INTRODUCTION

Increased availability of protein and peptide pharmaceuticals through recombinant DNA technology has produced, in turn, a demand for more sophisticated drug delivery systems able to maintain drug stability while improving efficacy and lowering toxicity. As a result, there have been numerous efforts made in altering the physical and chemical properties of proteins to make them more amenable to incorporation into drug delivery systems. Some researchers have focused on changing the primary structure of the protein in order to achieve improved performance. However, this approach generates a new compound with every mutation, each with its own efficacy and toxicity profile. In addition, mutagenesis would not allow for changes to be made after the active substance has been identified and characterized, making its application in a regulated industry improbable. Others have delved into the use of chemical modification, covalently attaching polymers, such as polyethylene glycol (PEG), to achieve conjugates with improved solubility and stability characteristics. This so-called pegylation method, while able to alter solubility and pharmacokinetic constants (1), often lacks specificity. In addition, it can lower long term stability (2), and the process is not reversible. In this review, we will describe a method for altering the solubility of a biopolymer (peptide, protein, or polynucleotide) without chemical modification. The method, termed hydrophobic ion pairing or HIP, is general, inexpensive and reversible. While using common detergents, it allows for the production of novel dosage forms, as well as provides real opportunities in nonaqueous enzymology, mass transport, and separation science. While this process can be and has been applied to a variety of ionic compounds, this review will focus on the use of HIP with biopolymers.

The high aqueous solubility of ionic compounds stems not simply from being charged, but from the fact that the counter ions tend to be small, hard (using hard and soft acid-base terminology), and easily solvated by water. If one was to replace the counter ion with a species of similar charge but less easily solvated, it is expected that the aqueous solubility would decrease. The HIP process involves stoichiometric replacement of polar counter ions (e.g., chloride, acetate, nitrate, etc.) with an ionic detergent of similar charge (see Figure 1). In much of the work to date, HIP has employed anionic detergents replacing these common anions, although replacement of cations with cationic detergents is just as feasible, but with greater limitations, such as the increased toxicity of cationic detergents and the difficulty in oblation of competing positive charges on proteins due to the high pKa of arginine and lysine side chains.

The ability of low levels of anionic detergents to reduce the aqueous solubility of proteins has been known for some time (3–5). However, until our work, it was not appreciated that the HIP process produced a concomitant increase in solubility in organic media. Furthermore, for many proteins, this dissolution in organic solvents occurs with retention of native-like structure and maintenance of enzymatic activity. Consequently, it is now possible to examine the structural integrity and solution behavior of proteins in nonaqueous environments without chemically modifying the protein. The same concepts can be
Hydrophobic Ion Pairing

\[ \text{NH}^+ \text{X}^- \rightarrow \text{anionic detergent} \rightarrow \text{NH}^+ \text{detergent} \]

\[ X = \text{Cl, OAc, NQ, etc.} \]

HIP COMPLEX FORMATION

Fig. 1. General scheme for formation of a hydrophobic ion paired complex.

applied to polynucleotides, using cationic detergents to replace counter ions, such as sodium and potassium. These HIP complexes display increased ability to cross membranes and solubility in organic solvents as nonpolar as methylene chloride.

PARTITIONING AND SOLUBILITY PROPERTIES OF HIP COMPLEXES

It has been widely recognized for some time that low levels of ionic detergents, such as sodium dodecyl sulfate (SDS), can lead to partitioning of proteins (3–5). These interactions should not be confused with the behavior of proteins in the presence of high concentrations of SDS, as used in gel electrophoresis (6). At higher concentrations, aqueous solubility is regained, presumably via micellar solubilization. These micelles are also capable of dispersing in a variety of organic compounds by inversion and formation of reverse micelles. Moreover, there appears to be an intermediate concentration range in which the SDS molecules can completely coat the surface of a protein (7,8). Under these conditions, the SDS to peptide ratio (on a molar basis) is 150–500:1. The complexes behave similarly to proteins in reverse micelles, except that the amount of unbound water in the micelle is significantly decreased (“collapsed” micelle). The HIP phenomenon occurs only in this low concentration regime; in all of the HIP complexes studied to date in our laboratory, the amount of anionic detergent required is approximately stoichiometric with the number of basic groups in the polypeptide. Therefore, the SDS to polypeptide ratio is about an order of magnitude less than for “coated” proteins or “collapsed” micelles, and care is taken to keep the concentrations well below the critical micelle concentration (cmc) for SDS under these conditions.

At the low SDS concentrations employed for HIP, the types of interactions are in a large part coulombic in nature, whereby one single positive charge on the protein interacts with one molecule of SDS. This is supported by the fact that partitioning of proteins using small amounts of SDS is almost always more efficient below the isoelectric point of the polypeptide. A study of the binding of azo dyes to gelatin using ultrafiltration by Gautam and Schott illustrates the forces involved in hydrophobic ion pairing (9). Binding isotherms were determined below the \( pK_a \) of the gelatin for sulfonated and carboxylated dyes of various sizes. Several conclusions drawn from this study may be highlighted to show the nature of the interactions. The isotherms have two linear segments, where the initial segment starts at the origin and abruptly changes to a horizontal plateau. The plateau approached stochiometric neutralization of basic groups in the gelatin in the case of sulfonated dyes at pH 5.

Presence of 0.15 M ammonium acetate lowered both initial slope of the isotherm and the plateau value, indicating that the acetate anions were having a coulombic screening effect upon the binding. Thermodynamic calculations indicate that ionic detergent binding is mainly enthalpy driven, despite negative entropy values, which is characteristic of coulombic interactions. Adachi and Harada noted the same type of phenomenon for cytochrome c (10). Cytochrome c was titrated with the anionic surfactant, Aerosol OT (AOT), a dioctylsulfosuccinate, at neutral pH. Some noteworthy observations were made. First, with no salt present, cytochrome c will completely precipitate at an 8:1 AOT:cytochrome c ratio, which is the overall charge of the protein at neutral pH. Second, addition of NaCl causes a shielding effect, with more AOT required for precipitation as salt concentration is increased. Third, the extent of precipitation decreases dramatically as the pH is adjusted upward toward the pI of the protein, which is 10.1. Precipitation with low amounts of SDS has been observed for protein pharmaceuticals as well. Arakawa et al. noted that precipitation of IL-2 occurs at intermediate concentrations of SDS which approximately corresponds to neutralization of the 14 basic residues of IL-2 (11). Lower molar ratios produced little or no precipitate, while large ratios much above the cmc resolubilized the precipitate, most likely due to micellar solubilization.

Although it has long been known that small amounts of SDS may cause precipitation of a protein, the hydrophobic nature of this precipitate had not been investigated until Powers et al. found that this precipitate can be partitioned into the less polar solvent, 1-octanol (12), as measured by the 1-octanol-water partition coefficient, \( P \). For the zwiterions, Gly-Phε, at neutral pH, the free carboxyl group remains negatively charged, and only a mild enhancement of log \( P \) is seen, but when the pH is adjusted to 3, the complex readily forms and log \( P \) is dramatically increased. Chemically blocking the C-terminus, forming Gly-Phε-NH2, produces an enhancement in partitioning similar to that of the low pH sample, again by removing the competing negative charge. They also investigated the partitioning of larger peptides, including neurotensin, AVP, and bradykinin into 1-octanol as a function of SDS concentration. At stoichiometric concentrations of SDS:charged groups, visible precipitates were formed, but these could readily be partitioned into the organic phase. The apparent partition coefficient increased by 2–4 orders of magnitude for these peptides upon the addition of simple anionic detergents, such as SDS.

Similar observations were made with the small protein, insulin. Insulin is a polypeptide of 51 amino acid residues, containing six basic groups, and six acidic groups. Near its isoelectric point of 5.5, insulin is quite insoluble, but adjustment of the pH to 2.5 protonates the acidic groups, and the only charged groups remaining are the six basic residues. Matsuura et al. examined the partitioning behavior of the HIP complex of insulin at pH 2.5 (13). The precipitation of the insulin reached a maximum at a 6:1 SDS:insulin ratio, as did the partition coefficient between water and 1-octanol, with the \( P \) value increasing by 3000-fold upon addition of SDS. The soluble SDS-insulin complex in 1-octanol was observed by both near and far-UV circular dichroism (CD) spectroscopy. The protein was found to contain nearly identical \( \alpha \)-helix content as that of the native enzyme. The near UV CD spectrum, indicative of tertiary structure, shows a similar, but less intense