Protein–solvent interaction

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Abstract

Protein folding and assembly can be manipulated in in vitro systems by co-solvents at high concentrations. A number of co-solvents that enhance protein stability and assembly have been shown to be excluded from the protein surface. Such co-solvent exclusion has been demonstrated by dialysis experiments and shown to be correlated with their effects on protein stability and assembly.

Keywords Protein folding and assembly · In vitro systems · Co-solvents · Preferentially excluded co-solvents · Dialysis equilibrium

Introduction

The folding and assembly of proteins are two essential processes for life. In in vivo systems, protein folding and assembly are mediated and regulated by specific ligands. In in vitro systems, protein folding and assembly can be manipulated by non-specific co-solvents. A variety of co-solvents have been shown to enhance the folding and stability of proteins and the assembly of such macromolecules as microtubule (Lee and Timasheff 1977), actin (Kasai et al. 1965; De La Cruz and Pollard 1995), tobacco mosaic virus (Lauffer and Stevens 1968) and flagellin (Wakabayashi et al. 1969). These co-solvents include salts, sugars, polyols, amino acids and amines. How do they affect protein folding and assembly? The co-solvents described above exert their effects at high concentrations, reflecting their weak interactions with proteins. Weak interactions have been measured by several thermodynamic techniques (Pittz and Timasheff 1978; Lee et al. 1979; Zhang et al. 1996), one of which is dialysis equilibrium. Dialysis equilibrium measurements have shown that the co-solvents that enhance protein folding and macromolecular interactions are excluded from the protein surface.

Dialysis measurements of co-solvent interaction have been mostly performed in S.N. Timasheff's laboratory, where I spent 5 years as a post-doctoral researcher (Lee et al. 1979; Timasheff 1992, 1993, 1998, 2002). As the resultant observations are a balance between binding and exclusion of co-solvents, the measured values are termed “preferential interactions,” meaning that it is a measurement of preference of the protein molecule for water and co-solvent (Timasheff and Inoue 1968; Timasheff 1992, 1993, 1998, 2002). When the preference is for water, the result is preferential hydration or preferential exclusion of the co-solvent. When the preference is for the co-solvent, the result is preferential co-solvent binding. Preferential co-solvent exclusion or preferential hydration has been successfully used to explain the effects of the co-solvent on protein stability and assembly (Timasheff 1992, 1993, 1998, 2002). The concept of preferentially excluded co-solvents has led to their applications for cryopreservation (Carpenter and Crowe 1988; Arakawa et al. 1990, 1991) and chromatography of pharmaceutical proteins (Lee et al. 2012; Gagnon et al. 2014; Yoshimoto et al. 2015). Here I review the excluded co-solvents and their effects on proteins in solution.

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Co-solvent effects

Protein stability

A large number of preferentially excluded co-solvents have been shown to stabilize proteins and also to decrease protein

solubility, leading to precipitation or crystallization of the protein. More importantly, some of them, including sugars and polyols (e.g. sucrose, trehalose, mannitol, sorbitol) play a critical role in the development of pharmaceutical protein therapeutics. It is customary to store reagent proteins in frozen state to minimize degradation. However, the freezing treatment itself is often detrimental to proteins. A range of widely different compounds have been used to reduce freezing damage to the proteins (Carpenter and Crowe 1988; Arakawa et al. 1990, 1991). It has been proposed that all of these compounds share a common property in that they are all excluded from the protein surface; namely, they show preferential exclusion from the native protein surface. Some of them are also used by certain organisms living in salty environments and are called “compatible solutes” or “osmolytes” (Yancey et al. 1982; Arakawa and Timasheff 1985b). They raise the osmotic pressure of the cytoplasm to protect cells against salty environments and do not bind to the cellular macromolecules. Nevertheless, they do enhance protein stability. A typical co-solvent for this is trimethylamine N-oxide (TMAO) (Arakawa and Timasheff 1985a, b) or trehalose (Mackenzie et al. 1988), both of which are excluded from the protein surface.

Chromatography

Ion exchange and hydroxyapatite chromatographies are normally operated under low to moderate salt concentrations. These chromatographies are often used as a polish step to remove aggregates of the product, i.e. the product-related materials, in the late stage of purification. Normally, aggregates bind more strongly to these resins than the monomers. It has been found that an excluded polymer, polyethylene glycol (PEG), enhances the binding of both monomers and aggregates, more so for the aggregates, leading to a greater separation between the monomers and aggregates than in the absence of PEG (Gagnon 2008; Yoshimoto et al. 2015). Such an enhancement of protein binding has led to a novel chromatography, steric exclusion chromatography (SXC) (Lee et al. 2012; Gagnon et al. 2014), that uses a unmodified bead or a monolith and a high molecular weight PEG, such as, for example, 6000 molecular weight PEG at 6%. This PEG solution generates high viscosity, such that a conventional column bead with pores may not work. To overcome this problem, a monolith column or a magnetic bead is used. Protein molecules accumulate or crystallize and form a multiple layer on the top surfaces in the presence of PEG. A decreasing PEG concentration is used to dissociate and elute the bound macromolecules. As PEG is more effective for aggregates, it should enhance the binding of larger solutes more effectively (Snyder et al. 2009). Thus, SXC has been successfully used to purify viruses and immunoglobulin M, which is a pentamer antibody (Lee et al. 2012).

Protein assembly

It is relevant to comment on protein solvent interactions in relation to solvent effects on the assembly of proteins as one of the topics of the papers contributing to this special issue. It is well documented in *in vitro* studies that assembled structures are stabilized (or destabilized) by co-solvents: for example, the stabilization of microtubules by 2-methylpentane-2,4-diol (Sheterline et al. 1977), glycerol (Erickson 1974a, b; Shelanski et al. 1973; Na and Timasheff 1981), dimethylhexandiol (Rebhun et al. 1974) and dimethyl sulfoxide (DMSO) (Rebhun and Sawada 1969); the stabilization of actin by sucrose (Kasai et al. 1965); and the stabilization of tobacco mosaic virus (TMV) by sucrose (Lauffer and Stevens 1968). Self-assembly of monomeric actin, flagellin, certain viral proteins and tubulin follows a two-step helical polymerization mechanism proposed by Oosawa and Kasai (1962) and Kasai (1969). The association constant, K , of the monomers to existing polymers is related to the concentration of the monomers, C_m , in equilibrium with the polymers as $K = (1/C_m)$ and can be modulated by co-solvents. Glycerol (Lee and Timasheff 1977), PEG (Lee and Lee 1979) and DMSO (Algaier and Himes 1989) have been shown to increase the K of biological polymers, including microtubules. In the absence of stabilizing additives, the equilibrium monomer concentration of tubulin was 8 mg/ml, meaning that the tubulin concentration needs to be at least 8 mg/ml before microtubule formation is observed. Microtubules are formed *in vivo* at much lower protein concentrations due to the binding of many microtubule stabilizing proteins (Sloboda et al. 1976), one of which is called tau (Sánchez et al. 2001). Extensive phosphorylation of tau is known to be associated with Alzheimer's disease and tauopathies (Iqbal et al. 2010; Ramkumar et al. 2017). Unique salts of organic ions, such as piperazine- N,N' -bis(2-ethanesulfonate) (PIPES), sodium glutamate, 2-(N -morpholine)ethanesulfonate (MES) and creatine phosphate, enhance the *in vitro* self-assembly of tubulin concentration in a dependent manner (Hamel and Lin 1981a, b; Hamel et al. 1981, 1982). Interestingly, in these studies these organic salts resulted in formation of not only microtubules but also of other polymer structures, indicating that they also affect the off-pathway self-association that generates ring structures. Unlike glycerol, these salts increase the ionic strength of the solution and thereby may alter electrostatic interactions between tubulin monomers that play a role in specific intermolecular interactions.

Actin polymerization is known to be strongly dependent on temperature and salt concentration. Kasai (1969) examined the effects of temperature and KCl on actin polymerization and found that both increasing temperature and KCl concentration enhanced actin polymerization, reaching a maximum at ~0.1 M at any temperature between 0 and 30 °C, followed by concentration-dependent decrease in polymerization. Nagy

and Jencks (1965) examined extensively the effects of co-solvents on actin assembly. These authors used solution viscosity as a measure of F-actin concentration. Table 1 lists the concentration of salts required to cause 50% reduction in viscosity of F-actin solution.

In these studies, potassium thiocyanate (KSCN) and sodium percholate (NaClO_4), known as salting-in salts, were observed to induce the depolymerization of F-actin at relatively low concentrations, while a relatively high concentration of sodium nitrate (NaNO_3), known to be neutral in salting-in or salting-out effects, was required for the same effect. To the contrary, salting-out sodium and ammonium sulfate, sodium phosphate (neutral pH) and potassium acetate (KCH_3COO) all resulted in no depolymerization or caused precipitation of F-actin at the highest concentration tested. These authors also examined various organic compounds and solvents, listed in Table 2. The protein-denaturing organic compounds formamide and urea were found to be effective in F-actin depolymerization. Guanidine hydrochloride (GdnHCl) caused denaturation at higher concentrations. More intriguing were the effects of methanol and ethanol at 4 M, which resulted in no depolymerization. These two organic solvents appear to act as a protein precipitant at these relatively low concentrations, which are insufficient to cause denaturation.

Polymeric flagella depolymerize into a subunit flagellin when treated with heat or acid. Once depolymerized, polymerization of flagellin is extremely slow at low protein concentrations. Wakabayashi et al. (1969) observed that salting-out salts, such as sodium and ammonium sulfate, sodium citrate, Na_2HPO_4 , accelerated flagella formation. In the presence of 1.2 M sulfate salts, the size of flagella filaments was reduced and the resultant short filaments precipitated. While NaCl was ineffective, salting-in divalent cation salts, such as MgCl_2 and Mg_2SO_4 , were also ineffective and instead resulted in depolymerization of existing flagella filaments (Wakabayashi et al. 1969).

TMV is composed of a single protein subunit and viral RNA. In the absence of the RNA, the protein assembles into helical oligomers and polymers with different architectures, depending on the solvent conditions (Durham 1972; Klug

Table 2 The effects of denaturing co-solvents on actin polymerization

Solvent	Concentration required for 50% reduction in viscosity of F-actin solution
Formamide	1.3 M
Urea	3.4 M
Guanidine hydrochloride (GdnHCl)	- ^a
Methanol	No change (4 M)
Ethanol	No change (4 M)

^a No depolymerization at 0.28 M. Precipitation and denaturation at concentrations of > 0.3 M

1999). Below pH 7, this viral protein self-assembles into a helical structure similar to that of microtubules, F-actin and flagella, independent of ionic strength. Above pH 6.5–7.0, a long single helix is no longer formed. Instead, the protein assembles into small oligomers, single-layer or stacked disks depending on the pH and ionic strength, all of which have a shape of closed rings (not a helical form). These disks aggregate at increasing KCl or $(\text{NH}_2)\text{SO}_4$ concentration.

Preferential interaction

The concept of preferential interaction was briefly described earlier in this article. It is a measure of the difference in co-solvent concentration between the bulk phase and the protein solution that are in dialysis equilibrium. A diagram of a typical case of preferential co-solvent interaction is shown in Fig. 1. Figure 1a depicts the condition in which the co-solvent concentration is identical inside and outside the dialysis membrane; namely, there is no difference in co-solvent concentration in the vicinity of protein surface compared to the concentration away from the protein surface. This does not necessarily mean that the protein molecule does not bind the co-solvent, but simply that the overall co-solvent concentration does not differ between the protein surface and the bulk phase. Figure 1b illustrates the case where the co-solvent concentration is greater inside the membrane; namely, there is excess co-solvent molecule relative to its concentration in the bulk phase. Such an excess concentration may encompass strong (e.g. stoichiometric) binding due to the subtle influences of the protein surface on the activity coefficient of the co-solvent molecules, leading to weak (e.g. transient) bindings. This represents the case of preferential co-solvent binding, as seen with various protein denaturants. The last case is depicted in Fig. 1c, in which there are no co-solvent molecules in the protein domain. This is an extreme case of total exclusion of the co-solvent molecules from the protein surface. We and others have observed that many co-solvents of widely different chemical structures show this exclusion behavior, such as sugars, polyols, amino acids, amines and salting-out salts

Table 1 The effects of co-solvents on actin polymerization

Salt	Concentration required for 50% reduction in viscosity of F-actin solution
KSCN	0.26 M
NaNO_3	1.8 M
NaClO_4	0.35 M
Na_2SO_4	No change
Na_2HPO_4	No change
KCH_3COO	No change
$(\text{NH}_4)_2\text{SO}_4$	No change

Fig. 1 Preferential interaction of protein with co-solvents. **a** Co-solvent concentration is identical inside and outside the dialysis membrane, i.e. a balanced condition. **b** Co-solvent concentration is higher inside the membrane, i.e. there is excess co-solvent molecule relative to its concentration in the bulk phase. This depicts the case of preferential co-solvent binding.

c No co-solvent molecules in the protein domain, i.e. an extreme case of total exclusion of the co-solvent molecules from the protein surface, resulting in preferential co-solvent exclusion. Square Protein molecule, circle co-solvent, dashed square dialysis membrane.

(Arakawa and Timasheff 1982a, b, 1985a, b; Gekko and Timasheff 1981; Lee and Timasheff 1977, 1981; Lee and Lee 1979). A few different mechanisms have been proposed to explain why they are excluded. Asakura and Oosaw (1954) proposed a “depletion effect” to explain the observation that polymers enhance actin polymerization; namely, the polymers are depleted from the protein surface, which is energetically unfavorable. Such unfavorable free energy is reduced when protein molecules self-associate, which reduces the surface area per protein molecule. The above depletion mechanism was expanded by A.P. Minton’s group as “excluded volume effect” (Minton 1980) and extended into low molecular weight co-solvents by Sheawin and Winzor (1988) and Schellman (2003).

A different theory, called “attraction pressure,” was proposed by Traube (1910). He proposed cohesive force of co-

solvents on water molecules, a force that is related to an increase in the surface tension of water. Namely, Traube showed that an increase in surface tension of water by the co-solvents correlates with their effects on decreasing protein solubility. A connection between the co-solvent binding and the surface tension was made much earlier by Gibbs (1878). When co-solvents bind to the air–water interface, they decrease the surface tension. When the co-solvents increase the surface tension, their interface concentration is depleted and hence they are excluded from the interface. This relationship was expanded into the “cavity theory” by Melander and Horvath (1977). As depicted in Fig. 2a, a cavity is created to accommodate a macromolecule in aqueous solution, overcoming the surface free energy. When co-solvents (Fig. 2b) increase the surface tension of the aqueous solution, this surface free energy becomes greater. Timasheff’s group extrapolated the co-solvent

Fig. 2 Surface tension effect and co-solvent exclusion. **a** Depiction of a cavity (square) to accommodate a protein molecule, overcoming the surface tension or “attraction pressure” (arrows). **b** When an excluded co-solvent (closed circle) is added into the water, the surface tension and “attraction pressure increase, leading to an energetically unfavorable state. **c** Unfolding of protein leads to a further increase in co-solvent exclusion (dashed arrows) and “attraction pressure” or surface tension effect (arrows). **d** Self-association of protein reduces co-solvent exclusion and “attraction pressure”

exclusion theory at the air–water interface proposed by Gibbs (1878) to the phenomenon at protein surface. Thus, those co-solvents that increase the surface tension should be excluded from the protein surface. Such a correlation was experimentally observed.

How does such a concept relate to the co-solvent effects on protein stability or assembly? A simple explanation is given in Fig. 2c for protein folding/unfolding and Fig. 2d for assembly/dimerization. When the protein molecule unfolds in the presence of excluded co-solvents (Fig. 2c), an increase in surface area should lead to a greater surface free energy and co-solvent exclusion, resulting in a more unfavorable situation. This in turn should lead to a more stable state, less surface area and co-solvent exclusion, stabilizing the native state through the addition of excluded co-solvents. In contrast, protein (self-)association (Fig. 2d) should lead to less surface area and co-solvent exclusion per protein, stabilizing the associated state.

Compliance with ethical standards

Conflict of interest Tsutomu Arakawa declares that he has no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by the author.

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