exchange and hydrophobic-interaction chromatography (HIC), which tend to operate under conditions favoring preservation of the native structure.

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It seems evident that two proteins are likely to show greater differences in their properties at interfaces in their native forms than in their denatured forms. Thus, chromatographic selectivity should also be greater when they are in their native forms. There are both empirical and theoretical reasons why substances that lower surface tension would be expected to facilitate protein elution in RPC and HIC[1–4]. We therefore set out to find for protein chromatography surfactants and conditions for their use that favor protein solubilization without denaturation. Scattered evidence[5,6] suggests that non-ionic (more precisely, zero-net-charge) surfactants are less likely to denature proteins than are ionic surfactants. Empirical evidence also supports the idea that submicellar concentrations of surfactants are much less likely to denature proteins than concentrations above the critical micelle concentration[7]. It also seems likely that surfactants with a relatively high CMC are less likely to denature proteins than those with a low CMC.

Two surfactants that appear to meet the above requirements are *n*-octylglucoside and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS). The latter, a dipolar ion derivative of cholic acid, was chosen for our initial studies. Its structure suggests the existence of strong constraints on the modes of hydrophobic interaction with proteins, with less likelihood of strong and denaturing interactions. We believe that the rigidity of the steroid ring system of CHAPS, coupled with the placement of its three hydroxyl groups, limit the kinds of hydrophobic interactions available to the molecule. This is in contrast with the structure of a surfactant with a flexible alkyl moiety, such as the alkylglycosides and their structural analogues.

We chose to carry out our preliminary studies with a hydrophobic interaction column and isocratic elution. Preliminary experiments showed a slow re-equilibration of the column following a change of surfactant concentration. This reinforced our decision to use isocratic elution rather than gradient elution for our initial studies. We adjusted the range of k' values of a variety of proteins by appropriate choice of the ammonium sulfate concentration of the eluent.

EXPERIMENTAL

Materials

Amylase (*E. coli*) and ribonuclease A (bovine, Lot 55F-8210) were purchased from Sigma (St. Louis, MO, U.S.A.), hen egg white lysozyme (Lot 7069) from Miles Laboratories (PTY) (South Africa) via Miles Laboratories (Elkhart, IN, U.S.A.), and bovine pancreatic trypsin inhibitor (Lot 104F-8035) from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). These proteins were used without further purification. CHAPS was prepared according to Hjelmeland[8] and twice recrystallized from methanol. Ammonium sulfate (ultra pure grade) was obtained from Schwartz-Mann (Cambridge, MA, U.S.A.) All other reagents were of ACS-certified analytical reagent grade. High-purity HPLC-grade water was prepared with a purification system from Mar Cor Medical Services (Harleysville, PA, U.S.A.).

Methods

The chromatographic system consists of two Waters (Milford, MA, U.S.A.) M6000A pumps, a Water 660 solvent programmer, a Rheodyne (Cotati, CA, U.S.A.)

Model 7125 injection valve with a 20-μl sample loop, a Waters Lambda-Max Model 480 spectrophotometer, and a Hewlett-Packard (Avondale, PA, U.S.A.) 3390A reporting integrator. The chromatographic column was a No. 1830 PolyPROPYL A, 5 μm, 200 × 4.6 mm column from Custom LC (Houston, TX, U.S.A.). The packing consisted of porous silica beads, coated with a propylamido derivative of immobilized poly(DL-aspartic acid). The column was thermostatted with a circulating water jacket maintained at 30.0 ± 0.2°C.

Ammonium sulfate solutions were prepared to contain 0.02 *M* potassium phosphate buffer adjusted to pH 6.2. Two such solutions, of identical composition, except that one also contained 1.6 m*M* CHAPS, were employed in a series of experiments with CHAPS concentrations ranging from 0 to 1.48 m*M*. The proteins were dissolved in the mobile phase at a concentration of *ca.* 1 mg/ml and filtered through a Millipore (Bedford, MA, U.S.A.) HA (0.45 μm) filter. The flow-rate was 1 ml/min. Samples (20 μl) were injected manually by the filled-loop technique. Surface tension measurements were carried out with a Fisher Scientific (Pittsburgh, PA, U.S.A.) Autotensiomat.

RESULTS AND DISCUSSION

First, the isocratic elution of several proteins was carried out with several concentrations of ammonium sulfate and of the two surfactants. Under any particular set of conditions, retention times were reproducible, but no consistent pattern was seen. The retention time of proteins was found to be either decreased or increased by the addition of surfactant. Because the CMC generally decreases with increasing concentration of a salting-out electrolyte[9] we proceeded to study retention as a function of CHAPS concentration at fixed ammonium sulfate concentration. The concentration of surfactant was kept below its CMC (*ca.* 1.7 m*M* for the solvent in Fig. 1). Fig. 1 shows the results for three proteins.

Lysozyme, ribonuclease A, and trypsin inhibitor were well separated at an ammonium sulfate concentration of 1.42 *M*, the values of k'_0 being 26, 0.92, and 5.4. The retention times of those proteins were reproducible (triplicates) to *ca.* ± 1% over the concentration range of CHAPS employed (0–1.5 m*M*). No substantial band-broadening was seen as the CHAPS concentration was increased. Because the heterogeneity of the amylase sample was too great to allow interpretation of the chromatograms, the results will not be reported. Believing that it should be instructive to compare the trends of k' with CHAPS concentration, we have normalized the retention parameters of the three proteins in the function k'/k'_0, where the denominator is the value found in the absence of CHAPS. In contrast with our expectation of decreased retention, CHAPS induced increases in k'/k'_0 for trypsin inhibitor and lysozyme. Further, while RNase A is more readily eluted in the presence of CHAPS, the dependence of k'/k'_0 is more complex than a monotonic function. Thus, there is no simple relationship between surface tension (Fig. 1, bottom) and solvent strength. It could be argued that it would be more sensible to use the interfacial tension between the mobile and stationary phase for correlating with solvent strength. However, to obtain suitable interfacial tension measurements is a substantial challenge. A more tractable approach appears to lie in the study of the equilibria outlined in Fig. 2, which will be discussed below.

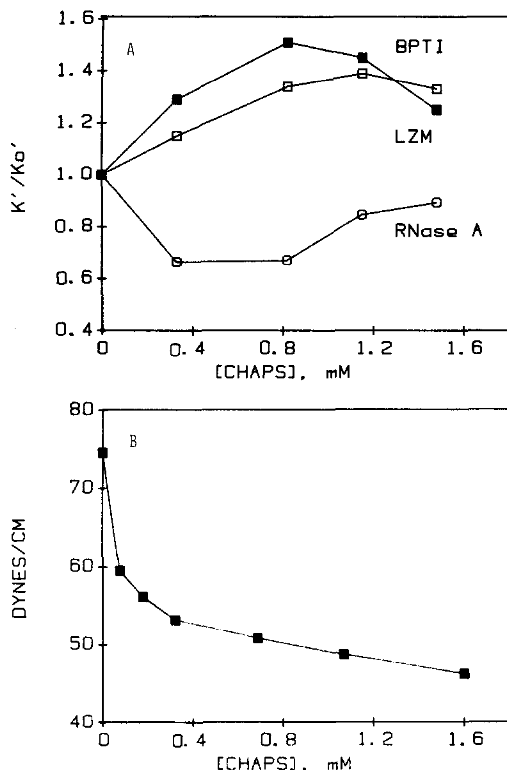

Fig. 1. (A) Effect of the surfactant CHAPS on retention of three proteins: ribonuclease A (RNase A), hen egg white lysozyme (LZM), and bovine pancreatic trypsin inhibitor (BPTI). HIC was carried out with 1.42 *M* ammonium sulfate, at 30.0 ± 0.2°C. The parameter k'/k'_0 is the ratio of the capacity factors determined in the presence (k') and absence (k'_0) of CHAPS. (B) Measured surface tension of the solvents used for the retention experiments of A.

Another expectation, that CHAPS-assisted elutions will change the selectivity, was realized. Although the normalized plots of Fig. 1 (upper) show similar trends for two of the three proteins, the effect of CHAPS is clearly different for each protein. These results support the idea that this surfactant can assist in chromatographic discrimination of similar proteins.

Fig. 2 is drawn to illustrate the principal processes in surfactant-mediated protein chromatography. This situation may be seen as formally analogous to proposals for ion-pairing mechanisms in the chromatography of small molecules[10]. However, some additional complexities should be noted. Even a small protein has the potential for associating with several ligands, in this case surfactant molecules. This means that formation of $Pr \cdot Srf_m$ is more accurately described by a series of equilibria involving the successive binding of 1,2 .... *m* surfactant molecules. Second, surfactant ligands will often form micelles. Third, the protein can undergo a profound structural disorganization, concomitant with the binding of *n* additional surfactant molecules. This structural disorganization is known as denaturation. Values of $(m + n)$ can be large, approaching one quarter of the number of amino acid residues in a polypeptide

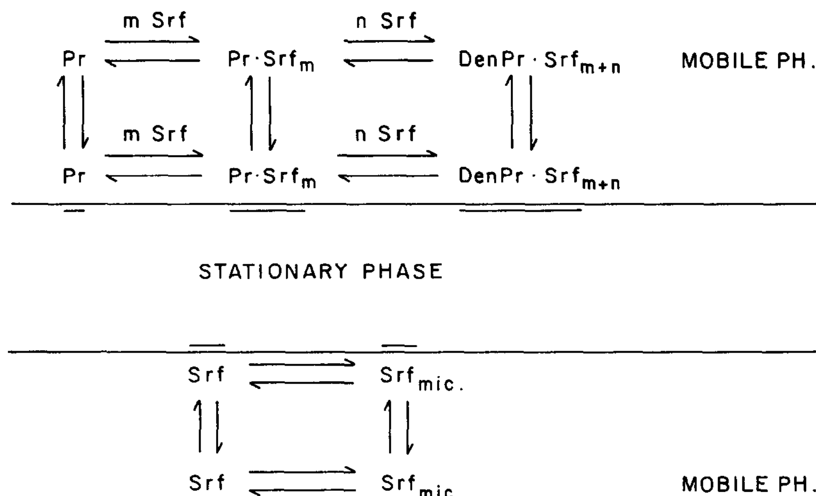

Fig. 2. Scheme for displaying some of the equilibria in surfactant-mediated protein chromatography. Species bound to the stationary phase are marked with double-line sub- or superscripts *e.g.*, Pr, Srf. Pr = native protein, Den · Pr = denatured protein, Srf = surfactant monomer, Srf_{mic} = micellar aggregate of surfactant. To reduce complexity, the drawing separates the lower set of surfactant interactions from those above the segment of stationary phase. No such physical separation is implied.

chain with the surfactant sodium dodecyl sulfate[11]. Fourth, in contrast with the general experience in the chromatography of small molecules, some of the equilibria in Fig. 2 may be sluggish. If the relaxation time for an equilibrium step is of the same order of magnitude as the residence time of the relevant species in the column, band-broadening or even substantial separation of slowly-equilibrating species may occur. For the sake of simplicity, we have not included in Fig. 2 the equilibria linked with H^+, nor the fact that the equilibria will depend to varying degrees on the concentration and type of other ions (salt effects). There is no doubt that many of the equilibria and kinetics in question will be responsive to changes in pH and salt. Likewise, a change in temperature can be expected to perturb some of these equilibria.

Examples of homogeneous solution equilibria corresponding to parts of Fig. 2 are readily available. Equilibrium optical measurements show the cooperative denaturation of several proteins with a series of alcohols commonly used as protein eluants in RPC[12]. Several such studies have appeared over the past 25 years[13–15]. Studies of the interactions between sodium dodecyl sulfate and over a dozen proteins show considerable variation in the binding isotherms of the proteins[16]. A noteworthy aspect of homogeneous solution denaturation is the slowness in approach to equilibrium that is often seen. Herskovits *et al.*[12] show some homogeneous solution examples of sluggish equilibria we mentioned above. Corbett and Roche[17] have shown that size-exclusion chromatography is capable of separating native and denatured myoglobin from an equilibrium solution of the two forms at low temperature. Benedek *et al.*[18] present a convincing case for the stationary-phase-catalyzed denaturation of RNase A. Parente and Wetlaufer[18] have observed partial separation of an equilibrium mixture of native and urea-denaturated chymotrypsinogen A, resolution increasing with the flow-rate.

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