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Review

Hydrophobic Ion Pairing: Altering the Solubility Properties of Biomolecules

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The high aqueous solubility of ionic compounds can be attributed to the ease of solvation of the counter ions. Replacement of the counter ions with ionic detergents dramatically alters the solubility properties of the molecule. Not only does the aqueous solubility drop precipitously, but the solubility in organic phases increases as well. Consequently, the partition coefficient changes by orders of magnitude. This ion pairing phenomenon, which we term hydrophobic ion pairing (HIP), has been extended to polyelectrolytes, such as proteins and polynucleotides. These materials form HIP complexes that dissolve in a range of organic solvents, often with retention of native structure and enzymatic activity. The HIP process has been used to purify protein mixtures, conduct enzymatic reactions in nonaqueous environments, increase structural stability, enhance bioavailability, and prepare new dosage forms.

KEY WORDS: ion pairing; ionic detergents; solubility; controlled release; proteins.

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

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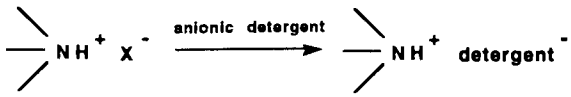

X = Cl,OAc,NO_3,etc.

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 precipitation 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 precipitation 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 carboxyated 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 stoichiometric 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 zwitterion, Gly-Phe, 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-Phe-NH_2, 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 α-helix content as that of the native enzyme. The near UV CD spectrum, indicative of tertiary structure, shows a similar, but less intense

spectra than that of hexameric Zn-insulin in water, suggesting a lower state of aggregation. In addition, the SDS-insulin complex was shown to be soluble (>0.9 mg/mL) in a number of organic solvents, including ethanol, N-methylpyrrolidinone, trimethyl phosphate, and 2-propanol, all of which are water miscible. Unfortunately, the complex was reported as insoluble in other water-miscible solvents such as dioxane and acetonitrile, as well as water-immiscible solvents such as ether, methylene chloride, and hexane. It appears that the SDS-insulin complex may be solubilized in relatively high polarity solvents, but not in ones with lower dielectric constants.

The work of Matsuura et al. (13) demonstrated that peptides and proteins containing only positive charges could be ion paired with SDS and dissolved in high dielectric organic media. However, many proteins may be unstable at the low pH necessary to protonate a majority of the acidic groups, but it has been found that elimination of all competing charges is not necessary. For example, subtilisin BPN' can be ion-paired with SDS by simply dissolving the enzyme to >1 mg/mL at a pH of 4.5–5.5 in a solution containing a small amount of $CaCl_2$ (1–2 mM), and adding an aqueous aliquot of SDS at a molar excess of about 50:1 (14). This causes precipitation of the protein, and the precipitate may be isolated by centrifugation, and subsequently redissolved in water miscible organic solvents such as short chain alcohols, trimethylphosphate, N,N-dimethylformamide (DMF). After redissolution in the organic solvent, the overall structure remains intact, as determined by CD.

Certainly, it would be desirable to expand the use of HIP to low dielectric milieu. This was reported for the first time in 1994 when Paradkar and Dordick reported that α-chymotrypsin could be extracted from an aqueous solution into isooctane by ion-pairing it with AOT (15). They were able to obtain concentrations in the mg/mL range, with a surfactant:protein ratio of only 30:1, a value too low for micellar incorporation. In addition, a dynamic light scattering experiment showed a mean molecular diameter of 6.8 nm, consistent with a complex of a single α-chymotrypsin molecule ion-paired with AOT (16).

Oligonucleotides may likewise be transferred to organic media via hydrophobic ion pairing. For oligonucleotides, the negatively charged phosphodiester bond can be ion paired with a cationic detergent. For example, using the dodecyl ester of arginine, we were able to increase the log P of adenosine triphosphate (ATP) from –3.2 to 1.1 in 1-octanol (unpublished results). As with proteins, transfer of polynucleotides into lower dielectric media may require a more hydrophobic tail on the detergent than a single alkyl chain, and a number of cationic lipids are commercially available. However, few of these reports discuss partitioning into organic solvents (17,18), and complexation rarely uses ion pairing terminology (18–20). Yet, there these DNA complexes do appear to enhance transfection (19,20), presumably by increasing permeability across membranes, a phenomenon described in Section V.

HIP IN NON-AQUEOUS ENZYMOLOGY

Since the discovery that enzymes may function in non-aqueous environments, there has been an increasing interest in utilizing enzymes in low water systems to conduct transformations difficult or impossible to achieve in an aqueous system. Since few proteins are soluble or stable in organic solvents, various approaches for creating an active and stable biocatalyst system have been attempted. Some of these techniques include simple suspension of a lyophilized powder, extensive chemical modification such as attachment of polyethylene glycol, heterogeneous carrier systems (such as microemulsions, reverse micelles, etc.), and mutagenesis of the enzyme. None of the aforementioned techniques truly approximates solubilization of the native enzyme in the organic medium.

As mentioned above, Dordick and Paradkar successfully demonstrated the application of the HIP technique for true solubilization of enzymes non-aqueous milieu, and measured the activity of α-chymotrypsin in isooctane (16). While less active than the enzyme in aqueous solution, the HIP complex does exhibit a significant level of activity. The same has been found for subtilisins (14,21). In either case, the level of activity was two to three orders of magnitude greater than lyophilized preparations suspended in organic solvents, the most common method of preparing enzymes for use in organic solvents. Clearly, HIP complexes represent a soluble alternative to less active suspensions.

Upon ion-pairing of the enzyme and its transfer to an organic solvent, the enzyme can be examined for structural integrity using spectroscopic techniques, such as CD or fluorescence spectroscopy (14,21). Retention of native-like structure should correspond to an increased level of enzymatic activity. This has been recently demonstrated with lyophilized preparations of HIP complexes. Ion pairing of subtilisin with SDS led to greater structural integrity for powders suspended in hexane, resulting in increased activity (22). Furthermore, subtle changes in the solvent composition may significantly affect the structure of the HIP protein complex. The utility of this approach can be seen in the case of α-chymotrypsin, where it was found to be native-like in non-polar solvents such as isooctane, decalin, and carbon tetrachloride. However, it was discovered that the more 'polar' methylene chloride left the enzyme with little or no organized structure as determined by CD. Indeed, the sample in methylene chloride showed less than 10% than that of the enzyme in carbon tetrachloride (14).

Dordick and co-workers have continued to use HIP to prepare a variety of enzyme systems (16,23–25) and conduct catalytic transformations. Recently, they reported using soluble HIP complexes to synthesize polymeric matrices with enzymes covalently attached, producing catalytically active polymers (24). The ability to prepare soluble active enzymes allows greater flexibility in using enzymes in nonaqueous environments, as well as higher levels of activity. It has now been found in a number of studies, soluble HIP complexes have greater activity by two to three orders of magnitude than the same enzyme suspended in the organic solvent (14,16,21,25).

SELECTIVE PRECIPITATION OF PROTEINS USING HIP

The ability of proteins to bind ionic detergents electrostatically raises the possibility that in a mixture of two proteins, one might be able to selectively bind to a highly charged species while not affecting a nearly neutral protein. Once the detergent binds, the solubility of that protein would drop, and selective precipitation would ensue. Therefore, HIP might have some value in pre-column purification of protein mixtures. This

would have a real advantage for formulations containing high concentrations of human serum albumin (HSA), added as a stabilizer. Many such formulations are currently on the market. The presence of large excesses of HSA makes it difficult to characterize the chemical integrity of the active ingredient, as it is usually present at concentrations up to 50-fold lower than HSA. The ability of HIP to selectively precipitate a protein pharmaceutical from a formulation with a large excess of HSA was demonstrated by Meyer et al. using interleukin-4 (IL-4) and HSA (26). The IL-4 was precipitated without affecting HSA, resulting in a pellet which was purer than that obtained by gel filtration chromatography. The degree of enrichment in IL-4 was more than 2000-fold.

ENHANCED TRANSPORT OF HIP COMPLEXES

Certainly, one attractive possibility for the use of HIP is to enhance transport of biomolecules across membranes. However, one must remember that HIP complexes are susceptible to dissociation if there is a sufficient concentration of polar ionic species present. As a result, the use of HIP complexes in the systemic circulation would be small. In fact, some HIP complexes have been shown to provide little, if any, increased transport under such conditions (27,28). Yet, HIP complexes have been noted to retain some degree of association, even under challenging conditions. It has been observed that association of cationic detergents with a DNA plasmid increases the level of transfection in cell culture, even without so-called helper lipids (unpublished results). This suggests that under conditions of restricted fluid and electrolyte flow, HIP complexes may provide some increased benefit by improving transport across biological barriers. For example, one report indicates that specific detergent-drug interactions enhances transport across the skin (29). Similar studies have been published regarding increased bioavailability when drugs are given rectally (30) and by inhalation (31) in the presence of fatty acid salts. Whether or not this is due to increased lipophilicity due to ion-pairing is unclear, but it is one possible mechanism. In fact, there have been reports of HIP complexation formation improving the pharmacokinetics of basic drug compounds (32,33). These reports suggest that the HIP complex may exist for an extended period of time, even in the gastrointestinal tract.

The observation that addition of ionic detergents accelerates transport in cell culture models of the gastrointestinal tract supports the hypothesis that HIP complexes exist, at least partially, even in relatively high ionic strength media (34–36). This may be the reason that some increased level of transfection is observed in cell culture when ion paired complexes are formed (19,20). In addition, Bromberg and Klibanov have shown that formation of a HIP complex is essential for transport of macromolecules across liquid membranes, and that the rate of transport was proportional to the partitioning of the HIP complex (17). The phenomenon was observed both for proteins and nucleic acid polymers.

ENHANCED STABILITY OF HIP COMPLEXES

Another striking feature of HIP complexes dissolved in organic solvents is that they can exhibit enhanced stability compared to that in aqueous solution. One way this is manifested is by increases in thermal melting temperature, or T_m. In the case of the SDS-insulin complex in 1-octanol, the apparent T_m increases from a value of approximately 65°C in water to 115°C in the organic solvent (13). Similarly, the complex of AOT with α-chymotrypsin dissolves in decalin. Upon heating, the far UV CD region (indicative of secondary structure) is nearly identical to the native protein, and no evidence is observed for unfolding, even after heating at 110°C for more than 1 hour (21). By contrast, the AOT-subtilisin BPN′ complex is reported to lose over 98% of its enzymatic activity after 30 minutes at 70°C (23). However, this same report states that if the enzyme is cooled to 25°C, a significant fraction of the activity is recovered. Furthermore, if the heat treated enzyme is extracted back into water a majority of the activity is recovered.

It is known that for dried proteins, the T_m increases dramatically as water content drops (37,38). Water content is a function of water activity of the system, and in the case of an ion-paired organic phase, the water activity would be close to unity unless steps were taken to dry the organic solvent, such as Dordick and coworkers routinely do by bubbling dry N_2 through the organic solvent after ion-pairing. Unfortunately, at this point it is unknown if the effect of changing the water activity of the system would have upon thermal stability of ion-paired materials compared to dried powders or dry powders suspended in organic media. In the case of SDS-insulin and 1-octanol mentioned earlier where the T_m was determined in water saturated octanol, which contains 1.7 M water, the measured T_m was 50°C higher for the insulin despite the high water content and activity.

In polar, water-miscible solvents, such as THF, protein suspensions are known to lose activity. One proposed mechanism for the inactivation is that the solvent strips off an essential hydration layer (39). It may be that HIP complexes of proteins in high dielectric media may also unfold by the same mechanism. Dordick and coworkers reported that the half life of ion-paired subtilisin BPN′ is less than one minute in THF (23). Other solvents may not necessarily be as detrimental to a protein as THF. For example, we found that subtilisin retains native-like secondary and tertiary characteristics when dissolved in short chain alcohols. Furthermore, in comparing stability of subtilisin in ethanol to that of water over the period of one week at 25°C, the water sample retains only 2% residual activity, while that in ethanol still retains approximately 60% of its original specific activity (15). Although the half-life of subtilisin is markedly increased in ethanol, the AOT-subtilisin complex in isooctane has a much greater half life, namely 1400 hours (23).

Formation of a HIP complex has also been found to stabilize enzymes in the lyophilized state (22). Whereas HIP complexes were found to have little structural difference in the solid state from the enzyme itself, there was a significant change when the powders were suspended in isooctane. Structural rearrangement occurs upon suspension in the organic solvent, even though it is quite nonpolar. The HIP complex of subtilisin retains more native-like structure in the isooctane, while the non-ion-paired form undergoes a greater degree of unfolding and a lower level of activity by about 15-fold.

USE OF HIP TO PREPARE COMPLEX DOSAGE FORMS

The work of Adjei and co-workers at Abbott Laboratories on LHRH compounds was to develop better dosage forms for

alternative routes of administration (nasal, pulmonary, etc.), and a number of patent applications were filed (40–42). However, none of the patent applications described the formulation efforts in terms of ion pairing and altering of solubility in organic phases. Only in subsequent publications by Manning and co-workers (12,13) and then by Adjei et al. (43), did a picture of general ion pairing phenomenon emerge. Soon after, Paradkar and Dordick described the use of even more hydrophobic detergents, such as AOT, to solubilize HIP complexes in nonpolar solvents such as alkanes (13,14). While the HIP process could have been relegated to a physical pharmacy curiosity, it is now apparent that the process provides some unique opportunities in enhancing drug delivery. Certainly, lowering aqueous solubility has some possible applications in taste masking. Also, this allows facile formation of suspensions, which should exhibit a controlled release profile based on the rate of ion exchange and resolubilization of the drug compound. However, the most promising application has been to couple the HIP process to advances in supercritical fluid technology.

The most common approach in the field of controlled release drug delivery is to distribute a pharmacologically active agent in a polymeric matrix. The most widely used class of polymers has been the biodegradable polyesters, poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their co-polymers (PLGA). Unfortunately, these polymers are not water-soluble, while most therapeutic agents are only readily soluble in aqueous solution. This is especially true for ionic compounds. This poses a significant challenge: how does one incorporate water-soluble drugs into water-insoluble polymers efficiently and evenly? To date, the approaches have focused on heterogeneous systems: emulsions, suspensions, and melts. With HIP it is possible to alter the solubility profile of an ionic compound, and render it soluble in the same solvents that dissolve the biodegradable polymers. Once the polymer and drug are dissolved in the same solvent, the mixture can be processed. The process of choice in our laboratories has been precipitation with a compressed antisolvent (PCA), where the antisolvent is a supercritical fluid. The combination of PCA and HIP has led to the facile production of drugs incorporated into microspheres of biodegradable polymers (44). Without HIP, the PCA process can only be used with hydrophobic compounds that dissolved directly in nonpolar organic solvents, and it is quite inefficient (45). The use of HIP expands our ability to incorporate ionic compounds into PLA/PGA materials, as well as other polymeric matrices.

SUMMARY

With the HIP process, it is possible to alter the solubility properties of any ionic compound, even polyelectrolytes, such as peptides, proteins, and polynucleotides. Formation of a HIP complex lowers aqueous solubility, while raising the solubility in organic media, often with retention of structure and function. It has been shown that HIP complexes display enhanced ability to cross biological barriers and increased stability. In addition, the HIP process can be used to separate proteins of different isoelectric points, prepare nonaqueous solutions of active enzymes, and provide an avenue for making new controlled release formulations.

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