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Review

Lyophilization and development of solid protein pharmaceuticals

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Abstract

Developing recombinant protein pharmaceuticals has proved to be very challenging because of both the complexity of protein production and purification, and the limited physical and chemical stability of proteins. To overcome the instability barrier, proteins often have to be made into solid forms to achieve an acceptable shelf life as pharmaceutical products. The most commonly used method for preparing solid protein pharmaceuticals is lyophilization (freeze-drying). Unfortunately, the lyophilization process generates both freezing and drying stresses, which can denature proteins to various degrees. Even after successful lyophilization with a protein stabilizer(s), proteins in solid state may still have limited long-term storage stability. In the past two decades, numerous studies have been conducted in the area of protein lyophilization technology, and instability/stabilization during lyophilization and long-term storage. Many critical issues have been identified. To have an up-to-date perspective of the lyophilization process and more importantly, its application in formulating solid protein pharmaceuticals, this article reviews the recent investigations and achievements in these exciting areas, especially in the past 10 years. Four interrelated topics are discussed: lyophilization and its denaturation stresses, cryo- and lyo-protection of proteins by excipients, design of a robust lyophilization cycle, and with emphasis, instability, stabilization, and formulation of solid protein pharmaceuticals.

Keywords: Aggregation; Cryoprotection; Denaturation; Excipient; Formulation; Freeze-drying; Glass transition; Stability; Lyoprotection; Residual moisture

1. Introduction

Developing recombinant protein pharmaceuticals has proved to be very challenging because of both the complexity of protein production and purification, and the limited physical and chemical stability of proteins. In fact, protein instability is one of the two major reasons why protein pharmaceuticals are administered traditionally through injection rather than taken orally like most small chemical drugs (Wang, 1996). To over-

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come the instability barrier, proteins often have to be made into solid forms to achieve an acceptable shelf life.

The most commonly used method for preparing solid protein pharmaceuticals is lyophilization (freeze-drying). However, this process generates a variety of freezing and drying stresses, such as solute concentration, formation of ice crystals, pH changes, etc. All of these stresses can denature proteins to various degrees. Thus, stabilizers are often required in a protein formulation to protect protein stability both during freezing and drying processes.

Even after successful lyophilization, the long-term storage stability of proteins may still be very limited, especially at high storage temperatures. In several cases, protein stability in solid state has been shown to be equal to, or even worse than, that in liquid state, depending on the storage temperature and formulation composition. For example, a major degradation pathway of human insulin-like growth factor I (hIGF-I) is oxidation of Met[59] and the oxidation rate in a freeze-dried formulation in air-filled vials is roughly the same as that in a solution at either 25 or 30°C (Fransson et al., 1996). Similarly, the oxidation rate of lyophilized interleukin 2 (IL-2) is the same as that in a liquid formulation containing 1 mg ml^{-1} IL-2, 0.5% hydroxypropyl-β-cyclodextrin (HP-β-CD), and 2% sucrose during storage at 4°C (Hora et al., 1992b). At a high water content ($>50\%$), the degradation rate of insulin is higher in a lyophilized formulation than in a solution with similar pH-rate profiles in both states (Strickley and Anderson, 1996). The glucose-induced formation of des-Ser relaxin in a lyophilized formulation is faster than in a solution during storage at 40°C (Li et al., 1996). These examples indicate that stabilizers are still required in lyophilized formulations to increase long-term storage stability.

In the past two decades, numerous studies have been conducted in the areas of protein freezing and drying, and instability and stabilization of proteins during lyophilization and long-term storage. Many critical issues have been identified in this period. These studies and achievements have been reviewed elsewhere with emphasis on physical and chemical instabilities and stabilization of proteins in aqueous and solid states (Manning et al., 1989; Cleland et al., 1993); chemical instability mechanisms of proteins in solid state (Lai and Topp, 1999); various factors affecting protein stability during freeze-thawing, freeze-drying, and storage of solid protein pharmaceuticals (Arakawa et al., 1993); and application of lyophilization in protein drug development (Pikal, 1990a,b; Skrabanja et al., 1994; Carpenter et al., 1997; Jennings, 1999). Nevertheless, it appears that several critical issues in the development of solid protein pharmaceuticals have not been fully examined, including various instability factors, stabilization, and formulation of solid protein pharmaceuticals.

To have an up-to-date perspective of the lyophilization process and more importantly, its application in formulating solid protein pharmaceuticals, this article reviews the recent investigations and achievements in these exciting areas, especially in the past 10 years. Four interrelated topics are discussed sequentially, lyophilization and its denaturation stresses; cryo- and lyo-protection of proteins by excipients; design of a robust lyophilization cycle; and with emphasis, instability, stabilization, and formulation of solid protein pharmaceuticals.

2. Lyophilization and its denaturation stresses

2.1. Lyophilization process

Lyophilization (freeze-drying) is the most common process for making solid protein pharmaceuticals (Cleland et al., 1993; Fox, 1995). This process consists of two major steps: freezing of a protein solution, and drying of the frozen solid under vacuum. The drying step is further divided into two phases: primary and secondary drying. The primary drying removes the frozen water and the secondary drying removes the non-frozen 'bound' water (Arakawa et al., 1993). The amount of non-frozen water for globular proteins is about 0.3–0.35 g g^{-1} protein, slightly less than the proteins' hydration shell (Rupley and Careri, 1991; Kuhlman et al., 1997). More detailed analy-

sis of each lyophilization step is provided in Section 4.

Lyophilization generates a variety of stresses, which tend to destabilize or unfold/denature an unprotected protein. Different proteins tolerate freezing and/or drying stresses to various degrees. Freeze-thawing of ovalbumin at neutral pH did not cause denaturation (Koseki et al., 1990). Repeated (three times) freeze-thawing of tissue-type plasminogen activator (tPA) did not cause any decrease in protein activity (Hsu et al., 1995). Some proteins can keep their activity both during freezing and drying processes, such as α_1-antitrypsin in phosphate–citrate buffer (Vemuri et al., 1994), porcine pancreatic elastase without excipients (Chang et al., 1993), and bovine pancreatic ribonuclease A (RNase A, 13.7 kD) in the presence or absence of phosphate (Townsend and DeLuca, 1990).

However, many proteins cannot stand freezing and/or drying stresses. Freeze-thawing caused loss of activity of lactate dehydrogenase (LDH) (Nema and Avis, 1992; Izutsu et al., 1994b; Andersson and Hatti-Kaul, 1999), 60% loss of L-asparaginase (10 μg ml^{-1}) activity in 50 mM sodium phosphate buffer (pH 7.4) (Izutsu et al., 1994a), and aggregation of recombinant hemoglobin (Kerwin et al., 1998). Freeze-drying caused 10% loss of the antigen-binding capacity of a mouse monoclonal antibody (MN12) (Ressing et al., 1992), more than 40% loss of bilirubin oxidase (BO) activity in the presence of dextran or polyvinylalcohol (PVA) (Nakai et al., 1998), loss of most β-galactosidase activity at 2 or 20 μg ml^{-1} (Izutsu et al., 1993, 1994a), complete loss of phosphofructokinase (PFK) and LDH activity in the absence of stabilizers (Carpenter et al., 1986, 1990; Prestrelski et al., 1993a; Anchordoquy and Carpenter, 1996), and dissociation of *Erwinia* L-asparaginase tetramer (135 kD) into four inactive subunits (34 kD each) in the absence of any protectants (Adams and Ramsay, 1996).

2.2. Denaturation stresses during lyophilization

The lyophilization process generates a variety of stresses to denature proteins. These include (1) low temperature stress; (2) freezing stresses, including formation of dendritic ice crystals, increased ionic strength, changed pH, and phase separation; and (3) drying stress (removing of the protein hydration shell).

2.2.1. Low temperature stress

The first quantitative study on low-temperature denaturation of a model protein was conducted presumably by Shikama and Yamazaki (1961). They demonstrated a specific temperature range in which ox liver catalase was denatured during freeze-thawing. Cold denaturation of catalase at 8.4 μg ml^{-1} in 10 mM phosphate buffer (pH 7.0) started at -6°C. Loss of catalase activity reached 20% at -12°C, remained at this level between -12°C and near -75°C, then decreased gradually from -75 to -120°C. There was almost no activity loss between -129 and -192°C. Similar results were also obtained for ovalbumin by Koseki et al. (1990). Incubation of frozen ovalbumin solution caused structural change of ovalbumin, as monitored by UV difference spectra, which increased with decreasing temperature between -10 and -40°C. Further decrease in incubation temperature to -80°C caused less structural change, and no change at -192°C. Perlman and Nguyen (1992) reported that interferon-γ(IFN-γ) aggregation in a liquid mannitol formulation was more severe at -20°C than at -70, 5 and 15°C during storage. To prevent freezing-induced complication in studying cold protein denaturation, cold and heat denaturation of RNase A has been conducted under high pressure (3 kbar). Under this condition, RNase A denatured below -22°C and above 40°C (Zhang et al., 1995). All these examples are clear indication of low temperature denaturation rather than a freezing or thawing effect.

The nature of cold denaturation has not been satisfactorily delineated. Since solubility of non-polar groups in water increases with decreasing temperature due to increased hydration of the non-polar groups, solvophobic interaction in proteins weakens with decreasing temperature (Dill et al., 1989; Graziano et al., 1997). The decreasing solvophobic interaction in proteins can reach a point where protein stability reaches zero, causing cold denaturation (Jaenicke, 1990). While

normal or thermal denaturation is entropy-driven, cold denaturation is enthalpy-driven (Dill et al., 1989; Shortle, 1996). Oligomeric proteins typically show cold denaturation, i.e. dissociation of subunit oligomers, since association is considered to be a consequence of hydrophobic interaction (Jaenicke, 1990; Wisniewski, 1998). Theoretically, the calculated free energy of unfolding (ΔG_{unf}) for proteins has a parabolic relationship with temperature. This means that a temperature of maximum stability exists, and both high and low temperature can destabilize a protein (Jaenicke, 1990; Kristjánsson and Kinsella, 1991).

2.2.2. Concentration effect

Freezing a protein solution rapidly increases the concentration of all solutes due to ice formation. For example, freezing a 0.9% NaCl solution to its eutectic temperature of -21°C can cause a 24-fold increase in its concentration (Franks, 1990). The calculated concentration of small carbohydrates in the maximally freeze-concentrated matrices (MFCS) is as high as 80% (Roos, 1993). Thus, all physical properties related to concentration may change, such as ionic strength and relative composition of solutes due to selective crystallization. These changes may potentially destabilize a protein.

Generally, lowering the temperature reduces the rate of chemical reactions. However, chemical reactions may actually accelerate in a partially frozen aqueous solution due to increased solute concentration (Pikal, 1999). Due to solute concentration, the rate of oligomerization of β-glutamic acid at -20°C was much faster than at 0 or 25°C in the presence of a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (Liu and Orgel, 1997).

The increase in the rate of a chemical reaction in a partially frozen state could reach several orders of magnitude relative to that in solution (Franks, 1990, 1994).

The reported oxygen concentration in a partially frozen solution at -3°C is as high as 1150 times that in solution at 0°C (Wisniewski, 1998). The increased oxygen concentration can readily oxidize sulphydryl groups in proteins. If a protein solution contains any contaminant proteases, concentration upon freezing may drastically accelerate protease-catalyzed protein degradation.

2.2.3. Formation of ice-water interface

Freezing a protein solution generates an ice-water interface. Proteins can be adsorbed to the interface, loosening the native fold of proteins and resulting in surface-induced denaturation (Strambini and Gabellieri, 1996). Rapid (quench) cooling generates a large ice-water interface while a smaller interface is induced by slow cooling (also see Section 4.2). Chang et al. (1996b) demonstrated that a single freeze–thaw cycle with quench cooling denatured six model proteins, including ciliary neurotrophic factor (CNTF), glutamate dehydrogenase (GDH), interleukin-1 receptor antagonist (IL-1ra), LDH, PFK, and tumor necrosis factor binding protein (TNFbp). The denaturation effect of quench cooling was greater or equivalent to that after 11 cycles of slow cooling, suggesting surface-induced denaturation. This denaturation mechanism was supported by a good correlation ($r = 0.99$) found between the degree of freeze-induced denaturation and that of artificially surface-induced denaturation. The surface was introduced by shaking the protein solution containing hydrophobic Teflon beads. In a similar study, a correlation coefficient of 0.93 was found between the tendency of freeze denaturation and surface-induced denaturation for eight model proteins, including aldolase, basic fibroblast growth factor (bFGF), GDH, IL-1ra, LDH, maleate dehydrogenase (MDH), PFK, and TNFbp (Kendrick et al., 1995b). However, there was no significant correlation ($r = 0.78$) between freeze denaturation and thermal denaturation temperature (Chang et al., 1996b).

2.2.4. pH changes during freezing

Many proteins are stable only in a narrow pH range, such as low molecular weight urokinase (LMW-UK) at pH 6–7 (Vrkljan et al., 1994). At extreme pHs, increased electrostatic repulsion between like charges in proteins tends to cause protein unfolding or denaturation (Goto and Fink, 1989; Volkin and Klibanov, 1989; Dill, 1990). Thus, the rate of protein aggregation is strongly affected by pH, such as aggregation of

interleukin 1β (IL-1β) (Gu et al., 1991), human relaxin (Li et al., 1995a), and bovine pancreatic RNase A (Townsend and DeLuca, 1990; Tsai et al., 1998). Moreover, the solution pH can significantly affect the rate of many chemical degradations in proteins (Wang, 1999).

Freezing a buffered protein solution may selectively crystallize one buffering species, causing pH changes. Na_2HPO_4 crystallizes more readily than NaH_2PO_4 because the solubility of the disodium form is considerably lower than that of the monosodium form. Because of this, a sodium phosphate buffer at pH 7 has a molar $[NaH_2PO_4]/[Na_2HPO_4]$ ratio of 0.72, but this ratio increases to 57 at the ternary eutectic temperature during freezing (Franks, 1990, 1993). This can lead to a significant pH drop during freezing, which then denatures pH-sensitive proteins. For example, freezing of a LDH solution caused protein denaturation due to a pH drop from 7.5 to 4.5 upon selective crystallization of Na_2HPO_4 (Anchordoquy and Carpenter, 1996). LDH is a pH-sensitive protein and a small drop in pH during freezing can partially denature the protein even in the presence of stabilizers such as sucrose and trehalose (Nema and Avis, 1992). The pH drop during freezing may also explain why freezing bovine and human IgG species in a sodium phosphate buffer caused formation of more aggregates than in potassium phosphate buffer, because potassium phosphate buffer does not show significant pH changes during freezing (Sarciaux et al., 1998).

The pH drop during freezing can potentially affect storage stability of lyophilized proteins. Lyophilized IL-1ra in a formulation containing phosphate buffer at pH 6.5 aggregated more rapidly than that containing citrate buffer at the same pH during storage at 8, 30 and 50°C (Chang et al., 1996c). Similarly, the pH drop of a succinate-containing formulation from 5 to 3–4 during freezing appeared to be the cause of less storage stability for lyophilized IFN-γ than that containing glycocholate buffer at the same pH (Lam et al., 1996).

2.2.5. Phase separation during freezing

Freezing polymer solutions may cause phase separation due to polymers' altered solubilities at low temperatures. Freezing-induced phase separation can easily occur in a solution containing two incompatible polymers such as dextran and Ficoll (Izutsu et al., 1996). During freezing of recombinant hemoglobin in a phosphate buffer containing 4% (w/w) PEG 3350, 4% (w/w) dextran T500, and 150 mM NaCl, liquid–liquid phase separation occurred and created a large excess of interface, denaturing the protein (Heller et al., 1997). Addition of 5% sucrose or trehalose could not reverse the denaturation effect in the system (Heller et al., 1999a).

Several strategies have been proposed to mitigate or prevent phase separation-induced protein denaturation during freezing. These include use of alternative salts (Heller et al., 1999a), adjustment of the relative composition of polymers to avoid or to rapidly pass over a temperature region where the system may result in liquid–liquid phase separation (Heller et al., 1999c), and chemical modification of the protein such as pegylation (Heller et al., 1999b).

2.2.6. Dehydration stresses

Proteins in an aqueous solution are fully hydrated. A fully hydrated protein has a monolayer of water covering the protein surface, which is termed the hydration shell (Rupley and Careri, 1991). The amount of water in full hydration is 0.3–0.35 g g^{-1} protein (Rupley and Careri, 1991; Kuhlman et al., 1997). Generally, the water content of a lyophilized protein product is less than 10%. Therefore, lyophilization removes part of the hydration shell. Removal of the hydration shell may disrupt the native state of a protein and cause denaturation. A hydrated protein, when exposed to a water-poor environment during dehydration, tends to transfer protons to ionized carboxyl groups and thus abolishes as many charges as possible in the protein (Rupley and Careri, 1991). The decreased charge density may facilitate protein–protein hydrophobic interaction, causing protein aggregation.

Water molecules can also be an integral part of an active site(s) in proteins. Removal of these functional water molecules during dehydration easily inactivates proteins. For example, dehydra-

tion of lysozyme caused loss of activity apparently due to removal of those water molecules residing functionally in the active site (Nagendra et al., 1998).

Lastly, dehydration during lyophilization may cause significant difference in moisture distribution in different locations of a product cake. The uneven moisture distribution may lead to possible localized overdrying, which may exacerbate dehydration-induced protein denaturation (Pikal and Shah, 1997).

2.3. Monitoring protein denaturation upon lyophilization

The most common method for monitoring protein denaturation upon lyophilization appears to be infrared (IR) spectroscopy, although other methods have been used such as mass spectroscopy (Bunk, 1997), and Raman spectroscopy (Belton and Gil, 1994). In the following section, IR methodology is discussed in monitoring protein denaturation upon lyophilization followed by a discussion on reversibility of protein denaturation.

2.3.1. Infrared (IR) spectroscopy

IR (or FTIR) is probably the most extensively used technique today for studying structural changes in proteins upon lyophilization (Susi and Byler, 1986; Dong et al., 1995; Carpenter et al., 1998, 1999). The lyophilization-induced structural changes can be monitored conveniently in the amide I, II, or III region. For lyophilized protein samples, residual water up to 10% (w/w) does not interfere significantly in the amide I region, a frequently used sensitive region for determination of secondary structures (Dong et al., 1995). However, IR studies on proteins in an aqueous solution need either subtraction of water absorption or solvent replacement with D_2O (Goormaghtigh et al., 1994). To make reliable subtraction, high protein concentrations (>10 mg ml^{-1}) are recommended to increase protein absorption signal, and a CaF_2 (or BaF_2) cell with a path length of 10 μm or less should be used to control the total sample absorbance within 1 (Cooper and Knutson, 1995).

Lyophilization may induce several potential changes in the IR spectra of proteins. Disruption of hydrogen bonds in proteins during lyophilization generally leads to an increase in frequency and a decrease in intensity of hydroxyl stretching bands (Carpenter and Crowe, 1989). Unfolding of proteins during lyophilization broadens and shifts (to higher wave numbers) amide I component peaks (Prestrelski et al., 1993b; Allison et al., 1996). Lyophilization often leads to an increase in β-sheet content with a concomitant decrease in α-helix content. Conversion of α-helix to β-sheet during lyophilization has been observed in many proteins such as tetanus toxoid (TT) in 10 mM sodium phosphate buffer (pH 7.3) (Costantino et al., 1996), recombinant human albumin (rHA) in different buffer solutions at different pHs (Costantino et al., 1995a), hGH at pH 7.8, and seven model proteins in water, including bovine pancreatic trypsin inhibitor (BPTI), chymotrypsinogen, horse myoglobin (Mb), horse heart cytochrome c (Cyt c), rHA, porcine insulin, and RNase A (Griebenow and Klibanov, 1995).

An increase in β-sheet content during lyophilization is often an indication of protein aggregation and/or increased intermolecular interaction (Yeo et al., 1994; Griebenow and Klibanov, 1995; Overcashier et al., 1997). Lyophilization-induced increase in β-sheet content seems to be a rather general phenomenon as lyophilization or air-drying of unordered poly-L-lysine induced structural transition to a highly ordered β-sheet (Prestrelski et al., 1993b; Wolkers et al., 1998b). Such transition has also been observed in proteins during lyophilization such as human insulin in water (pH 7.1) (Pikal and Rigsbee, 1997). The β-sheet structure after lyophilization shows a higher degree of intermolecular hydrogen bonding because polar groups must satisfy their H-bonding requirement by intra- or intermolecular interaction upon removal of water. The intermolecular β-sheet is characterized by two major IR bands at about 1617 and 1697 cm^{-1} in solid state, which can be used to monitor protein denaturation (Allison et al., 1996). Similarly, the relative intensity of α-helix band also can be used

in this regard (Yang et al., 1999; Heller et al., 1999b).

The extent of changes in overall IR spectrum of a protein upon lyophilization reflects the degree of protein denaturation. The changes relative to a reference spectrum can be measured using a correlation coefficient (r) as defined by Prestrelski et al. (1993a), or the extent of spectral area overlap (Heimburg and Marsh, 1993; Allison et al., 1996; Kendrick et al., 1996). Using the correlation coefficient, Prestrelski et al. (1993b) were able to measure the relative freeze-drying stability of several model proteins, including bFGF, bovine α-lactalbumin, bovine α-casein, IFN-γ, and recombinant granulocyte colony-stimulating factor (rG-CSF) in the presence of different sugars. Nevertheless, Griebenow and Klibanov (1995), after analyzing secondary structures of seven model proteins upon lyophilization, concluded that the correlation coefficient was not highly sensitive to structural alterations in proteins. Instead, comparison of overlapping area-normalized second-derivative or deconvoluted spectra seemed more reliable and objective.

Recently, IR has been used in real-time monitoring of freezing and dehydration stresses on proteins during lyophilization. By this method, glucose at 10% was shown to protect lysozyme both during the freezing and drying processes (Remmele et al., 1997).

2.3.2. Reversibility of freezing- or lyophilization-induced protein denaturation

Many proteins denature to various extents upon freezing, especially at low concentrations (< 0.1 mg ml^{-1}). Freezing-induced denaturation may or may not be reversible. Freezing lysozyme or IL-1ra caused reversible denaturation (Kendrick et al., 1995a). In contrast, recombinant factor XIII (rFXIII, 166 kD) was irreversibly denatured upon freezing, and loss of native rFXIII at 1 mg ml^{-1} increased linearly with the number of freeze–thaw cycles (Kreilgaard et al., 1998b).

Similarly, lyophilization-induced denaturation can be either reversible or irreversible. In the absence of stabilizers, PFK at 25 μg ml^{-1} at pH 7.5 and 8.0 was fully and irreversibly inactivated upon lyophilization (Carpenter et al., 1993), while loss of BO activity in a PVA-containing formulation was at least partially reversible (Nakai et al., 1998). Using IR spectroscopy, Prestrelski et al. (1993b) demonstrated that lyophilization-induced structural changes were irreversible for bFGF, IFN-γ, and bovine α-casein, but essentially reversible for G-CSF and bovine α-lactalbumin. The extensive aggregation and precipitation of IFN-γ and casein upon rehydration confirmed the irreversibility in structural changes. Therefore, lyophilization of proteins may lead to three types of behavior, (1) no change in protein conformation; (2) reversible denaturation; or (3) irreversible denaturation.

In many cases, IR-monitored structural changes during lyophilization seem to be reversible. Griebenow and Klibanov (1995) demonstrated that lyophilization (dehydration) caused significant changes in the secondary structures of seven model proteins in the amide III region (1220–1330 cm^{-1}), including BPTI, chymotrypsinogen, Mb, Cyt c, rHA, insulin, and RNase A. The structure of almost all proteins became more ordered upon lyophilization with a decrease in the unordered structures. Nevertheless, all these structural changes were reversible upon reconstitution. Other examples of reversible changes in the secondary structures of proteins upon lyophilization include rHA (Costantino et al., 1995a), *Humicola lanuginosa* lipase (Kreilgaard et al., 1999), IL-2 (Prestrelski et al., 1995), and lysozyme (Allison et al., 1999).

3. Cryo- and lyo-protection of proteins by stabilizers

As discussed before, both freezing and dehydration can induce protein denaturation. To protect a protein from freezing (cryoprotection) and/or dehydration (lyoprotection) denaturation, a protein stabilizer(s) may be used. These stabilizers are also referenced as chemical additives (Li et al., 1995b), co-solutes (Arakawa et al. 1993), co-solvents (Timasheff, 1993, 1998), or excipients (Wong and Parascrampuria, 1997; Wang, 1999). In the following section, a variety of protein

stabilizers are presented for cryo- and lyo-protection, followed by discussions of their possible stabilization mechanisms.

3.1. Stabilizers for cryo- and lyo-protection

Nature protects life from freezing or osmotic shock by accumulating selected compounds to high concentrations (>1 M) within organisms. These accumulated compounds are known as cryoprotectants and osmolytes, which are preferentially excluded from surfaces of proteins and act as structure stabilizers (Timasheff, 1993). However, since the dehydration stress is different from those of freezing, many effective cryoprotectants or protein stabilizers in solution do not stabilize proteins during dehydration (drying). Some even destabilize proteins during lyophilization. For example, $CaCl_2$ stabilized elastase (20 mg ml^{-1}) in 10 mM sodium acetate (pH 5.0), but caused the lyophilized protein cake to collapse and lose activity (Chang et al., 1993).

Similarly, effective lyophilization stabilizers (lyoprotectants) may or may not stabilize proteins effectively during freezing. Therefore, in cases when a single stabilizer does not serve as both a cryoprotectant and a lyoprotectant, two (or more) stabilizers may have to be used to protect proteins from denaturation during lyophilization.

3.1.1. Sugars/polyols

Many sugars or polyols are frequently used nonspecific protein stabilizers in solution and during freeze-thawing and freeze-drying. They have been used both as effective cryoprotectants and remarkable lyoprotectants. In fact, their function as lyoprotectants for proteins has long been adopted by nature. Anhydrobiotic organisms (water content $<1\%$) commonly contain high concentrations (up to 50%) of disaccharides, particularly sucrose or trehalose, to protect themselves (Crowe et al., 1992, 1998).

The level of stabilization afforded by sugars or polyols generally depends on their concentrations. A concentration of 0.3 M has been suggested to be the minimum to achieve significant stabilization (Arakawa et al., 1993). This has been found to be true in many cases during freeze-thawing. For example, freezing rabbit muscle LDH in water caused 64% loss of protein activity, and in the presence of 5, 10 or 34.2% sucrose, the respective losses were 27, 12, and 0% (Nema and Avis, 1992). Other sugars or polyols that can protect LDH during freeze-thawing to different degrees include lactose, glycerol, xylitol, sorbitol, and mannitol, at 0.5–1 M (Carpenter et al., 1990). Increasing trehalose concentration gradually increased the recovery of PFK activity during freeze-thawing and the recovery reached a maximum of 90% at about 300 mg ml^{-1} (Carpenter et al., 1990). A similar stabilizing trend was also observed for sucrose, maltose, glucose, or inositol (Carpenter et al., 1986).

Since freezing is part of the freeze-drying process, high concentrations of sugars or polyols are often necessary for lyoprotection. These examples include the protection of chymotrypsinogen in the presence of 300 mM sucrose (Allison et al., 1996), complete inhibition of acidic fibroblast growth factor (aFGF) aggregation by 2% sucrose (Volkin and Middaugh, 1996), increase in glucose-6-phosphate dehydrogenase (G6PDH) activity from 40 to about 90% by 5.5% sugar mixture (glucose:sucrose = 1:10, w/w) (Sun et al., 1998), complete recovery of LDH by either 7% sucrose or 7% raffinose, a trisaccharide (Moreira et al., 1998), significant improvement of PFK recovery by 400 mM trehalose (Carpenter et al., 1993), and complete protection of four restriction enzymes by 15% trehalose (Colaco et al., 1992). More examples can be found in Table 2.

Lower concentrations of sugars or polyols may or may not have any significant effect. At 5 to 100 mM, neither trehalose nor glucose could protect LDH or PFK to a significant level during lyophilization (Carpenter et al., 1993). To determine the minimum sugar concentration that offers the maximum stabilization effect, Tanaka et al. (1991) studied the lyoprotective effect of saccharides on the denaturation of catalase during lyophilization. They demonstrated that saccharides protected the protein by direct interaction with the protein and a concentration of saccharides sufficient to form a monomolecular layer on the protein surface was the minimum to achieve the maximum stabilization. Therefore, the stabi-

lization of catalase was found to depend not on the bulk concentration of maltose but on the weight ratio of maltose to catalase (Tanaka et al., 1991). Maximum stabilization of catalase was at a ratio of about 0.4. A recent study showed that maximum protection (about 75% recovery) of L-asparaginase at 1.45 mg ml^{-1} during lyophilization was reached at a saccharide concentration of about 0.5 mg ml^{-1}, which was about the calculated monosaccharide concentration required to interact with all exposed highly polar residues of the protein (Ward et al., 1999). At this concentration, the weight ratio of saccharide to L-asparaginase is 0.34, which is coincidentally very close to that of maltose to catalase.

On the other hand, increasing sugar/polyol concentration to a certain level may eventually reach a limit of stabilization or even destabilize a protein during freeze-drying. For example, actin was maximally stabilized during lyophilization in the presence of 5% (w/v) sucrose and a further increase in sucrose concentration to 10% did not improve the protein stability significantly, which was apparently attributable to sticky, pliable, and collapsed formulation structure (Allison et al., 1998). Increasing trehalose concentration to 150 mg ml^{-1} in a PFK formulation (at 50 μg ml^{-1}, pH 8.0) increased the freeze-drying recovery of PFK activity to about 65%, but further increases in trehalose concentration caused a gradual decrease in recovery of the protein activity (Carpenter and Crowe, 1989). At a trehalose concentration of 400 mg ml^{-1}, basically no PFK activity was left after freeze-drying. Since trehalose at 400 mg ml^{-1} protected about 90% of the protein activity after freeze-thawing, the destabilization of PFK at high concentrations of trehalose occurred in the dehydration step, possibly due to crystallization of trehalose, preventing requisite hydrogen bonding to the dried protein (see Section 3.2) (Carpenter and Crowe, 1989). A similar trend was observed in the stabilization of several other proteins during lyophilization in the presence of increasing concentrations of excipients, including mannitol for L-asparaginase (10 μg ml^{-1}) in 50 mM sodium phosphate buffer (pH 7.4) (Izutsu et al., 1994b), mannitol for β-galactosidase (2 μg ml^{-1}) in 10 mM sodium phosphate buffer (pH 7.4) (Table 2), mannitol for LDH in 50 mM sodium phosphate buffer (pH 7.4) (Izutsu et al., 1994b), and myo-inositol for PFK during freeze-thawing and freeze-drying (Table 2). Again, the decreased protein recovery is probably due to crystallization of these excipients at high concentrations.

The level of protein protection afforded by different sugars or polyols can be either similar or significantly different, depending on the formulation composition, concentration and physical properties of the stabilizer, and its compatibility with the protein. Ward et al. (1999) found that several saccharides, including trehalose, lactose, maltose, sucrose, glucose, and mannitol, displayed similar level of protection towards tetrameric L-asparaginase (1.45 mg ml^{-1}) during lyophilization at saccharide concentrations up to 0.1%. At 2%, glucose or lactose protected L-asparaginase from dissociation during freeze-drying, but mannitol did not, possibly due to its crystallization and loss of intimate interaction with the protein (Adams and Ramsay, 1996). Probably for the same reason, mannitol at 88 mM inhibited the formation of insoluble hGH aggregates in phosphate buffer (pH 7.4) at a freezing rate of 50°C min^{-1}, but accelerated hGH aggregation at lower freezing rates of 0.5 and 5°C min^{-1} (Eckhardt et al., 1991). In a different study, however, Tanaka et al. (1991) demonstrated that both mannitol and sorbitol could increase the recovery of catalase activity during lyophilization to a similar level as that offorded by maltose. They also showed that different sugars (maltose, glucose, and maltotriose) at 1 mg ml^{-1} could increase the recovery of catalase activity to the same level (from 35 to 90%), but maltopentaose, maltohexaose, and maltoheptaose were not as effective (Tanaka et al., 1991). The ineffectiveness of larger saccharides suggests that protein stabilization by sugars may depend on their glucoside chain lengths, and a long chain length may interfere with intermolecular hydrogen-bonding between stabilizing sugars and proteins.

In many cases, disaccharides appear to be the most effective and universal stabilizers among sugars and polyols (Arakawa et al., 1993; Carpenter et al., 1997). For example, the disaccharides

trehalose, sucrose, maltose, and lactose, were all essentially equivalent to or more effective than monosaccharides such as glucose in stabilizing PFK during lyophilization (Crowe et al., 1993b). Trehalose at 400 mM increased the recovery of PFK activity to greater than 60% during lyophilization whereas glucose at the same concentration only recovered less than 5% of the protein activity (Carpenter et al., 1993). Similarly, the activity of H^+-ATPase upon lyophilization was increased from 4 to 100, 91, and 84% in the presence of disaccharides trehalose, maltose, and sucrose, respectively, but only 72 and 37% in the presence of monosaccharides glucose and galactose at 20 mg sugar per mg protein (Sampedro et al., 1998).

Among disaccharides, sucrose and trehalose appear to be the most commonly used. In comparison to sucrose, trehalose seems to be a preferable lyoprotectant for biomolecules, because it has a higher glass transition temperature (Crowe et al., 1992, 1996). The higher glass transition temperature of trehalose arises at least partly from the formation of trehalose–protein–water microcrystals, preventing water plasticizing the amorphous phase (Librizzi et al., 1999). Other properties of trehalose are also considered to be advantageous, which include (1) less hygroscopicity, (2) an absence of internal hydrogen bonds, which allows more flexible formation of hydrogen bonds with proteins, and (3) very low chemical reactivity (Roser, 1991). To support these arguments, Roser (1991) demonstrated that 35 air-dried restriction and DNA-modifying enzymes are maximally stabilized by 0.3 M trehalose in comparison to other non-reducing sugars, including sucrose, sorbitol, mannitol, galactitol, etc. as well as reducing sugars, including glucose, mannose, galactose, maltose, lactose, etc. These advantages of using trehalose were later challenged by Levine and Slade (1992), who contended that sucrose could be equally effective in protecting biomolecules. In reality, the relative stabilization effect of these two sugars seems to be depend on both the protein and sugar concentration. For example, trehalose at 30 mg ml^{-1} was more effective in inhibiting IL-6 aggregation during lyophilization than sucrose at the same concentration (Lueckel et al., 1998b), while sucrose was a better stabilizer than trehalose during freeze-drying of *Humicola lanuginosa* lipase, a hydrophobic protein (Kreilgaard et al., 1999). Trehalose at 20 mg mg^{-1} protein was more effective than sucrose in stabilizing H^+-ATPase during lyophilization, but at ≤ 10 mg mg^{-1} protein, sucrose was more effective (Sampedro et al., 1998). In stabilizing G6PDH during lyophilization, both the glucose/trehalose (1:10, w/w) and glucose/sucrose (1:10, w/w) systems were shown to be equally effective (Sun and Davidson, 1998).

Not all proteins can be stabilized by sugars/polyols. This is still an unsolved puzzle (Carpenter et al., 1999). For example, sucrose at concentrations from 0.1 to 0.5 M showed little effect on the aggregation of recombinant hemoglobin in PBS during freeze–thaw cycles (Kerwin et al., 1998). Addition of 5% sucrose in MN12 formulation did not show significant stabilizing effect during lyophilization (Ressing et al., 1992). Trehalose at 5% actually increased the loss of LDH activity in water from 64 to 74% during freezing (Nema and Avis, 1992). Although the pH of the trehalose solution decreased during freezing, the pH change during freezing could not explain the destabilization of LDH by trehalose because sucrose, which stabilized LDH, also caused the same pH change. Therefore, the type of sugar and its subunit orientation might have caused the difference in stabilizing LDH (Carpenter et al., 1986).

In a few cases, sugars have to be used with another excipient(s) to achieve satisfactory protein stabilization. Carpenter et al. (1986) demonstrated that freezing rabbit skeletal muscle PFK in liquid nitrogen for 30 s completely inactivated the protein. Inclusion of 1 mM $ZnSO_4$ or 50 mM sugars (trehalose, sucrose, or maltose) helped to retain less than 13 or 10% of the initial protein activity after freeze-thawing, while a combination of 1 mM $ZnSO_4$ and 50 mM trehalose (sucrose or maltose) resulted in retention of more than 80% protein activity. More than 85% of protein activity was recovered when $ZnSO_4$ was used with glucose or inositol. Thus, sugars and metal ions had a synergistic effect in stabilizing PFK during freezing. Similarly, neither 10 mM sugar (trehalose, lactose or mannitol) nor 1% PEG could

improve the lyophilization recovery (46%) of LDH at 2 mg ml^{-1} in phosphate buffer (pH 7.5). However, combined use of 1% PEG and 10 mM lactose completely protected the protein from inactivation (Prestrelski et al., 1993a). Also, combined use of 1% PEG and sugar (>25 mM trehalose or glucose) almost completely protected the activity of PFK during lyophilization (Carpenter et al., 1993). PEG in these formulations served as a lyoprotectant, while the sugars were used against dehydration denaturation.

3.1.2. Polymers

Polymers have been used to stabilize proteins in solution and during freeze-thawing and freeze-drying (Arakawa et al., 1993). One of the favorable polymers used in the history of protein drug development was serum albumin. It has been used both as a cryoprotectant and lyoprotectant. For example, bovine serum albumin (BSA) at 1% completely protected the activity of rabbit muscle LDH in water during freezing (Nema and Avis, 1992). At much lower concentrations between 0.05 and 0.1% (w/v), human serum albumin (HSA), due to its effective inhibition of protein surface adsorption and general stabilization of proteins during lyophilization, was used in formulating freeze-dried hydrophobic cytokines, such as interleukin-1α (IL-1α), IL-1β, IL-3, and macrophage colony stimulating factor (MCSF) (Dawson, 1992). Increasing BSA concentrations to 0.05% gradually increased the activity recovery of LDH at 25 μg ml^{-1} from about 30 to 100% during freeze-thawing and to about 80% during freeze-drying (Anchordoquy and Carpenter, 1996). Many protein products on the market, such as Betaseron®, Epogen®, Kogenate®, and Recombinate™ contain albumin (Physicians' Desk Reference, 1999). However, the ever-increasing concern about the potential contamination of serum albumin with blood-borne pathogens limits its future application in protein products. Therefore, rHA has been recommended recently to replace serum albumin as a protein stabilizer (Tarelli et al., 1998). Nevertheless, the ultimate solution is to develop albumin-free formulations for protein pharmaceuticals.

In addition to albumin, other polymers also have been used. The level of protein stabilization afforded by these polymers depends on structure and concentration of both the polymer and the protein. For example, dextran (5%), PVA (2.5%), hydroxypropyl methylcellulose (HPMC) (1%), or gelatin (0.5%) reduced the loss of rabbit muscle LDH activity in water during freezing from 64 to 24%, 24, 18, and 9%, respectively (Nema and Avis, 1992). LDH activity was also protected during lyophilization in the presence of different concentrations of polyethyleneimine (Andersson and Hatti-Kaul, 1999). While ovalbumin at 0.01% had little effect on the stability of catalase (8.4 μg ml^{-1} in 10 mM phosphate buffer, pH 7.0) during freezing, gelatin at the same concentration completely protected the protein activity (Shikama and Yamazaki, 1961). Polyvinylpyrrolidone (PVP) (40 kD) increased both the freeze-thawing and freeze-drying recovery of LDH in a concentration-dependent manner (Anchordoquy and Carpenter, 1996). Addition of 2% dextran (192 kD) into a sucrose-containing actin formulation significantly increased the protein stability during lyophilization (Allison et al., 1998). Hydroxyethyl cellulose (HEC) at 1% completely inhibited lyophilization-induced aggregation of aFGF at 100 μg ml^{-1} in PBS containing 33 μg ml^{-1} heparin, although reconstitution time was increased significantly (Volkin and Middaugh, 1996).

Stabilization of proteins by polymers can generally be attributed to one or more of these polymer properties: preferential exclusion, surface activity, steric hindrance of protein–protein interactions, and/or increased solution viscosity limiting protein structural movement. In recent years, additional properties of polymers have been implicated in stabilizing proteins during freeze-thawing and freeze-drying. Polymers such as dextran have been reported to stabilize proteins by raising the glass transition temperature of a protein formulation significantly and by inhibiting crystallization of small stabilizing excipients such as sucrose (Skrabanja et al., 1994). PEG 3350 or dextran T500 at 4% (w/w) has been found to inhibit a pH drop during freezing of a phosphate-buffered solution by inhibiting crystallization of disodium

phosphate (Heller et al., 1997). Probably by the same mechanism, BSA or PVP (40 kD) at 10% dramatically inhibited the pH drop during freezing of a buffered LDH solution (Anchordoquy and Carpenter, 1996). At least partly due to this inhibition effect, both BSA and PVP increased the freeze–thaw recovery of the protein in a concentration-dependent manner. The inhibition of crystallization of small molecules is apparently due to polymer-induced viscosity increase (Slade et al., 1989).

On the other hand, polymers may cause phase separation during freezing, adversely affecting protein stability (see Section 2.2). Certain polymers may destabilize proteins during lyophilization due to steric hindrance, preventing efficient hydrogen bonding with proteins. Dextran (40 kD) at concentrations of up to 100 mg ml^{-1} failed to inhibit dehydration-induced unfolding of lysozyme because of its inability to form adequate hydrogen bonding with the protein (Allison et al., 1999). Similarly, this compound could hardly prevent formation of β-sheets in poly-L-lysine during dehydration (Wolkers et al., 1998b). In fact, Dextran (162 kD) at 5% (w/v) was shown to destabilize *Humicola lanuginosa* lipase during lyophilization, as determined by IR (Kreilgaard et al., 1999).

3.1.3. Protein itself

Protein aggregation in solution is generally concentration-dependent. It has been suggested that increasing protein concentration to higher than 0.02 mg ml^{-1} may facilitate potential protein aggregation (Ruddon and Bedows, 1997). Increasing protein concentration increases aggregation of many proteins in solution, such as LMW-UK in the range 0.2–0.9 mg ml^{-1} (Vrkljan et al., 1994), IL-1β in the range 100–500 μg ml^{-1} (Gu et al., 1991), apomyoglobin in the range 4–12 mg ml^{-1} in the presence of 2.4 M urea (De Young et al., 1993), and insulin (Brange et al., 1992a).

In contrast, proteins at higher concentrations are often more resistant against both freezing- and lyophilization-induced protein denaturation/aggregation. The activity recovery of many labile proteins after freeze-thawing correlates directly with initial protein concentration (Allison et al., 1996). For example, increasing initial concentration of rhFXIII from 1 to 10 mg ml^{-1} increased the recovery of native rhFXIII during repeated freeze-thawing (Kreilgaard et al., 1998b). The recovery of LDH activity gradually increased from 6% at a protein concentration of 10 μg ml^{-1} to about 65% at concentrations above 175 μg ml^{-1} after freeze-thawing (Carpenter et al., 1990). Up to about 90% LDH activity was recovered when the concentration was increased to 500 μg ml^{-1} (Anchordoquy and Carpenter, 1996). Koseki et al. (1990) demonstrated that increasing the ovalbumin concentration in the range 0.5–2.5 mg ml^{-1} at pH 1.9 decreased the structural changes of the freeze-treated (-40°C) protein, as measured by UV.

Similarly, the lyophilization recovery of PFK activity at 25 and 40 μg ml^{-1} was 34 and 64%, respectively, in the presence of 200 mM trehalose (Carpenter et al., 1987). Increasing the concentration of rabbit muscle LDH from 10 to 500 μg ml^{-1} gradually increased the activity recovery from less than 20% to about 60% during lyophilization (Anchordoquy and Carpenter, 1996). Increasing the concentration of bovine and human IgG species markedly decreased lyophilization-induced protein aggregation (Sarciaux et al., 1998). Certain proteins, however, do not show this concentration-dependent protection. The percentage of lyophilization-induced denaturation of catalase in the absence of a stabilizer was determined to be about 65%, independent of the protein concentration in the range 1–5000 μg ml^{-1} (Tanaka et al., 1991).

The mechanisms of proteins' self-stabilization during freezing and/or lyophilization have not been clearly delineated. Proteins are polymers, and therefore, at least some of the above-discussed stabilization mechanisms for polymers may be applicable to proteins' self-stabilization. Recently, two hypotheses have been reiterated to explain the concentration-dependent protein stabilization upon freezing (Allison et al., 1996). First, unfolding of proteins at high concentrations during freezing may be temporarily inhibited by steric repulsion of neighboring protein molecules. Second, the surface area of ice-water interface formed upon freezing is finite, which limits the

amount of protein to be accumulated and denatured at the interface. In addition, favorable protein–protein interactions (possible formation of dimers or multimers) may contribute to the increased protein stability at high concentrations, as observed for thermophilic proteins (Mozhaev and Martinek, 1984).

3.1.4. Non-aqueous solvents

Non-aqueous solvents generally destabilize proteins in solution. At low concentrations certain non-aqueous solvents may have a stabilizing effect. These stabilizing non-aqueous solvents include polyhydric alcohols such as PEGs, ethylene glycol, and glycerol and some polar and aprotic solvents such as dimethylsulphoxide (DMSO) and dimethylformamide (DMF) (Volkin and Klibanov, 1989; Carpenter et al., 1991).

In fact, polyhydric alcohols are among the commonly used and effective cryoprotectants. For example, in the presence of 0.2 M PEG 400, the loss of rabbit muscle LDH activity upon freezing was reduced from 64 to 15% (Nema and Avis, 1992). LDH can also be protected from freeze-thawing denaturation to different degrees by ethylene glycol or 2-methyl-2,4-pentanediol (Carpenter et al., 1990). PEG at 1–10% (w/v) completely protected both LDH and PFK at 25 μg ml^{-1} (at pH 7.5 and 8.0, respectively) during freeze-thawing, although they were not effective stabilizers during freeze-drying (Carpenter et al., 1993). Glycerol at 0.3% (v/v) prevented freezing denaturation of ovalbumin (0.1%) (Koseki et al., 1990) and at 1 M, increased the recovery of catalase activity upon freezing from 80 to 95% (Shikama and Yamazaki, 1961).

Cryoprotection of proteins by these non-aqueous solvents may be pH-dependent. Ethylene glycol stabilized RNase A at pH 2.3 but destabilized it at pH 5.5 (Arakawa et al., 1991). This is partly because proteins may tolerate freezing denaturation to different degrees at different pHs.

3.1.5. Surfactants

The formation of ice-water interfaces during freezing may cause surface denaturation of proteins (see Section 2.2). Surfactants may drop surface tension of protein solutions and reduce the driving force of protein adsorption and/or aggregation at these interfaces. Low concentrations of nonionic surfactants are often sufficient to serve this purpose due to their relatively low critical micelle concentrations (CMC) (Bam et al., 1995). Other stabilization mechanisms were also proposed, such as assistance in protein refolding during thawing and protein binding, which may inhibit protein–protein interactions (Carpenter et al., 1999).

Tween 80 is one of the commonly used surfactants for protein stabilization during freezing. Tween 80 at concentrations of $\geq 0.01\%$ protected both LDH and GDH from denaturation during quench freezing and thawing (Chang et al., 1996b). The freeze–thaw recovery of LDH activity was increased from 36 to 57 and 65% in the presence of 0.002 and 0.005% Tween 80, respectively (Nema and Avis, 1992). Maximum freeze–thaw recovery (about 80%) of LDH activity was reached in the presence of 0.05% Tween 80. Tween 80 at concentrations from 0.005 to 0.01% also protected several other proteins from freezing denaturation, including TNFbp, IL-1ra, bFGF, MDH, aldolase, and PFK (Kendrick et al., 1995b).

Other nonionic and ionic surfactants have also been reported in cryoprotection of proteins. The following surfactants protected LDH from freezing denaturation to various degrees, Brij 35; Brij 30 (polyoxyethylene lauryl ether); Lubrol-px; Triton X-10; Pluronic F127 (polyoxyethylene-polyoxypropylene copolymer); and SDS (Nema and Avis, 1992; Chang et al., 1996b). SDS at 0.5 mM increased the activity recovery of catalase (8.4 μg ml^{-1} in 10 mM phosphate buffer, pH 7.0) from 80 to 90% upon freezing (Shikama and Yamazaki, 1961).

3.1.6. Amino acids

Certain amino acids can be used as cryoprotectants and/or lyoprotectants. For example, freezing rabbit skeletal muscle PFK in liquid nitrogen for 30 s inactivated the protein completely, and several amino acids, including glycine, proline, or 4 hydroxyproline, significantly increased the recovery of the protein activity (Carpenter et al., 1986). Glycine at low concentrations has been shown to

suppress the pH change in 10 or 100 mM sodium phosphate buffer during freezing (Pikal and Carpenter, 1998). Therefore, amino acids may protect proteins from freezing denaturation at least partly by reducing the rate and extent of buffer salt crystallization.

As lyoprotectants, several amino acids increased the lyophilization recovery of LDH from 22 to about 39–100%, including proline, L-serine, sodium glutamate, alanine, glycine, lysine hydrochloride, sarcosine, γ-aminobutyric acid (Carpenter et al., 1990). Glycine alone or in combination with mannitol inhibited aggregation of an antibody-vinca conjugate during lyophilization (Roy et al., 1992). LDH activity was increased by 20% during vacuum-drying in the presence of phenylalanine:arginine:H_3PO_4 (1:1:0.5 molar ratio) (Mattern et al., 1999).

3.1.7. Miscellaneous excipients

Salts and amines have been used as cryoprotectants. LDH activity can be protected to various degrees upon freezing in the presence of potassium phosphate, sodium acetate, ammonium sulfate, magnesium sulfate, sodium sulfate, trimethylamine N-oxide, or betaine (Carpenter et al., 1990). Increasing the potassium phosphate concentration from 10 mM to 1 M increased the recovery of LDH upon freezing from less than 20% to more than 80% (Arakawa et al., 1993).

Metal ions can protect certain proteins during lyophilization. In the presence of 100 mM sugars such as trehalose, maltose, sucrose, glucose or galactose, some divalent metal ions improved the recovery of PFK activity (at 40 μg ml^{-1} in 1 mM sodium borate, pH 7.8) during lyophilization in a concentration-dependent manner. The relative effectiveness of these metal ions was apparently in the following order: $Zn^{2+} > Cu^{2+} > Ca^{2+}$, $Mn^{2+} > Mg^{2+}$ (Carpenter et al., 1987).

The activity of LDH can be protected to different degrees during lyophilization in the presence of some amphiphilic excipients, including HP-β-CD, 3-[(3-cholamidepropyl)-dimethylammonio]-1-propanesulfate (CHAPS), sodium cholate, sucrose monolaurate (Izutsu et al., 1995). Combinations of sucrose and these amphiphilic excipients increased the protein stability synergistically.

Recently, Ramos et al. (1997) demonstrated that 2-*O*-β-mannosylglycerate at 500 mM increased the freeze-drying recovery of LDH activity (at 50 ug ml^{-1}) from 12 to 85% while trehalose only increased the recovery to 54%.

3.2. Mechanisms of protein stabilization during lyophilization

Since freezing and drying stresses imposed on proteins during lyophilization are different, mechanisms of protein stabilization by excipients are not the same in the two stages of lyophilization.

3.2.1. Mechanisms of cryoprotection

One of the most widely accepted protein stabilization mechanisms in liquid state is preferential interaction. Preferential interaction means that a protein prefers to interact with either water or an excipient(s) in an aqueous solution. In the presence of a stabilizing excipient, the protein prefers to interact with water (preferential hydration) and the excipient is preferentially excluded from the domain of the protein (preferential exclusion). In this case, proportionally more water molecules and fewer excipient molecules are found at the surface of the protein than in the bulk. Therefore, preferential exclusion of an excipient is usually associated with an increase in the surface tension of water. Detailed discussion of this stabilization mechanism can be found elsewhere (Arakawa et al., 1991, 1993; Timasheff, 1993; Lin and Timasheff, 1996; Timasheff, 1998).

The preferential interaction mechanism applies equally well during freeze–thaw processes (Carpenter et al., 1991; Arakawa et al., 1993; Crowe et al., 1993b). Protein stabilizers, which are excluded from protein surface in solution, can also stabilize proteins during freezing. Nema and Avis (1992) examined the stabilizing effect of 13 cryoprotectants on the recovery of rabbit muscle LDH activity, including trehalose (5%), mannitol (5%), sucrose (5, 10, 34.2%), Brij 30 (polyoxyethylene lauryl ether, 0.05%), Tween 80 (0.002–1%), Pluronic F127 (1%), HPMC (1%), PVP (2.5%), PEG 400 (0.2 M), gelatin (0.5%), BSA (1%), β-cyclodextrin (0.9%) and dextran (5%). They found that the cryoptotectants that increased the

stability of LDH in solution at room temperature also improved the recovery of protein activity after freeze–thaw. However, no apparent correlation was found between the increase in surface tension induced by the cryoprotectants and their protective effect on protein recovery during freeze–thaw cycles. Based on this study, several other stabilization mechanisms were postulated, including modification of the size of ice crystals, reduction (instead of elevation) of surface tension, and restriction of diffusion of reacting molecules. Supposedly, reduction of surface tension is how most surfactants stabilize proteins during freezing.

Besides polymers, many cryoprotectants can increase the viscosity of a solution, restricting diffusion of reacting molecules. In fact, the difference in solution viscosity has explained why trehalose is apparently more effective than sucrose, maltose, glucose, or fructose in stabilizing liquid pyrophosphatase and G6DPH (Sola-Penna and Meyer-Fernandes, 1998). On top of this, concentration of all solutes during freezing increases the solution viscosity rapidly. Therefore, the rate of a chemical reaction may increase initially due to concentration of all solutes but then drops gradually as the viscosity increases (Pikal, 1999). The rate of a chemical reaction is minimized at the glassy state when the viscosity is increased to 10^{12} Pa·s (Angell, 1995). In addition to viscosity increase, some of these cryoprotectants stabilize proteins by suppressing pH changes during freezing (Anchordoquy and Carpenter, 1996).

The preferential interaction mechanism does not fully explain protein cryoprotection by polymers or by proteins themselves at high concentrations. These different mechanisms have been addressed in Section 3.1.

3.2.2. Mechanisms of lyoprotection

During lyophilization, the preferential interaction mechanism is no longer applicable because the hydration shell of proteins is removed (Carpenter et al., 1993; Crowe et al., 1993b; Allison et al., 1996). Thus, many excipients that stabilize proteins in solution do not offer the same effect during lyophilization. For example, KCl at 500 mM effectively protected LDH from thermal inactivation at 50°C, but did not protect the protein activity at all during lyophilization (Ramos et al., 1997).

One major mechanism of protein stabilization by lyoprotectants is the formation of an amorphous glass during lyophilization (Roser, 1991; Franks, 1994; Fox, 1995). Formation of a glass increases the viscosity to 1012 Pa s (1013 P) (Angell, 1995). It is the extreme viscosity at the glassy state, that increases protein stability by slowing down interconversion of conformational substates and conformational relaxation of a protein (Hagen et al., 1995, 1996). This stabilization mechanism explains the retention of G6PDH activity during freeze-drying (Sun et al., 1998). Amorphous materials are structurally more similar to a liquid than crystalline materials (Taylor and Zografi, 1998b). Freeze-dried amorphous insulin is far more stable than crystalline insulin against deamidation and dimer formation at different water contents up to 15% (Pikal and Rigsbee, 1997). Izutsu et al. (1995) studied the effect of amphiphilic excipients on freeze-drying of LDH and found that only those that remain amorphous in the solid state protected the enzyme during freeze-drying. These excipients, including HP-β-CD, CHAPS, sodium cholate, sucrose monolaurate, showed a concentration-dependent stabilization effect during freeze-drying.

A glass can be roughly divided into two types: fragile and strong. The viscosity of a fragile glass increases more deeply than a stronger glass for a given temperature drop below the glass transition temperature (Angell, 1995). Therefore, excipients forming fragile glasses are better stabilizing agents (Hatley, 1997). Both sucrose and trehalose can form a fragile glass (Hatley, 1997; Duddu et al., 1997).

Another interrelated stabilization mechanism is the water replacement hypothesis (Crowe et al., 1993a; Allison et al., 1996, 1998). This mechanism involves the formation of hydrogen bonds between a protein and an excipient(s) at the end of the drying process to satisfy the hydrogen bonding requirement of polar groups on the protein surface (Carpenter and Crowe, 1989; Carpenter et al., 1990). These excipients preserve the native structures of proteins by serving as water substitutes (Carpenter et al., 1990; Arakawa et al., 1991;

Carpenter et al., 1993; Prestrelski et al., 1995). In this way, intra- or interprotein hydrogen bonding may be prevented during dehydration (Leslie et al., 1995; Cardona et al., 1997). Therefore, stabilization of proteins requires hydrogen bonding with an excipient(s) during freeze-drying or dehydration (Carpenter and Crowe, 1989; Arakawa et al., 1991; Carpenter et al., 1991; Crowe et al., 1998).

Since an amorphous state of proteins and stabilizers allows maximal H-bonding between protein and stabilizer molecules, crystallization of any amorphous protein stabilizers during lyophilization often causes protein destabilization due to inefficient hydrogen bonding. Mannitol can easily be crystallized and its crystallization is apparently responsible for the destabilization of some proteins during lyophilization. The aggregation of IL-6 during lyophilization could not be inhibited effectively in a formulation containing only mannitol (Lueckel et al., 1998b). In the presence of 1% PEG, increasing the mannitol concentration above 10 mM reduced the activity of LDH and PFK, possibly due to crystallization of mannitol (Carpenter et al., 1993). Mannitol at 300 mM destabilized *Humicola lanuginosa* lipase during lyophilization and DSC analysis indicated that 85% mannitol was crystallized during lyophilization (Kreilgaard et al., 1999).

Although it was debatable whether or not hydrogen bond was indeed formed between trehalose and lysozyme upon lyophilization (Belton and Gil, 1994), many studies have confirmed hydrogen bonding by IR spectroscopy between carbohydrates and freeze-dried proteins, such as lysozyme, BSA, and PFK (Carpenter and Crowe, 1989; Crowe et al., 1993b; Remmele et al., 1997; Allison et al. 1999) and bFGF, γ-IFN, recombinant G-CSF, bovine α-lactalbumin, and bovine α-casein (Prestrelski et al., 1993b). The degree of structural protection of lysozyme by sucrose and trehalose spectra was shown to correlate with the extent of hydrogen bonding between the sugars and the protein (Allison et al., 1999). Hydrogen bonding has also been demonstrated between sucrose and other non-protein polymers, such as poly-L-lysine (Wolkers et al., 1998b), and PVP (Taylor and Zografi, 1998b). Different excipients may form hydrogen bonds with proteins to different extents because of their structural differences. Sucrose has been found to form hydrogen bonds with lysozyme to a greater extent than trehalose (Allison et al., 1999) and with PVP than both trehalose and raffinose (Taylor and Zografi, 1998b). The difference among sugars in stabilization of proteins may be partially due to the difference in the extent and intimacy of hydrogen bond formation.

In addition to glass formation, many excipients, especially polymers, can stabilize proteins by increasing T_g of protein formulations, since higher T_gs generally result in more stable protein formulations during lyophilization. For example, Costantino et al. (1998b) examined six stabilizers (lactose, trehalose, cellobiose, mannitol, sorbitol, and methyl α-D-mannopyranoside) during lyophilization of rhGH and found that the higher the T_g of the stabilized formulation, the greater the degree of structural (such as α-helix) preservation in the co-lyophilizate with less protein aggregation. In general, larger carbohydrates form a glass more readily with a higher T_g than smaller ones, but have more steric hindrance interfering with intimate hydrogen bonding with a dried protein (Crowe et al., 1993b). Therefore, selection of such an excipient needs balancing both the formation of a glass with a high T_g and intimacy of hydrogen bonding.

Other mechanisms of protein stabilization also seem operable. Sugars may stabilize proteins by inhibiting crystallization of other excipients such as PEGs during lyophilization (Izutsu et al., 1995), by inhibiting acute lyophilization-induced protein unfolding such as rhIL-1ra (Chang et al., 1996a), or by preserving a protein's internal mobility such as sperm whale Mb (Sastry and Agmon, 1997). Polyelectrolytes can stabilize a protein during lyophilization by forming multiple electrostatic interactions with the protein (Gibson, 1996).

4. Design of a robust lyophilization cycle — a step-by-step analysis

The purpose of designing a robust lyophilization cycle for protein pharmaceuticals is to obtain a consistent, stable, and esthetically acceptable product. To achieve this goal, a number of

parameters that directly determine or characterize a lyophilization cycle need to be determined or defined. These parameters should include glass transition temperature (T'_g)/collapse temperature (T_{col}), cooling rate, drying rate, and residual moisture content.

4.1. Characterization of protein formulations prior to lyophilization

In addition to glass transition temperature (T'_g)/collapse temperature (T_{col}), several other critical temperatures, including crystallization temperature (T_{cry}), eutectic temperature (T_{eut}), and devitrification temperature (T_{dev}), should be determined in order to design a robust lyophilization cycle. These temperatures are mostly determined by thermal analysis such as DSC, electrical resistance measurements, and direct microscopic observation.

4.1.1. Glass transition temperature (T'_g) and collapse temperature (T_{col})

Ice formation during cooling of a protein solution concentrates all solutes. Solute concentration eventually changes the solution from a viscous liquid to a brittle glass, which contains about 20–50% water (Pikal, 1990b; Hatley et al., 1996). The temperature of this reversible transition for the maximally freeze-concentrated solution is termed glass transition temperature, T'_g. This temperature is also called the temperature of vitreous transformation (Rey, 1999). T'_g is used to differentiate this transition from the softening point of a true glass transition, T_g of a pure polymer. T'_g is one of the most important parameters for optimization of a lyophilization process (Franks, 1990).

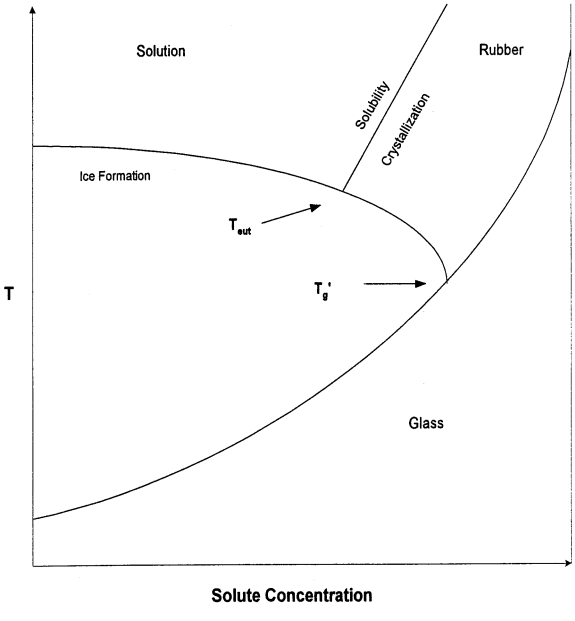

Fig. 1. A theoretical phase diagram showing ice formation, solute crystallization, eutectic point, and glass transition during freezing.

The collapse temperature (T_{col}) is the temperature at which the interstitial water in the frozen matrix becomes significantly mobile (Jennings, 1999). T_{col} is closely related to T'_g. In fact, T_{col} has been considered to be equivalent to T_g of an amorphous system or to the eutectic melting temperature of a crystalline system (Slade et al., 1989; Pikal, 1990a,b). Recent literature indicates that the T_{col} of many small carbohydrates is consistently higher than their T'_g by about 12 K (Sun, 1997). The discrepancy between T'_g and T_{col} for polymers seems even larger (Roos and Karel, 1991). This is because the decrease in viscosity at T'_g may not be sufficient enough to cause structural collapse (Bindschaedler, 1999). For reference, Table 1 lists T'_gs and T_{col}s of some commonly used excipients and buffers.

4.1.2. Crystallization temperature (T_{cry})

When the temperature of an aqueous protein formulation drops below 0°C, water usually crystallizes out first. Then, the crystalline component, which usually has the least solubility in the formulation, may crystallize out. This temperature is termed crystallization temperature.

4.1.3. Eutectic crystallization/melting temperature (T_{eut})

When the temperature of an aqueous protein formulation further decreases after crystallization of the least soluble component, this component and water crystallize out at the same time as a mixture. This temperature is termed eutectic crystallization/melting temperature. The relationship between T_{eut} and T'_g is shown in Fig. 1. Due to

Table 1
Glass transition and collapse temperatures (°C) of buffers, excipients and proteins

Compounds	T'_g	T_{col}	T_g	References
Buffering agents				
Citric acid	−54[a], −53[b]		11[b]	[a]Chang and Randall, 1992; [b]Lu and Zografi, 1997
Hepes	−63			Chang and Randall, 1992
Histidine	−33[a], −32[b]			[a]Chang and Randall, 1992; [b]Österberg and Wadsten, 1999
Potassium acetate	−76			Franks, 1993
Postassium citrate	−62			Franks, 1993
Potassium phosphate (KH_2PO_4)		−55		Chang and Randall, 1992
Sodium acetate	−64			Chang and Randall, 1992
Sodium bicarbonate	−52			Franks, 1993
Sodium citrate	−41			Chang and Randall, 1992
Sodium phosphate (NaH_2PO_4)	−45			Chang and Randall, 1992
Tris base	−55			Franks, 1993
Tris–HCl	−65			Chang and Randall, 1992
Excipients, low MW				
β-Alanine	−65			Franks, 1993
Arabinose			3	Roos, 1993
Arginine			42	Mattern et al., 1999
Cellobiose			77	Franks, 1990; Costantino et al., 1998b
Fructose	−42[b]		10[a], 13[b]	[a]Roos, 1993; [b]Wisniewski, 1998
Fucose			31	Roos, 1993
Galactose	−41[a]		31[b], [c]38	[a]Franks, 1990; [b]Levine and Slade, 1992; [c]Roos, 1993
Glucose	−43[a]	−41[d]	4[c], 32[b], 39[a]	[a]Franks, 1990; [b]Levine and Slade, 1992; [c]Prestrelski et al., 1995; [d]Adams and Ramsay, 1996
Glutamic acid	−17			Chang and Randall, 1992
Glycerol	−65[a]		−92[b], −93[a]	[a]Franks, 1990; [b]Bell et al., 1995
Glycine	−37			Chang and Fischer, 1995
Histidine	[b]−32		[a]37	[a]Mattern et al., 1999; [b]Österberg and Wadsten, 1999
Lactose		−30.5[b]	101[a], 114[c]	[a]Levine and Slade, 1992; [b]Adams and Ramsay, 1996; [c]Taylor and Zografi, 1998b
Lysine			68	Mattern et al., 1999
Maltose	−30[a]		43[a], 82[b], 100[c]	[a]Franks, 1990; [b]Levine and Slade, 1992; [c]Taylor and Zografi, 1998b
Maltotriose	−23			Hatley and Franks, 1991
Mannitol	−33[a], −27[b]		13[c]	[a]Meredith et al., 1996; [b]Lueckel et al., 1998a; [c]Kim et al., 1998

Table 1 (*Continued*)

Compounds	T'_g	T_{col}	T_g	References
Mannose	−41[a]		30[a], 33[b]	[a]Franks, 1990; [b]Levine and Slade, 1992
Melibiose			91	Roos, 1993
Octulose			21	Wolkers et al., 1998a
Raffinose		−26[c]	108[b], 114[a]	[a]Taylor and Zografi, 1998b; [b]Wolkers et al., 1998a; [c]Willemer, 1999
Ribose	−47[b]		−10[b], −13[a]	[a]Roos, 1993; [b]Wisniewski, 1998
Sodium chloride	< −60			Franks, 1993
Sorbitol (glucitol)	−44[a,b]	−54[c]	−3[a]	[a]Franks, 1990; [b]Kerr et al., 1993; [c]Adams and Ramsay, 1996
Sucrose	−32[f]	−31[d], −40[h]	31[c], 61[a], 72[e], 77[b], 75[g]	[a]Levine and Slade, 1992; [b]Hancock et al., 1995; [c]Prestrelski et al., 1995; [d]Adams and Ramsay, 1996; [e]Costantino et al., 1998c; [f]Kasraian et al., 1998; [f]Lueckel et al., 1998a; [g]Taylor and Zografi, 1998b; [h]Overcashier et al., 1999
Trehalose		−29[c], −34[h]	78[a], 46[b], 117[d], 115[c], 105[f], 118[g]	[a]Levine and Slade, 1992; [b]Prestrelski et al., 1995; [c]Adams and Ramsay, 1996; [d]Duddu and Dal Monte, 1997; [e]Miller et al., 1997; [f]Lueckel et al., 1998a; [g]Taylor and Zografi, 1998b; [h]Overcashier et al., 1999
Water	−133[a], −137[b]			[a]Bell et al., 1995; [b]Miller et al., 1997
Xylitol	−47[b]		−23[a], −39[b]	[b]Franks, 1990; [a]Roos, 1993
Xylose	−48[a]		9[a], 14[b]	[a]Franks, 1990; [b]Roos, 1993
Excipients, high MW				
Cellulose			227	Hancock and Zografi, 1994
β-Cyclodextrin			108	Prestrelski et al., 1995
Dextran (10 kD)		−10[b]	91[a]	[a]Prestrelski et al., 1995; [b]Willemer, 1999
Dextran (40 kD)			94[a], 101[b]	[a]te Booy et al., 1992; [b]Prestrelski et al., 1995
Dextran (70 kD)		−11		Adams and Ramsay, 1996
Ficoll		−20		Pikal, 1990b; Izutsu et al., 1996
Gelatin		−8		Pikal, 1990b
Hydroxypropylmethyl-cellulose			155	Hancock and Zografi, 1994
Hydroxyethyl starch	−12[a]	> −5[b]	> 110[c]	[a]Crowe et al., 1993b; [b]Pikal, 1990b; [c]Crowe et al., 1998
Maltodextrin 860			169	Taylor and Zografi, 1998b
Methocel		−9		Willemer, 1999
PEG (6 kD)		−13		Willemer, 1999
Polydextrose	−27			Kerr et al., 1993
PVP K15 (10 kD)		−27[b]	100[a]	[a]Bell et al., 1995; [b]Izutsu et al., 1996
PVP (40 kD)		−24		Adams and Ramsay, 1996
PVP K30 (40 kD)			180	Bell et al., 1995
PVP K90 (1000 kD)			185[a], 176[b]	[a]Hancock et al., 1995; [b]Taylor and Zografi, 1998b
Sephadex G 200		−10		Willemer, 1999
Starch			227	Hancock and Zografi, 1994
Proteins				
BSA	−13[a], −11[b]			[a]Slade et al., 1989; [b]Chang and Randall, 1992

Table 1 (*Continued*)

Compounds	T'_g	T_{col}	T_g	References
α-Casein	−12.5			Slade et al., 1989
Globulins (egg)	−10.5			Slade et al., 1989
HSA		−9.5		Pikal, 1990b
α-Lactalbumin (Sigma)	−10.5			Slade et al., 1989
LDH	−9			Chang and Randall, 1992
Lysozyme	−16.5[a], −13[b]			[a]Slade et al., 1989; [b]Chang and Randall, 1992
rhuMAb HER2		−20		Overcashier et al., 1999
Myoglobin	−15			Chang and Randall, 1992
Ovalbumin	−11[a]	−10[b]		[a]Chang and Randall, 1992; [b]Willemer, 1999
RNase A	−12			Chang and Randall, 1992

excipient interaction(s), many multicomponent protein formulations do not exhibit T_{eut} (Hatley et al., 1996).

4.1.3.1. Devitrification temperature (T_{dev}). When the temperature of the glassy maximally freeze-concentrated solution (MFCS) increases, an endothermic glass transition first occurs. Further increase in temperature above T'_g may lead to an exothermic event, corresponding to the recrystallization of a component such as mannitol (Meredith et al., 1996). This temperature is termed devitrification temperature. Devitrification is a process by which a metastable glass forms a stable crystalline phase on heating above T'_g (Slade et al., 1989; Chang and Randall, 1992; Rey, 1999). Recrystallization can occur if a solution has been cooled rapidly, arresting crystal nucleation and/or growth. To detect recrystallization, the heating rate should be slower than the critical heating rate, which is defined as the minimum heating rate fast enough to prevent devitrification of the unfrozen fraction of a solution (Chang and Randall, 1992).

4.2. Freezing — the first step in lyophilization

The freezing step during lyophilization is considered to be at least as important as the drying step due to its potential effect on proteins (Willemer, 1992). One critical parameter that needs to be defined during freezing is the cooling rate. The cooling rate, v, can be defined as

$$v = \frac{\delta T(r,t)}{\delta t},$$

where, $T(r,t)$ is the temperature field, a function of both time, t, and location, r. Therefore, the rate varies temporally and spatially (Hartmann et al., 1991). In general, a faster freezing rate generates small ice crystals (Eckhardt et al., 1991; Willemer, 1992; Wisniewski, 1998). This is because water is super-cooled and crystallization into ice occurs rapidly, producing small ice crystals (Pikal, 1990a; House and Mariner, 1996). Conversely, a slower cooling rate generates larger ice crystals. The size of crystals determines the pore size to be created during subsequent drying. Large ice crystals create large pores, leading to rapid water sublimation during primary drying (Willemer, 1992), but the secondary drying may slow down due to smaller surface areas, limiting water desorption during secondary drying (Bindschaedler, 1999). To keep a balance, a moderate degree of supercooling (10–15°C) has been recommended (Pikal, 1990a).

The rate of freezing-induced protein denaturation is a complex function of both cooling rate and final temperature (Franks, 1990). The effect of cooling rates on the stability of proteins varies significantly. For example, increasing the freezing rate from 0.5 to 50°C min^{-1} did not significantly affect the formation of soluble aggregates of rGH at 2 mg ml^{-1} in 5 mM phosphate buffer (pH 7.4 or 7.8), but the formation of insoluble aggregates (particulates) increased sharply with increasing cooling rates, even in the presence of up to 250 mM mannitol (Eckhardt et al., 1991). Rapid freezing also caused formation of more aggregates for bovine and human IgG than slow freezing (Sarciaux et al., 1998). This may result from the formation of smaller ice crystals and larger ice-water interfaces at higher freezing rates, leading to a greater extent of surface-induced protein denaturation. On the contrary, faster freezing caused less loss of LDH activity (Nema and Avis, 1992) and less change in the secondary structure of hemoglobin in a PEG/dextran solution (Heller et al., 1999a). The lower loss of protein activity is likely because faster freezing may prevent extensive crystal growth, which may substantially hinder solute concentration-induced protein denaturation. Therefore, stability of proteins may be affected differently at different freezing rates depending on protein denaturation mechanisms.

It should be noted that freezing rate may have a potential impact on the storage stability of lyophilized proteins. Hsu et al. (1995) demonstrated that faster cooling during lyophilization of tPA resulted in a product cake with larger internal surface area, which led to formation of more opalescent (insoluble) particulates upon long-term

storage at 50°C. The rate of formation of opalescent particulates during storage correlated well ($r = 0.995$) with internal surface area of the lyophilized tPA product cake.

As discussed in Section 2.2, buffer species may crystallize out selectively and cause pH shifting during freezing. Therefore, it is preferable to keep all buffer species amorphous during freezing of pH-sensitive proteins. A faster cooling rate may prevent nucleation and subsequent crystallization (Franks, 1993). Each buffer salt has its own critical cooling rate, which is defined as the minimum cooling rate fast enough to prevent crystallization of a solute (Chang and Randall, 1992). Crystallization will not occur when the cooling rate is higher than the critical cooling rate. If a protein solution is cooled rapidly with liquid nitrogen, it is likely that buffer salts will remain amorphous. Otherwise, selective crystallization and a subsequent pH change may occur (Chang et al., 1996b).

Freezing rate influences the extent of crystallization of a formulation excipient, such as mannitol (Hsu et al., 1996). Accordingly, the duration of subsequent thermal treatment after freezing can be affected (see next section). In addition, different freezing rates may favor formation of certain crystalline forms of an excipient, which may potentially affect protein stability and reconstitution. It has been observed that a slower rate of crystallization tends to favor formation of γ-glycine, whereas rapid crystallization seems to favor formation of the β-polymorph (Akers et al., 1995). Different polymorphs of mannitol were also obtained at different concentrations during freezing (Izutsu et al., 1993). Recently, Kim et al. (1998) demonstrated that slow freezing (about 0.2°C min^{-1}) of 10% (w/v) mannitol produced a mixture of α and β-polymorphs and fast freezing (by liquid nitrogen) of the same solution produced δ-form. The reconstitution time (with water) was 36 and 78 s, respectively, for the fast-freeze and slow-freeze dried mannitol samples.

What is the ideal location in a product vial where the cooling rate should be measured? Hartmann et al. (1991) demonstrated that the cooling rate in a flat plate-shaped freezing container, vertically submerged into liquid nitrogen, generally became higher from the surface to the center of the container. They found that the optimum location where the cooling rate should be measured, was 1/3 away from the center and 2/3 away from the inside surface of the sample container. The cooling rate at this point represented $> 80\%$ of the entire sample volume. The geometrical center was apparently the worst location (the least representative) for measuring cooling rate. Although the optimum location for measuring cooling rate in a cylindrical vial has not been determined, it may not be at the center based on the above study.

4.3. Thermal treatment prior to drying

Very often, a thermal treatment step, annealing, is included before the primary drying step. There are at least two reasons for this. First, during the freezing step, a crystalline component may not be completely crystallized. Complete crystallization may be necessary if this component is to provide necessary cake structure or if the protein is more stable after complete crystallization. Recrystallization can be promoted by heat treatment at temperatures above T'_g of the formulation. The duration of the treatment depends on the composition of the formulation and the heating rate through T'_g (Getlin, 1991). Second, removal from the amorphous phase of a crystalline component which has a low T'_g, such as glycine ($T'_g = -42$°C), can increase the T'_g of the amorphous phase (Carpenter et al., 1997). The increased T'_g can allow more efficient primary drying at a higher temperature. For example, annealing at -20°C in a glycine:sucrose (1:1 weight ratio) formulation increased the T'_g from -44 to -33°C and produced a formulation cake of better appearance and higher mechanical strength (Lueckel et al., 1998a,b). Similarly, incorporation of an annealing step in the lyophilization of a monoclonal antibody crystallized glycine and enabled a higher drying temperature (Ma et al., 1998).

However, an annealing step may have an adverse effect on the stability of a protein due to crystallization of an amorphous stabilizer, losing hydrogen bond interaction with the protein (also

see Sections 3.2 and 5.2). Such cases occurred to several proteins during lyophilization, including β-galactosidase (2 μg ml^{-1}) in mannitol formulation during lyophilization (Izutsu et al., 1993), interleukin-6 (IL-6) in sucrose:glycine (1:1 at 20 mg ml^{-1}) formulation (Lueckel et al., 1998b), and LDH in mannitol formulation (Izutsu et al., 1994b). Annealing at −7°C for 1 or 12 h was also found to destabilize hemoglobin in PEG/dextrin (1:1 weight ratio) system during lyophilization, as monitored by IR (Heller et al., 1999c).

4.4. Drying

The drying step is divided into two phases: primary and secondary drying. The primary drying removes the frozen water (sublimation of ice) and the secondary drying removes non-frozen 'bound' water (desorption of water). The amount of non-frozen water for globular proteins is about 0.3–0.35 g g^{-1} protein, slightly less than the proteins' hydration shell (Rupley and Careri, 1991; Kuhlman et al., 1997).

Different models have been reported to describe the drying/sublimation rate during lyophilization (Jennings, 1999). When the shelf temperature is fixed, the drying/sublimation rate, v, of a frozen solid can be expressed as:

$$v = \frac{A_p(P_p - P_0)}{R_p}$$

in which A_p is the cross sectional area of a product, P_p is product vapor pressure at the sublimation front, P_0 is partial vapor pressure in a product vial, and R_p is resistance of a dried product layer to vapor flow (Nail and Johnson, 1992). R_p may be different under different freezing conditions. Since A_p, P_p, and R_p at a fixed freezing rate are usually fixed for a particular protein formulation in a chosen container, drying rate can be changed only through adjustment of P_0.

As indicated in the above equation, drying rate is inversely proportional to R_p. If R_p changes during the drying process, the drying rate and the product temperature will change accordingly. During lyophilization, continuous drying may lead to formation of an increasingly dry product layer (Overcashier et al., 1999). This formulation-dependent dry layer hinders diffusion of water vapor, increases R_p, and causes product temperature to rise, as observed for *Erwinia* L-asparaginase during lyophilization (Adams and Ramsay, 1996). Any temperature rise during lyophilization may potentially cause product collapse. In this case, the shelf temperature needs to be reduced accordingly to prevent product collapse or melt. On the other hand, small-scale product collapse during lyophilization may decrease R_p (Overcashier et al., 1999).

Several other factors, such as ice morphology, crystal size distribution (see Section 4.2), and formulation composition, also affect the drying rate or time. Excipients in protein formulation may interact with proteins and reduce the availability of water-binding sites in proteins. At the same time, excipients themselves may interact with water molecules. Thus, the water-binding force of the formulation components and the associated amount of monolayer water molecules covering the protein formulation will be different depending on the formulation composition, as demonstrated in the rhGH:sugar or rhIGF-I:sugar formulations (Costantino et al., 1998c). Consequently, the drying time will be different depending on formulation composition. In addition, composition-dependent formation of any excipient hydrates during lyophilization would invariably reduce the drying rate, as implicated in the formation of a mannitol hydrate during lyophilization (Yu et al., 1999).

The driving force for water sublimation during lyophilization is the temperature difference between the product and the condenser. The commonly used condenser temperature is −60°C, allowing a minimum of 20°C lower than the product temperature during primary drying (Franks, 1990). During secondary drying the condenser temperature can be set even lower, such as −80°C, for formulations that require very low residual moisture (Bindschaedler, 1999). To achieve a high drying rate, product temperature is frequently set as high as possible. Since the product temperature is controlled by the shelf temperature, effective heat transfer between shelf and product is essential, which is affected by the degree of vacuum in the drying chamber. A mod-

erate increase in chamber pressure often increase the drying rate due to more effective heat transfer, leading to a higher product temperature (Bindschaedler, 1999). In the development of a lyophilized vaccine formulation, the convection of heat between shelf and product was shown to be more effective under a vacuum of 100–120 mTorr than under maximum vacuum (15–25 mTorr) (House and Mariner, 1996). For this reason, drying of the formulation was not conducted under maximum vacuum. Similarly, it has been reported that the maximum drying rate at a chamber pressure of 400 μmHg is more than twice that at 100 μmHg at a shelf temperature of 40°C (Nail and Johnson, 1992). In a more recent report, it was shown that at a constant shelf temperature of 25°C, the specific sublimation rate was 0.19, 0.16, and 0.11 g h^{-1} cm^{-2} at chamber pressures of 300, 200, and 100 mTorr, respectively, for a protein formulation containing trehalose, histidine, and polysorbate 20 (Overcashier et al., 1999). Therefore, a balanced degree of vacuum in the drying chamber is needed to achieve the desired drying rate. It has been suggested that a chamber pressure at one-fourth to one-half of the saturated vapor pressure over ice usually lead to a high sublimation rate (Bindschaedler, 1999).

During lyophilization, complete sample collapse results in both a lower rate of water sublimation/desorption and an inferior product. In addition, collapsed materials may crystallize more easily than non-collapsed materials during storage (Darcy and Buckton, 1997). To prevent product collapse, the product temperature must be kept below the glass transition temperature (T'_g) (or T_{col}) of the formulation or below the eutectic melting temperature (T_{eut}) of any crystalline component. On the other hand, primary drying should be operated at a temperature as close as possible to these temperature limits for high efficiency. Therefore, to have an efficient drying step and to reduce probability of product collapse, a formulation should be designed such that its T'_g is as high as possible. To ensure a high T'_g during secondary drying, the primary drying cycle should be completely finished so that only a minimum amount of bound water is left in the formulation. There are other advantages of drying at a relatively high temperature. Sheehan and Liapis (1998) recently modeled primary and secondary drying of pharmaceutical products in vials. They found that controlling heat input close to melting and scorch (thermally damaging) temperature constraints resulted in not only faster drying time but also more uniform distribution of temperature and bound water in the formulation at the end of secondary drying. The scorch temperature was defined as the temperature of the top surface that shows thermal damage and the melting temperature of the frozen phase was considered to be about 10°C below the melting point of the ice.

The end of the primary drying process is when all the frozen water is removed and the rate of water sublimation is significantly reduced. Several methods can be used in monitoring the completion of the primary drying cycle. A simple method is to observe the changes in product temperature (or chamber pressure) during freeze-drying. The end of the primary drying process is when the product temperature approaches the shelf temperature, evidenced by a significant change in the slope of the product temperature trace due to a reduced sublimation rate. To prevent premature ending, an extra 2–3 h may be added to the drying cycle. A more objective method is the pressure rise test. By disconnecting the vacuum source, the chamber pressure should rise at a rate depending on the amount of moisture in the product. The end of the drying process would be when the rate of pressure rise is below a specified value. Another method for determining the end of the primary drying process is the measurement of the heat transfer rate (Jennings and Duan, 1995; Oetjen, 1999). Timely detection of the end of the primary drying cycle by this method have resulted in a more efficient drying process (Jennings and Duan, 1995). The duration of the secondary drying cycle is dictated by the required moisture content in the final product (see next section).

To improve drying efficiency, a single-drying step may be designed for certain proteins. Chang and Fischer (1995) developed such a cycle for rhIL-1ra formulation. In this single cycle, the shelf temperature was set for the secondary drying and the product temperature was controlled to a certain level by adjusting the chamber tempera-

ture. Freeze-drying of 1 ml of rhIL-1ra formulation could be completed within 6 h with a final moisture level of 0.4%. However, the cycle-associated heterogeneity in temperature and moisture distribution within the product may cause potential damage to other labile proteins.

4.5. Residual moisture

The desired residual moisture level in a lyophilized product dictates duration of the secondary drying step. An electronic hygrometer or a residual gas analyzer may be used to determine residual moisture level during lyophilization and thus, the end-point of secondary drying (Nail and Johnson, 1992). The aforementioned pressure rise test or the measurement of heat transfer rate can also be used for determination of the end of the secondary drying cycle. If these methods are not available, the minimum duration of drying may have to be determined systematically using different combinations of shelf temperatures and durations (Greiff, 1992). Moisture content of lyophilized formulations can be determined by several methods, including loss-on-drying, Karl Fischer titration, thermal gravimetric analysis (TGA), gas chromatography (GC), or near IR. The reproducibility of moisture determination by the first three methods was found to be similar for several biological products (May et al., 1989).

What is the residual moisture in lyophilized product? Proteins have both strong and weak binding sites to accommodate unfrozen water. The weak binding sites include mostly carbonyl backbone plus hydrophilic –OH and –NH– groups, while the strong binding sites include those ionizable groups in amino acids such as Glu, Asp, Lys, and Arg (Careri et al., 1979). For lysozyme, strongly bound water molecules amount up to 10% (g g^{-1} protein) at 38°C (Careri et al., 1979). Since the residual moisture content for lyophilized protein products is usually below 10%, secondary drying removes weakly bound and some of strongly bound water molecules. Therefore, the residual moisture is a small portion of strongly bound water molecules in proteins.

Residual moisture causes a variety of instabilities in dried proteins and in many cases, the effect is complex (see Section 5.2). Usually, a lower moisture content leads to a more stable protein product, although there may not be any significant difference in protein stability between near-zero and an intermediate moisture content of about 1% (Pikal, 1990a). On the other hand, if a lyophilized formulation needs additional viral inactivation by dry heat, its moisture content needs to be high enough to achieve efficient and effective inactivation (Savage et al., 1998). As a general rule, a moisture content in a lyophilized protein formulation should not exceed 2% (Daukas and Trappler, 1998). This general rule seems applicable at least to a couple of protein formulations. Lyophilized bFGF with sugars was stable as long as the moisture content was below 2% (Wu et al., 1998). Lyophilized monoclonal antibody cA2 IgG remained stable with a moisture content of 2.2% or less (Katakam et al., 1998). However, lyophilized BSA and bovine γ-globulin (BGG) formulations were more stable at a water content of about 10% than at $<1\%$ (Yoshioka et al., 1997). The complicated effect of water on stability of solid protein formulations is discussed in Section 5.2.

To find the moisture content that confers the maximal stability for a lyophilized protein product, long-term stability studies should be conducted on protein formulations with different moisture contents. Only these real-time stability studies can determine the optimal moisture content for the final protein product.

5. Instability, stabilization, and formulation of solid protein pharmaceuticals

Lyophilized proteins may lose activity rapidly during storage, even though they may be stable during lyophilization. For instance, porcine pancreatic elastase without excipients retained its full activity after freeze-drying, but lost about 70% activity in 2 weeks at 40°C and 79% RH (Chang et al., 1993). Therefore, lyophilized proteins still need stabilization in the solid state to survive long-term storage as pharmaceuticals. In the following section, instability pathways of proteins in solid state during storage are first described in

brief, followed by a detailed description of factors affecting stability of solid proteins and various stabilization strategies. In the final section, several aspects are discussed in relation to formulation of acceptable solid protein pharmaceuticals.

5.1. Instability of solid proteins during storage

A variety of instability mechanisms have been reported of lyophilized proteins during storage. These include aggregation (a major physical instability) and different chemical degradations such as deamidation, browning reaction, oxidation, hydrolysis, and disulfide bond formation/exchange.

5.1.1. Protein aggregation

Aggregation is one of the major instabilities for lyophilized protein pharmaceuticals during storage (Costantino et al., 1998d). Many lyophilized proteins form aggregates easily during storage under accelerated conditions, such as BSA at 37 or 60°C (Liu et al., 1990; Jordan et al., 1994), rHA at 37°C and 96% RH (Costantino et al., 1995a,b), porcine pancreatic elastase at 40°C and 79% RH (Chang et al., 1993), *Humicola lanuginosa* lipase at 40 or 60°C (Kreilgaard et al., 1999), IL-2 at 45°C (Zhang et al., 1996) or 65°C (Kenney et al., 1986), rhIL-1ra at 50°C (Chang et al., 1996a), insulin at 50°C and 96% RH (Costantino et al., 1994b), tPA at 50°C (Hsu et al., 1995), and tumor necrosis factor (TNF) at 37°C (Hora et al., 1992a). Some proteins form aggregates during storage under ambient conditions. These include aFGF (Volkin and Middaugh, 1996), β-galactosidase (Yoshioka et al., 1993), hGH (Pikal et al., 1992; Costantino et al., 1998b), and antibody-vinca conjugate (Roy et al., 1992).

Protein aggregation can be physical, chemical, or both. Physical (non-covalent) interaction is the cause of protein aggregation for tetanus toxoid (Costantino et al., 1994a), ovalbumin (chicken egg albumin), and glucose oxidase (Liu et al., 1990). Disulfide bond formation or exchange is a major chemical (covalent) pathway leading to protein aggregation. Proteins that aggregate by this mechanism include β-galactosidase (Yoshioka et al., 1993), insulin (Costantino et al., 1994b; Strickley and Anderson, 1996; Pikal and Rigsbee, 1997), rHA (Costantino et al., 1995b), BSA and β-lactoglobulin (Liu et al., 1990; Jordan et al., 1994). Both covalent (disulfide bond) and non-covalent formation of dimers and trimers were observed for a spray-dried anti-IgE monoclonal antibody (Andya et al., 1999).

Often, both soluble and insoluble protein aggregates can form at the same time during storage. This is the case for hGH (Pikal et al., 1992; Costantino et al., 1998b), *Humicola lanuginosa* lipase (Kreilgaard et al., 1999), and tPA (Hsu et al., 1995). The relative amounts of soluble and insoluble protein aggregates may change with storage conditions such as lysozyme aggregation under different relative humidities (Separovic et al., 1998). Both physical and chemical aggregation can lead to formation of insoluble aggregates such as moisture-induced aggregation of lyophilized insulin during storage (Costantino et al., 1994b).

5.1.2. Chemical degradations

Generally speaking, chemical degradations of proteins in solid state have not been reported as extensively as in liquid state. Nevertheless, several chemical degradation pathways have been observed in lyophilized proteins during storage. In some cases, multiple degradation processes proceed simultaneously in a protein such as lyophilized rGH, which undergoes methionine oxidation, asparagine deamidation, and irreversible aggregation during storage (Pikal et al., 1992). Detailed mechanisms of chemical degradations in solid proteins and peptides have recently been reviewed (Lai and Topp, 1999).

Chemical degradations may not affect the activity of proteins, depending on the location of the transformed residue(s). Due to the terminal location of Met^{B4} and Met^{B25} in recombinant human relaxin, oxidation of these two residues did not change the protein bioactivity (Nguyen et al., 1993). The Met^{1} mono-oxidized recombinant human leptin (16 kD) did not show any detectable changes in tertiary structure and retained its full potency, as compared with the native form (Liu et al., 1998). Other protein degradation products having essentially the same biological activity as the intact proteins include deamidated insulin

(Brange et al., 1992b), oxidized IL-2 (Kenney et al., 1986), and deamidated rIL-2 (Sasaoki et al., 1992).

5.1.3. Deamidation

Deamidation is one of the major degradation pathways in lyophilized proteins during storage. Asn and Gln are the two amino acids susceptible to deamidation in proteins. Many cases of deamidation have been reported in lyophilized proteins. For example, both lyophilized rGH and bFGF deamidated during storage (Pikal et al., 1992; Wu et al., 1998). Insulin, lyophilized from a solution of pH 3–5, deamidated via a cyclic anhydride intermediate at C-terminal Asn^{A21} in addition to covalent dimerization during storage (Strickley and Anderson, 1996). The combined formation of deamidated insulin and insulin dimers was shown to be a linear function of square root of time (Pikal and Rigsbee, 1997). Storing lyophilized IL-1ra (in 2% glycine, 1% sucrose, and 10 mM sodium citrate buffer) at 50°C caused protein deamidation in addition to aggregation (Chang et al., 1996a,c).

5.1.4. Maillard reaction

Reducing sugars such as glucose can react with lysine and arginine residues in proteins to form carbohydrate adduct via the Maillard reaction, which is also called the browning reaction (Paulsen and Pflughaupt, 1980). The Maillard reaction has been a subject of extensive investigation mainly in the food industry (Chuyen, 1998). In the development of solid protein pharmaceuticals, this reaction has also been observed in several lyophilized proteins during storage, including aFGF (Volkin and Middaugh, 1996), bFGF (Wu et al., 1998), human relaxin (Li et al., 1996), IgG (Hekman et al., 1995), and porcine pancreatic elastase (Chang et al., 1993). This glycation reaction also occurred to an anti-IgE monoclonal antibody co-spray-dried with lactose during storage (Andya et al., 1999).

The browning reaction can result in significant inactivation of a lyophilized protein during storage. Storing lyophilized invertase in the presence of raffinose, lactose, or maltose at 95°C for 7 days led to significant browning and the color intensity of the reconstituted protein solution correlated positively with the activity loss of the protein (Schebor et al., 1997). The lactose or maltose-induced browning reaction may explain why these two sugars were less effective than sucrose, a non-reducing sugar, in stabilizing vacuum-dried restriction enzyme *Eco*RI during storage at 45°C (Rossi et al., 1997).

Although sucrose is a non-reducing sugar, it can be easily hydrolyzed into two reducing sugars, especially at low pHs during storage, not only in liquid state (Reyes et al., 1982; Buera et al., 1987) but also in solid state (te Booy et al., 1992; Skrabanja et al., 1994). It has been demonstrated that the rate of color formation in a freeze-dried sucrose/lysine formulation at pH 2.5 at 40°C was close to that in a glucose/lysine formulation, partly due to the catalytic effect of the amino acid on sucrose hydrolysis (O'Brien, 1996). Therefore, lyophilized proteins in a sucrose-containing formulation may still have the potential to experience the browning reaction. This is the case for lyophilized IL-6 during storage (Lueckel et al., 1998b).

5.1.5. Oxidation

The side chains of Met, Cys, His, Trp, and Tyr residues are potential sites of oxidation (Manning et al., 1989). Methionine residues in proteins can easily be oxidized by atmospheric oxygen. Lyophilized hGH was easily oxidized in a vial containing only 0.4% oxygen during storage at 25°C (Pikal et al., 1991, 1992). The methionine oxidation in hGH during storage has been proved to be insensitive to moisture. Other examples of methionine oxidation in proteins include lyophilized hIGF-I (Fransson et al., 1996) and IL-2 (Kenney et al., 1986; Hora et al., 1992b).

The free sulphydryl groups of cysteines in solid proteins can also be easily oxidized to form disulfide bridges during storage. This is one of the major chemical mechanisms of covalent protein aggregation as mentioned above.

5.1.6. Hydrolysis

Although moisture content is usually low in lyophilized protein formulations, hydrolysis can still occur during storage. Hydrolysis of bFGF has been observed recently in a lyophilized sugar

formulation (Wu et al., 1998). Li et al. (1996) found that the loss of relaxin activity (48%) in a lyophilized glucose formulation during storage at 40°C for 2 weeks was significantly higher than that (8%) in either mannitol or trehalose formulation. The loss of protein activity was apparently due to glucose-induced elimination of serine at the C-terminal of the B chain in relaxin to form des-Ser relaxin.

5.2. Factors affecting stability of solid proteins

Several factors can affect the stability of solid protein pharmaceuticals. These include storage temperature, glass transition temperature, formulation pH, residual moisture content, type and concentration of formulation excipients, and crystallization of amorphous excipients.

5.2.1. Storage temperature

Storage temperature is probably one of the most important factors affecting protein stability in solid state. Many lyophilized proteins show increased loss of activity at high temperatures, such as restriction enzyme PstI in a trehalose formulation and neutral lactase in a PVP formulation at temperatures between 37 and 70°C (Colaco et al., 1992; Mazzobre et al., 1997). Unfortunately, due to the structural complexities of proteins, the temperature effect on stability of solid proteins cannot be simply described by a single instability mechanism, although in general, the higher the temperature, the lower the protein stability, both physically and chemically. It should be noted that fluctuating storage temperatures may be more detrimental to a lyophilized protein than a single high storage temperature (Ford and Dawson, 1994).

High temperatures accelerate physical aggregation of proteins in solid state. This can be ascribed to an increased mobility of protein molecules at high temperatures, which facilitates protein–protein interactions. Increasing temperatures increased aggregation of lyophilized rhIL-1ra between 8 and 50°C (Chang et al., 1996a,c), aggregation of vacuum-dried LDH between 3 and 60°C, and aggregation of vacuum-dried rhG–CSF between 3 and 80°C (Mattern et al., 1997, 1999). High temperatures also accelerate chemical degradations of proteins in solid state, such as deamidation of rhIL-1ra at temperatures between 8 and 50°C (Chang et al., 1996a,c) and dimerization of TNF at temperatures between 25 and 80°C (Hora et al., 1992a).

Increasing temperature also affects protein stability indirectly. Since proteins in a solid formulation are stabilized in an amorphous phase, crystallization of an amorphous component(s) may destabilize proteins. Crystallization of an amorphous component in a protein formulation can occur slowly during storage below T_g and rapidly above T_g (Sun, 1997). The effect of crystallization on protein stability will be discussed in a following section.

5.2.2. Glass transition temperature (T_g)

Glass transition temperature (T_g) of protein formulations is considered to be one of the major determinants of protein stability (Hatley and Franks, 1991). T_g of a polymer is defined as the transition temperature between the rubbery (or liquid-like) and glassy (solid-like) states. Microscopically, a polymer chain may have cooperative localized motion above T_g, and below T_g, only individual atoms are able to make small excursions about their equilibrium positions (Ravve, 1967). The greater mobility above T_g is due to a greater free volume and higher degree of both translational and rotational freedom (Slade et al., 1989). Therefore, formation of a glassy state should result in a drop in molecular diffusion/motion and thus, increased protein stability (Duddu and Dal Monte, 1997).

DSC is the most widely used method for determination of T_g (Crowe et al., 1998). However, the T_g of a protein formulation may not be easily detectable or accurately determined by DSC due to its poor reproducibility, limited detectability, formulation heterogeneity, and contribution of secondary (or β-) relaxation processes (Fan et al., 1994; Bell et al., 1995; Pikal and Rigsbee, 1997; Yoshioka et al., 1997, 1998). Several other factors can affect T_g and/or its determination, such as the condition of glass formation, moisture content, temperature ramping rate, thermal history of samples, or presence of multiple temperature transi-

tions (Franks, 1994; Her and Nail, 1994; Sartor et al., 1994; Shamblin et al., 1998; Lueckel et al., 1998a). These factors may partly explain why T_gs are so different for the same compound, as reported by different investigators (Table 1).

Generally, the higher the glass transition temperature, the more stable the protein formulation. Therefore, the glass transition temperature may be used with caution as a guiding parameter to screen protein stabilizers and formulations. Is there a minimum T_g, which can considered to be adequate to achieve long-term stability for solid protein pharmaceuticals? It has been recommended that the T_g of a stable protein product should be at least 20°C above ambient storage temperature (Franks, 1994). In other words, the product T_g should be higher than 40°C to achieve long-term stability and to tolerate shipping stresses (Carpenter et al., 1997). When T_g is above this temperature, protein molecular mobility is restricted, thus minimizing protein reactivity (Pikal et al., 1991).

Is there any significant difference in molecular mobility immediately below and above T_g, which leads to a significant difference in protein stability? Oksanen and Zografi (1993) found that there was no critical (sharp) change in diffusion coefficient (mobility) of water in PVP around T_g and significant mobility existed below T_g. Significant molecular mobility was also found of indomethacin, PVP, and sucrose at temperatures immediately below T_g (Hancock et al., 1995). Therefore, significant protein degradation does occur below T_g, such as the degradation of lyophilized human insulin via formation of a cyclic anhydride intermediate at C-terminal Asn^{A21} (Strickley and Anderson, 1996). In a recent study, thermal inactivation of lactase in a lyophilized PVP formulation was investigated at temperatures between 37 and 70°C and the inactivation rate constant did not show a step change around the T_g (55°C) of the formulation (Mazzobre et al., 1997).

How well can T_g be used to predict stability of solid protein pharmaceuticals? Mixed results have been obtained. The storage stability of freeze-dried IL-2 correlated positively with T_g of the formulations in the presence of stabilizers of different molecular weights, including glucose (180 D), sucrose (342 D), trehalose (342 D), raffinose (505 D), stachyose (667 D), β-cyclodextrin (1135 D), dextran 12 (10 kD), and dextran 40 (39 kD) (Prestrelski et al., 1995). Positive correlation was also observed between the stability of freeze-dried invertase at 90°C and the glass transition temperature of polymer excipients including maltodextrin and PVPs (PVP 10, 40, and 360) (Schebor et al., 1996). The denaturation temperature (T_m) of several dried proteins, including β-lactoglobulin, ovalbumin, lysozyme, somatotropin, and ribonuclease *A*, correlated positively with T_g of the sugar or polyol additives (Bell et al., 1995; Bell and Hageman, 1996). The thermal stability of proteins in seed axes strongly depends on T_g of the intracellular glass (Sun et al., 1998). However, there are many examples where formulations of a lower T_g are more stable than those of a higher T_g, especially when sugar formulations are compared with polymer formulations. For example, the T_g values of both sucrose and trehalose are significantly lower than those of maltodextrin and PVP (40 kD) but both sugars were more effective than the polymers in stabilizing vacuum-dried restriction enzyme *Eco*RI during storage at 37 or 45°C (Rossi et al., 1997). Similarly, the T_g of freeze-dried invertase formulations containing trehalose, maltodextrin, or PVP was 63, 124, and 119°C, respectively, and the remaining activity of invertase in the respective formulations after incubation at 90°C for 6 h was 74, 47, and 56% (Cardona et al., 1997). The inferiority of polymers in stabilizing proteins has been attributed to their inefficient hydrogen bonding with proteins (see Section 3.1). In a different study, Chang et al. (1996a) found that the T_g of a lyophilized rhIL-1ra formulation containing either phosphate or citrate was 26 and 46°C, respectively, but both deamidation and aggregation of the protein in phosphate-containing formulation were significantly slower during storage at 0°C. They also found that increasing the sucrose concentration from 1 to 10% (w/v) in rhIL-1ra formulation (2% glycine and 10 mM citrate, pH 6.5) decreased the T_g of the lyophilized formulation from 68.5 to 64.6°C, but both aggregation and deamidation of the protein were significantly slower during storage at 50°C.

Why does T_g sometimes fail to predict protein stability? The reason is that T_g is a critical temperature of molecular mobility of amorphous materials, but may not be a direct indicator of molecular mobility (Yoshioka et al., 1998). For instance, the T_g of a sucrose-containing chimeric monoclonal antibody formulation was lower than that of a trehalose-containing formulation, but the molecular mobility in the sucrose-containing protein formulation was apparently lower than that in the trehalose-containing formulation at low temperatures (<12°C), possibly due to the differences in glass fragilities of the two formulations (Duddu et al., 1997). Probably due to the lower molecular mobility, protein aggregation in the sucrose formulation during storage at 5°C was slightly lower than that in the trehalose formulation. Therefore, T_g may not be a direct indicator of protein mobility. Instead, the Kauzmann temperature (T_K), which is the extrapolated isoentropy temperature below both T_m and T_g, has been suggested as being more of a molecular mobility indicator (Hancock and Zografi, 1997; Hancock et al., 1998). This is because T_K is frequently coincided with the zero mobility temperature (T_0) (Angell, 1995).

Recent studies indicate that T_{mc} appears more closely related to protein stability than T_g. T_{mc} was defined as the molecular mobility-changing temperature at which protein or excipient protons in lyophilized formulations begin to exhibit Lorentzian relaxation process resulting from higher molecular mobility in addition to Gaussian relaxation process resulting from lower molecular mobility (Yoshioka et al., 1997, 1998, 1999). At T_{mc}, protein formulations transition from a non-liquidized state to a microscopically liquidized state. T_{mc} has been found to be more closely related to stability of lyophilized γ-globulin than T_g (Yoshioka et al., 1997, 1998). The molecular mobility of proteins can be determined by NMR or dielectric analysis (Pearson and Smith, 1998).

5.2.3. Formulation pH

The pH of a protein solution for lyophilization often affects the stability of dried protein products during long-term storage. The solution pH of a solid formulation upon reconstitution is considered to be a measure of the solid-state microenvironment 'pH' (Strickley and Anderson, 1997). Therefore, solid-state acidity/basicity may still affect protein stability, both physically and chemically. For example, the formation of non-dissociable aggregates during storing lyophilized RNase depended on the pH of the protein solution for lyophilization in the following order, pH $10.0 > 4.0 > 6.4 >$ water (Townsend and DeLuca, 1990). At a storage temperature of 25°C, aggregation of a lyophilized antibody-vinca conjugate from a solution of pH 8.5 was more than that from pH 7.1 or 6.1 (Roy et al., 1992). Both the remaining activity and dimer formation of lyophilized TNF during storage varied with solution pHs from 4.0 to 10.0 (Hora et al., 1992a). Some lyophilized proteins, however, are insensitive to pH changes. Lyophilization of ovalbumin from solutions of different pHs (3.0, 5.5, 7.3, and 9.0) had little effect on the formation of non-covalent protein aggregates during storage (Liu et al., 1990).

In a few cases, the acidity/basicity of a solid formulation affects chemical stability of a protein. The rate and product distribution (between covalent dimer and deamidated form) of insulin degradation in a lyophilized formulation depended on the pH of the reconstituted protein solution (Strickley and Anderson, 1996, 1997). The rate of deamidation of lyophilized IL-1ra (2% glycine and 1% sucrose in 10 mM sodium citrate buffer) at 50°C depended on the solution pH in the following order, pH $7.0 > 6.5 > 6.0 > 5.5$ (Chang et al., 1996c).

One of the major chemical aggregation mechanisms is formation of intermolecular disulfide bonds, which requires the presence of a free thiolate ion, the reactive group. A free thiolate ion can be brought about upon ionization of a free thiol group under alkaline conditions or via β-elimination of an intact disulfide, a reaction that is also accelerated under alkaline conditions. Therefore, the solution pH significantly affects protein aggregation through these mechanisms. For example, insulin lyophilized at 1 mg ml^{-1} at pH 10 aggregated completely in just 1 day at 50°C and 96% RH, whereas insulin lyophilized at pH 7.3 exhibited aggregation of less than 50% in 3

weeks under the same conditions (Costantino et al., 1994b). rHA lyophilized at pH 4.0 was much more stable than that lyophilized at pH 7.3 or 9.0 against moisture-induced aggregation (Costantino et al., 1995a).

The 'pH' of a solid protein formulation also has indirect effects on protein stability. The pH-induced change in the hygroscopicity of a protein is such an effect. At relatively low relative humidity ($\leq 75\%$), the equilibrated moisture content of a lyophilized insulin formulation is similar at pH 3.1 and 5.0 at 35°C, but at higher relative humidity, the low-pH formulation is much more hygroscopic than that of high pH (Strickley and Anderson, 1996). Sucrose at lower pHs may hydrolyze readily to form fructose and glucose, which can react with proteins via the Maillard reaction (see Section 5.1). It was found that the actual sucrose content of freeze-dried sucrose from a solution of pH 3 (in citrate and phosphate buffer) dropped to 79% at 4°C in 1 month while that at pH 9 remained above 95%, apparently due to the difference in the rate of sucrose hydrolysis at different pHs (te Booy et al., 1992; Skrabanja et al., 1994).

5.2.4. Moisture content

The residual moisture content after lyophilization often controls long-term protein stability, both physically and chemically (Franks, 1990; Hatley and Franks, 1991). The moisture content of a lyophilized protein formulation may change significantly during storage due to a variety of factors, such as stopper moisture release and leakage (Section 5.4), crystallization of an amorphous excipient (next section), or moisture release from an excipient hydrate (Yu et al., 1999). Water can affect protein stability both indirectly as a plasticizer or reaction medium and directly as a reactant or a product (Shalaev and Zografi, 1996).

Indirectly as a plasticizer, water drastically decreases glass transition temperature of proteins, polymers or other formulation excipients (Slade et al., 1989; Roos and Karel, 1991; Buera et al., 1992; te Booy et al., 1992; Roos, 1993; Wolkers et al., 1998a). The quantitative effect of water on glass transition temperature can be easily estimated by the Gordon–Taylor equation (Hancock and Zografi, 1994). Depression of T_g by water may reach 10° or more for each percent of moisture retained, especially at low-level moisture contents (Angell, 1995; Hatley, 1997; Rossi et al., 1997). Therefore, a lyophilized protein may easily adsorb sufficient amounts of moisture during storage to reduce its T_g below the storage temperature, to accelerate its instability, and to cause possible product collapse (Oksanen and Zografi, 1990). For example, dimerization of lyophilized insulin increases significantly when water content is high enough to cause significant decrease in glass transition temperature and cake collapse (Strickley and Anderson, 1997). High moisture content also facilitates crystallization of formulation excipients such as various sugars, which will be discussed in the next section.

Generally, increasing moisture content of a lyophilized protein increases the deterioration rate of proteins. Sorbed water increases the free volume of a lyophilized protein, promoting molecular mobility (Towns, 1995; Shamblin et al., 1998). The denaturation temperature (161°C) of dried somatotropin decreased with increasing moisture content until it reached a plateau of 65°C at moisture contents of $>28\%$ (Bell et al., 1995). Increasing moisture content of lyophilized tPA from 4.6 to 7.6 or 18.0% increased loss of protein activity during storage at 50°C (Hsu et al., 1991). Increasing the moisture content of a lyophilized invertase formulation from 2.1 to 10.9% gradually decreased the stability of the protein upon incubation at 90°C for 10 h (Cardona et al., 1997). Increasing relative humidity accelerated activity loss of vacuum-dried restriction enzyme *Eco*RI in both sucrose and trehalose formulations during storage at 45°C (Rossi et al., 1997). Protein aggregation often increases with increasing relative humidity during storage, such as insulin (Costantino et al., 1994b) and lysozyme (Separovic et al., 1998). The aggregation-induced loss of activity of bovine pancreas RNase was faster at 9.8% moisture than at 1.9% (Townsend and DeLuca, 1990). Moisture uptake was also the cause of reduced α-helical conformation and increased β-structure (a common theme of protein aggregation) of spray-dried rhG–CSF or recombinant consensus interferon (rConIFN) (French et al., 1995).

The increased deterioration in lyophilized proteins at high moisture contents may result from increased chemical degradations. The formation of degradation products of insulin in lyophilized formulations was directly proportional to the moisture content of the formulation from 3 to 52% (Strickley and Anderson, 1996, 1997). The increased rate of a chemical reaction at high water contents is due to increased mobility of water involved in the reaction and its positive effect on the mobility of proteins (Hageman, 1988; Towns, 1995). The diffusion coefficient (mobility) of water in PVP at 25°C can increase exponentially with increased motion of PVP side chains as the water content increases (Oksanen and Zografi, 1993). In fact, the rate of hydrogen exchange in lysozyme powder containing 0.2 g water g^{-1} protein (about 17% moisture) is the same as that in a dilute solution (Rupley and Careri, 1991).

In many cases, however, the effect of moisture on protein stability is a complex function. Chang et al. (1996c) studied the stability of lyophilized rhIL-1ra (from 50 mg ml^{-1} rhIL-1ra, 2% Glycine, and 1% sucrose in 10 mM citrate buffer at pH 6.5) at moisture levels between 0.5 and 3.2% (w/w) at 30°C. They found that the least stable formulation had a moisture level of around 0.8% and the more stable formulations had a moisture level of either ≤ 0.5 or 3.2%. In a few studies, a clear bell-shaped relationship was demonstrated between protein stability and moisture content. For example, maximum aggregation (78%) of lyophilized tetanus toxoid occurred at a water content of about 36% during storage at 37°C for 10 days, and less aggregation was observed at water contents either below or above that level (Schwendeman et al., 1995). Aggregation of both (lyophilized) rHA and BSA during storage at 37°C had a bell-shaped relationship as a function of water content with maximum aggregation at about 32 and 28% moisture, respectively (Liu et al., 1990; Costantino et al., 1995b). Similar bell-shaped aggregation dependence on water content was also observed for ovalbumin (chicken egg albumin), glucose oxidase, bovine β-lactoglobulin (Liu et al., 1990), and insulin (Katakam and Banga, 1995; Separovic et al., 1998).

Changing moisture content in a protein formulation may change degradation mechanisms and thus, the overall rate of degradation. The mechanism of aggregation of lyophilized tetanus toxoid (150 kD) was different under different relative humidities during storage. Under 80% RH, all the aggregates were formed by non-disulfide covalent bonding after storing the lyophilized protein at 37°C for 10 days, but under 97% RH, 55% of the aggregates were formed by hydrophobic interaction and scrambling of disulfide bonds (Schwendeman et al., 1996). Hekman et al. (1995) found that increasing moisture content of lyophilized diethylenetriaminepentaacetic anhydride (DTPA)-conjugated IgG above 20% could change the major degradation pathway from Maillard reaction to one which also involved precipitation during storage.

The effect of residual moisture on protein stability can be strongly influenced by several other factors, including temperature, excipient composition, and moisture distribution within a product cake. Pikal et al. (1992) demonstrated that lyophilized rGH, in the presence of glycine and mannitol, aggregated almost linearly with increasing moisture content from 0.7 to 2.5% during storage at 40°C, but the curve was bell-shaped at 25°C. The rate of hGH degradation in the lyophilized formulation varied greatly with formulation composition as well as with storage temperature. They also found that hGH degradation in a lyophilized formulation containing glycine and mannitol was more sensitive to high levels of moisture or headspace oxygen than in a formulation without these excipients (Pikal et al., 1992). Moisture distribution within a protein product is usually quite uneven and this may result in a biphasic loss of activity (initial fast phase and remaining slow phase) during storage (Franks, 1990).

5.2.5. Type and concentration of formulation excipients

Many formulation excipients stabilize proteins in solid state in a concentration-dependent manner (see Section 5.3). On the other hand, overuse of an excipient(s) may eventually destabilize a protein. Even sugars/polyols, the universal protein

stabilizers, may destabilize a protein if their quantities are not appropriately used in a protein formulation (see next section and Section 5.3).

Certain excipients, that are used to stabilize proteins in liquid state or during lyophilization, may destabilize proteins in solid state. Ascorbic acid is a frequently used antioxidant for small drugs, but at 5 mM, it reduced the storage stability of lyophilized elastase (20 mg ml^{-1} in 10 mM sodium acetate, pH 5.0) drastically at 40°C and 79% RH (Chang et al., 1993).

Lyophilized protein formulations often contain a buffering agent(s). Different proteins may need different buffering agents for maximum stabilization in solid state. For example, histidine has been shown to be the best buffer agent for minimizing aggregation of lyophilized recombinant factor IX (rFIX) during storage at 30°C among all the buffering agents examined, including sodium phosphate, potassium phosphate, and Tris (Bush et al., 1998). Sodium phosphate has been shown to be a better buffering agent than sodium citrate and sodium maleate in maintaining the solubility of lyophilized rIFN-β-1b (Betaseron®) upon reconstitution (Lin et al., 1996). In a different study, glycocholate buffer was found to be better than succinate buffer in stabilizing lyophilized IFN-γ as about 50% IFN-γ activity was lost in a lyophilized succinate-containing formulation during storage at 25°C for 4 weeks, while about 28% activity was lost in glycocholate-containing formulation (Lam et al., 1996).

The buffer concentration also influences storage stability of lyophilized proteins. The loss of activity of lyophilized bovine pancreatic RNase in sodium phosphate buffer (pH 6.4) during storage at 45°C increased with increasing buffer concentration in the range of 0, 0.02, and 0.2 M (Townsend and DeLuca, 1990). One possible cause is that buffering agents may change T_g of lyophilized formulations. The T_g of lyophilized rhIL-1ra formulation containing 1% sucrose, 4% mannitol, and 2% glycine decreased from 46 to 26°C when the buffering agent sodium citrate was replaced with sodium phosphate (Chang et al., 1996a). A lower T_g generally leads to a less stable formulation (see Section 5.2).

5.2.6. Crystallization of amorphous excipients

The potential for crystallization of amorphous excipients in a lyophilized protein formulation during storage always exists because the crystalline state is more stable thermodynamically (Hancock and Zografi, 1997). Crystallization usually destabilizes a protein due to a loss of intimate excipient interaction with proteins and a possible decrease in T_g of the amorphous phase. The decrease in T_g arises from increased moisture content of the amorphous phase because the relative amount of the amorphous material(s) is reduced after crystallization and the total amount of moisture in a sealed container does not change. However, if the amorphous excipient crystallizes as hydrates, such as trehalose dihydrate or raffinose pentahydrates, the T_g of the amorphous phase can actually increase (Aldous et al., 1995).

There are many cases of protein destabilization due to excipient crystallization. Inositol crystallization in a lyophilized β-galactosidase formulation destabilized the protein during storage (Izutsu et al., 1994a). The formation of bFGF degradants in a lyophilized formulation under 'acidic' condition was accelerated by sucrose crystallization (Wu et al., 1998). Mannitol crystallization was apparently the cause of a drastic decrease in storage stability for co-spray-dried anti-IgE monoclonal antibody at 5 or 30°C (Costantino et al., 1998a). Crystallization of excipients also causes possible 'pH' shifting, affecting protein stability as observed in lyophilized bFGF during storage (Wu et al., 1998).

Many sugar/polyol excipients have a tendency to crystallize during storage. The rate of crystallization increases with increasing temperature or relative humidity (Schmitt et al., 1999). Immediate crystallization of disaccharides can occur when these sugars are heated to about 50°C above their T_gs (Cardona et al., 1997). Lyophilized sucrose in a LDH formulation (in 10 mM PBS, pH 7.2) is mainly amorphous but tends to crystallize with increasing residual water content or during heating or storage at elevated temperatures (Moreira et al., 1998). Spray-dried amorphous lactose, sucrose, or trehalose can easily crystallize at 25°C and under RH of $\geq 52\%$ (Naini et al., 1998). Both lyophilized and spray-dried rhDNase–lac-

tose mixture could easily crystallize upon exposure to high humidity ($\geq 60\%$) (Chan and Gonda, 1998; Chan et al., 1999). An amorphous sucrose formulation could change to a crystalline form completely in 1 month at 60°C (te Booy et al., 1992). Because of sucrose crystallization, trehalose stabilized lyophilized rFXIII and *Humicola lanuginosa* lipase much more effectively than sucrose during storage at 60°C (Kreilgaard et al., 1998a, 1999). In a more detailed study, it was demonstrated that increasing moisture content dropped roughly linearly the crystallization temperature of sucrose or recombinant human somatotropin (rbSt) (Sarciaux and Hageman, 1997). Therefore, moisture content in a protein formulation should be minimized to prevent crystallization of an excipient(s).

Crystallization tendency of amorphous excipients is strongly affected by their relative amount in a protein formulation. For example, amorphous mannitol, sorbitol, or methyl α-D-mannopyranoside in a rhGH formulation at an excipient:rhGH molar ratio of less than 131:1 did not crystallize easily during storage at 50°C for 4 weeks, but crystallization occurred at 1000:1 molar ratio (Costantino et al., 1998b). Increasing the relative content of sucrose significantly decreased its crystallization temperature in a lyophilized sucrose:rhGH formulation (Costantino et al., 1998c) and facilitated its crystallization under moisture in a spray-dried sucrose:trypsinogen formulation (Tzannis and Prestrelski, 1999b). Mannitol at 10 or 20% stabilized a co-spray-dried anti-IgE monoclonal antibody during storage at 5 or 30°C, but mannitol at 30% crystallized and drastically destabilized the protein during storage (Costantino et al., 1998a). Similarly, inositol at concentrations between 50 and 160 mM in a lyophilized β-galactosidase formulation did not crystallize during storage at 70°C for 7 days but crystallized at concentrations above 250 mM (Izutsu et al., 1994a). DSC data indicated that the crystallization temperature of inositol decreased from 110 to 60°C when the inositol concentration in the formulation increased from 50 to 500 mM.

Crystallization tendency of amorphous excipients also changes depending on the type and concentration of co-existing formulation excipients (also see Section 5.4). Inclusion of sodium phosphate in a spray-dried formulation containing mannitol and an anti-IgE monoclonal antibody inhibited mannitol crystallization, which in turn reduced solid-state protein aggregation during storage at 5 or 30°C (Costantino et al., 1998a). Addition of phosphate and citrate in a lyophilized sucrose formulation inhibited the crystallization of sucrose as demonstrated by DSC (te Booy et al., 1992).

Many polymer excipients (or proteins) have been shown to inhibit the crystallization of small carbohydrate excipients. Maltodextrins or PVP effectively retard the crystallization of sucrose (Roos and Karel, 1991; Shamblin et al., 1996). Dextran or carboxymethyl cellulose sodium salt (CMC-Na) effectively inhibits the crystallization of inositol (Izutsu et al., 1994a). PVP can reduce the tendency of moisture-induced crystallization of lyophilized sucrose (Shamblin and Zografi, 1999). Both catalase and insulin can inhibit lactose crystallization in a co-spray-dried amorphous mixture upon exposure to short-term elevated humidity (Forbes et al., 1998).

To examine excipient crystallization in a protein formulation during storage, X-ray diffractometry or IR can be used (Izutsu et al., 1994a; Kreilgaard et al., 1999). Recently, Raman spectroscopy has been shown to detect 1% amorphous or crystalline content and proved to be another useful method for crystallinity detection (Taylor and Zografi, 1998a).

5.2.7. Reconstitution medium

The reconstitution step may potentially affect protein stability by several mechanisms. First, rapid reconstitution with water may not allow a dried protein to rehydrate as slowly as the dehydration step (Cleland et al., 1993). Therefore, a protein may not be able to refold to its native form during reconstitution, causing denaturation and/or aggregation. Second, the pH of water-reconstituted protein formulations may be different from those before lyophilization, possibly due to loss of some formulation components. For example, three insulin solutions have pHs of 2.0, 3.0 and 4.0 before lyophilization, and the respective pHs of the reconstituted lyophilized insulin were

found to be 3.1, 3.3 and 4.1, presumably due to loss of HCl during lyophilization (Strickley and Anderson, 1996). Lyophilization-induced evaporation of acetic acid can also cause similar pH changes (Hatley et al., 1996). Last, many proteins are temperature-sensitive and the temperature of the reconstitution medium can make a significant difference. For instance, the activity of H^+-ATPase was completely recovered when it was rehydrated at 20°C but the recovered activity was reduced to 62% when it was rehydrated at 30°C (Sampedro et al., 1998).

To alleviate these problems, addition of a surfactant and/or a stabilizer(s) in the reconstitution medium or adjustment of medium pH may be required. For example, inclusion of Tween 20 in the reconstitution medium decreased aggregation of interferon-γ compared with reconstitution with water alone (Webb et al., 1998). Addition of maltose in the reconstitution buffer increased activity recovery of the dried restriction endonuclease *Hin*dIII (Uritani et al., 1995). The amount of aggregates in reconstituted IL-2 formulation could be reduced by inclusion of one of the following excipients in the medium: heparin, dextran sulfate, glycine, lysine–HCl, polylysine, Tween 20, or HP-β-CD (Zhang et al., 1996). Lowering reconstitution solution pH from 7.0 to 4.0 also lowered the amount of aggregates after reconstitution. About 11% of recombinant human keratinocyte growth factor (rhKGF) formed aggregates immediately after reconstitution of the lyophilized KGF with water, but only about 1.5% formed aggregates after reconstitution with water containing 0.05% (w/v) heparin (16 kD) or sucrose octasulfate (SOS) (Zhang et al., 1995). Other stabilizers that inhibited reconstitution-induced aggregation included dextran sulfate, fucoidan, pentosan polysulfate, chondroitin sulfate, myo-inositol sulfate, sulfated β-cyclodextrin, polyphosphoric acid, NaCl, $(NH)_2SO_4$, etc.

5.3. Stabilization of solid proteins by excipients

Solid protein pharmaceuticals may need to be stabilized in two stages, during lyophilization (preparation); and during long-term storage. Protein stabilization during lyophilization has been discussed in Section 3. The following section focuses on protein stabilization for long-term storage.

5.3.1. Stabilization mechanisms

Stabilization mechanisms for lyophilized proteins during long-term storage are similar to those for protein lyoprotection (see Section 3.2). These stabilization mechanisms include formation of an amorphous glassy state, water replacement by excipients, and hydrogen bonding between excipients and proteins (Fox, 1995). It has been shown that excipients capable of replacing water molecules upon dehydration better preserve the native structure of proteins, resulting in enhanced stability (Prestrelski et al., 1995). Nevertheless, it is now clear that glass formation alone is not sufficient for protein stabilization in solid state. The supporting evidence for this argument is that glass-forming polymers fail to stabilize or even destabilize solid proteins during storage (Colaco et al., 1992; Nakai et al., 1998; Lueckel et al., 1998b). Therefore, a combination of these mechanisms is required for maximum protein stabilization in solid state (Crowe et al., 1996, 1998).

In addition to the above-mentioned mechanisms, other stabilization hypotheses have also been proposed. One of them is the prevention of protein–protein interaction and aggregation by physical dilution and separation of protein molecules. The reduced aggregation of lyophilized BSA in the presence of NaCl, sodium phosphate, carboxymethyl-cellulose, dextran, DEAE-dextran, or PEG on incubation at 37°C has been attributed to a general dilution effect (Liu et al., 1990). This mechanism has also been suggested in stabilization of other proteins during storage such as rHA by dextran (Costantino et al., 1995a), IL-1ra by sucrose (Chang et al., 1996c), and insulin by trehalose (inhibition of covalent dimerization) at 35°C (Strickley and Anderson, 1997).

Another stabilization hypothesis is excipient–water interactions. Costantino et al. (1995a) demonstrated that rHA co-lyophilized with NaCl (NaCl:protein = 1:6 on a weight basis) did not exhibit any aggregation after a 4-day incubation period at 37°C and 96% RH, while the protein without the excipient lost over 80% solubility after

just 1 day under the same conditions. Since inclusion of NaCl did not induce any significant change in the secondary structure of rHA after lyophilization, the stabilization effect of NaCl was apparently due to water uptake by NaCl in the vicinity of rHA, which facilitated protein refolding into its native and more stable conformation. This stabilization mechanism was clearly demonstrated in a similar study, in which it was found that the effectiveness of several excipients (D-glucaric acid, D-gluconic acid, sorbitol, sodium chloride, etc.) in inhibiting aggregation of lyophilized rHA roughly correlated with their water uptake under 96% RH (Costantino et al., 1995b). The greater the excipient's affinity for water, generally, the greater the stabilizing effect. However, if a protein is very sensitive to moisture, this stabilization mechanism is not applicable. For example, the aggregation of moisture-sensitive TT in a lyophilized formulation containing NaCl, D-sorbitol, or PEG 20 000 (at an excipient/protein weight ratio of 1:5) did not correlate with water uptake in these formulations during storage at 37°C and 86% RH (Schwendeman et al., 1995).

Polymers may stabilize proteins by increasing T_g of a solid protein formulation, because polymers usually have higher T_gs due to their high molecular weights (te Booy et al., 1992; Prestrelski et al., 1995). The T_gs of maltodextrin and PVP were found to increase linearly with their molecular weights, as described by the Fox and Flory equation (Roos and Karel, 1991; Buera et al., 1992). Because of this effect, the T_g of dextran-formulated γ-globulin formulations increased significantly with increasing molecular weight of dextran from 10 to 510 kD (Yoshioka et al., 1997). In addition, polymers (or proteins) may indirectly stabilize proteins by inhibiting the crystallization of other stabilizing excipients in a solid formulation (see Section 5.2).

5.3.2. Sugars/polyols

Sugars or polyols have commonly been used to stabilize lyophilized proteins for long-term storage, such as sucrose, trehalose, mannitol, sorbitol, etc. Sucrose has been shown to prevent aFGF aggregation completely at 2% during storage at 25°C for 1 year (Volkin and Middaugh, 1996), to inhibit the aggregation rate of rhIL-1ra significantly in a concentration range of 0–10% (w/v) (Chang et al., 1996a,c), and to stabilize rFXIII at 100 mM during storage at 40°C (Kreilgaard et al., 1998a). Sucrose has also been used to improve the storage stability of factor IX (FIX) (Bush et al., 1998) and rIFN-β-1b (Betaseron®) (Lin et al., 1996). Trehalose increased the stability of lyophilized invertase during incubation at 90°C (Schebor et al., 1996; Cardona et al., 1997). Both sucrose and trehalose inhibited the dimer formation in TNF during storage at 37°C (Hora et al., 1992a). Trehalose or lactose at or above 300:1 (excipient:protein) molar ratio inhibited aggregation of a spray-dried anti-IgE monoclonal antibody effectively during storage at 30°C under both 11 and 38% RH (Andya et al., 1999). Mannitol has been shown to decrease aggregation of lyophilized TT during storage at 37°C and 86% RH (Costantino et al., 1996) and lyophilized antibody-vinca conjugate during storage at 25°C (Roy et al., 1992). Sorbitol could prevent aggregation of rHA completely at 1:1 (excipient:protein) weight ratio during storage at 37°C for 4 days (Costantino et al., 1995b) and reduce aggregation of lyophilized TT effectively at 1:5 weight ratio during storage at 37°C and 86% RH for 6 days (Schwendeman et al., 1995). Inositol was able to protect lyophilized β-galactosidase in a concentration-dependent manner during storage at temperatures below 50°C (Izutsu et al., 1994a). A variety of mono-, di-, and trisaccharides or polyols protected lyophilized restriction enzyme PstI to various degrees during storage at 37°C (Colaco et al., 1992). More examples can be found in Table 2.

As used in lyoprotection, sucrose and trehalose seem to be the most commonly used disaccharides for protection of solid proteins during long-term storage. Their relative effect in stabilizing solid proteins is still a much-debated subject. Clearly, trehalose has several advantages over sucrose, as discussed in Section 3.1. In addition, formation of a dihydrate during storage of freeze-dried trehalose has been demonstrated to prevent moisture-induced decrease in glass transition temperature, which usually destabilizes proteins (Aldous et al., 1995; Crowe et al., 1996). These advantages might explain why trehalose was

Table 2
Examples of protein instability and stabilization by excipients

Proteins	Formulation compositions	Study conditions	Remaining Activity (or as stated)	References
Actin	10 mg ml^{-1} actin in 1 mM Tris (pH 8.0), 0.1 mM ATP, 0.25 mM 2 mercaptoethanol, 0.1 mM $CaCl_2$, and 0.0025% NaN_3	Freeze-drying	~24%	Allison et al., 1998
	+1% (w/v) sucrose		33%	
	+5% (w/v) sucrose		~67%	
ADH (baker's yeast)	50 μg ml^{-1} in 20 mM potassium phosphate buffer (pH 7.6)	Freeze-drying	55%	Ramos et al., 1997
	+500 mM KCl		34%	
	+500 mM trehalose		98%	
	+500 mM mannosylglycerate		89%	
Bilirubin oxidase	0.4 mg ml^{-1} in 0.2 mg ml^{-1} Triton X-100 and 40 mg ml^{-1} dextran	Freeze-drying	~56%	Nakai et al., 1998
	0.4 mg ml^{-1} in 0.2 mg ml^{-1} Triton X-100 and 40 mg ml^{-1} PVA		~58%	
	0.4 mg ml^{-1} in 0.2 mg ml^{-1} Triton X-100 and 40 mg ml^{-1} trehalose		~85%	
Catalase (ox liver)	8.4 μg ml^{-1} in 10 mM phosphate buffer (pH 7.0)	Freezing for 10′ at temperatures between −15 and −70°C	80%	Shikama and Yamazaki, 1961
	+1 M glycerol		95%	
	+0.5 mM sodium dodecyl sulfate		90%	
Catalase (beef liver)	1 mg ml^{-1} in 50 mM phosphate buffer	Freeze-drying	~35%	Tanaka et al., 1991
	+0.2 mg ml^{-1} maltose		~70%	
	+1 mg ml^{-1} maltose		~90%	
	+1.3 mg $^{-1}$ml maltose		~90%	
Elastase	Lyophilized formulation at 20 mg ml^{-1} in 10 mM sodium acetate (pH 5.0)	Incubation at 40°C, 79% RH for 2 weeks	33%	Chang et al., 1993
	+ 10% sucrose		86%	
	+ 10% dextran 40		82%.	
β-Galactosidase	20 μg ml^{-1} in 200 mM phosphate buffer (7.4) and 100 mM inositol	Freeze-drying	47%	Izutsu et al., 1994a
	+400 mM inositol		~90%	
	+400 mM inositol and 1 mg ml^{-1} dextran		~100%	
	+400 mM inositol and 1 mg ml^{-1} CMC-Na		~100%	
β-Galactosidase	2 μg ml^{-1} in 10 mM sodium phosphate, (pH 7.4)	Freeze-drying	~5%	Izutsu et al., 1993
	+50 mM mannitol		~95%	
	+200 mM mannitol		~70%	
	+500 mM mannitol		~25%	

Table 2 (*Continued*)

Proteins	Formulation compositions	Study conditions	Remaining Activity (or as stated)	References
β-Galactosidase	2 μg ml^{-1} in 200 mM sodium phosphate, pH 7.4	Freeze-drying	~5%	Izutsu et al., 1993
	+50 mM mannitol		~40%	
	+200 mM mannitol		~95%	
	+500 mM mannitol		~95%	
rFXIII	2 mg ml^{-1} in 0.1 mM EDTA and 10 mM Tris buffer (pH 8)	Freeze-drying	82%	Kreilgaard et al., 1998a
	+100 mM mannitol		90%	
	+1% (w/v) PEG		115%	
	+3.5% (w/v) dextran		109%	
	+100 mM trehalose		117%	
	+100 mM sucrose		111%	
	+0.002% (w/v) Tween 20		106%	
GDH	50 μg ml^{-1} in 20 mM potassium phosphate buffer (pH 7.5)	Freeze-drying	65%	Ramos et al., 1997
	+500 mM KCl		32%	
	+500 mM trehalose		86%	
	+500 mM mannosylglycerate		95%	
hGH	Lyophilized formulation at 2 mg ml^{-1}	Incubation at 50°C	Rate constant = 4.5 per day	Costantino et al., 1998b
	+mannitol at 31:1 molar ratio		0.004 per day	
	+trehalose at 31:1 molar ratio		0.002 per day	
	+cellobiose at 31:1 molar ratio		0.0003 per day	
H^+-ATPase	1 mg $ml-^1$ in 1 mM EGTA-Tris buffer (pH 7.0)	Freeze-drying	4%	Sampedro et al., 1998
	+20 mg trehalose per mg protein		100%	
	+20 mg maltose per mg protein		91%	
	+20 mg sucrose per mg protein		84%	
	+20 mg glucose per mg protein		72%	
	+20 mg galactose per mg protein		37%	
rhIL-1ra	Lyophilized formulation at 10 mg ml^{-1} rhIL-1ra, 2% glycine and 10 mM sodium citrate (pH 6.5)	Incubation at 50°C	Degradation at 1.8% per week, aggregation at 0.08 (A_{500nm}) per week	Chang et al., 1996c
	+1% sucrose (w/v)		0.15% and 0.008, respectively	
	+1% maltose		6.4% and 0.003, respectively	
	+1% sorbitol		2.3% and 0.04, respectively	
	+1% trehalose		0.43% and 0.005, respectively	
IL-2	Lyophilized formulation at 0.8 mg ml^{-1} in MES buffer (pH 7)	Incubation at 45°C for 4 weeks	47%	Prestrelski et al., 1995
	+0.5% sucrose		86%	
	+5% mannitol		28%	

Table 2 (*Continued*)

Proteins	Formulation compositions	Study conditions	Remaining Activity (or as stated)	References
Insulin	Lyophilized formulation at 1 mg ml^{-1} (pH 4)	Incubation at 35°C for 7 days	0.04 mg $ml-^{1}$ degradant (18% dimers)	Strickley and Anderson, 1997
	+5 mg ml^{-1} trehalose		0.02 mg ml^{-1} degradant (1% dimers)	
LDH	About 0.5 mg ml^{-1} (0.25 mg ml^{-1} rabbit muscle and 0.25 mg ml^{-1} porcine heart LDH) in 0.1 M NaCl and 10 mM potassium phosphate, pH 7.5)	Freeze-thawing	76%	Anchordoquy and Carpenter, 1996
	+0.1% (w/v) BSA		80%	
	+1.0% (w/v) BSA		100%	
	+0.1% (w/v) PVP		79%	
	+1.0% (w/v) PVP		98%	
LDH (rabbit muscle)	25 μg ml^{-1} in water	Freeze-thawing	19%	Aldén and Magnusson, 1997
	+ 1% PEG		79%	
LDH (rabbit muscle)	9 μg ml^{-1} in water	Freeze-thawing	36%	Nema and Avis, 1992
	+1% (w/v) methocel		82%	
	+1% (w/v) Pluronic F127		87%	
	+2.5% (w/v) PVP		76%	
	+0.2 M PEG 400		85%	
	+0.5% (w/v) gelatin		91%	
	+1% (w/v) BSA		100%	
	+0.9% (w/v) β-cyclodextrin		31%	
	+5% (w/v) dextran		76%	
	+5% (w/v) trehalose		26%	
	+5% (w/v) mannitol		6%	
	+5% (w/v) sucrose		73%	
	+10% (w/v) sucrose		88%	
	+34.2% (w/v, 1 M) sucrose		102%	
	+0.05% (w/v) Brij 30		100%	
	+0.002% Tween 80		57%	
	+0.005% Tween 80		65%	
	+0.05% Tween 80		79%	
	+0.1% Tween 80		81%	
	+0.5% Tween 80		80%	
	+1% Tween 80		83%	
LDH	2 μg ml^{-1} in 50 mM sodium phosphate buffer (pH 7.4)	Freeze-drying	~9%	Izutsu et al., 1995

Table 2 (*Continued*)

Proteins	Formulation compositions	Study conditions	Remaining Activity (or as stated)	References
	+400 mM mannitol		~14%	
	+200 mM sucrose		~30%	
	+10 mg ml^{-1} CHAPS		~76%	
	+10 mg ml^{-1} HP-β-CD		~73%	
	+10 mg ml^{-1} sodium cholate		~60%	
	+10 mg ml^{-1} PEG 3000		~30%	
	+10 mg ml^{-1} PEG 20 000		~38%	
LDH	About 0.5 mg ml^{-1} (0.25 mg ml^{-1} rabbit muscle and 0.25 mg ml^{-1} porcine heart LDH) in 0.1 M NaCl and 10 mM potassium phosphate, (pH 7.5)	Freeze-drying	66%	Anchordoquy and Carpenter, 1996
	+0.1% (w/v) BSA		65%	
	+1.0% (w/v) BSA		86%	
	+10.0% (w/v) BSA		92%	
	+0.1% (w/v) PVP		77%	
	+1.0% (w/v) PVP		70%	
	+1.0% (w/v) PVP		97%	
LDH	50 μg ml^{-1} in 50 mM Tris–HCl (pH 7.2)	Freeze-drying	27%	Andersson and Hatti-Kaul, 1999
	+0.01% polyethyleneimine (low MW)		43%	
	+0.1% polyethyleneimine (low MW)		47%	
	+1% polyethyleneimine (low MW)		69%	
	+0.01% polyethyleneimine (high MW)		52%	
	+0.1% polyethyleneimine (high MW)		64%	
	+1% polyethyleneimine (high M.W.)		69%	
LDH	Lyophilized formulation at 50 μg ml^{-1} in 50 mM Tris–HCl (pH 7.2)	Incubation at 36°C for 1 week	6%	Andersson and Hatti-Kaul, 1999
	+0.01% polyethyleneimine (low MW)		27%	
	+0.1% polyethyleneimine (low MW)		31%	
	+1% polyethyleneimine (low MW)		59%	
	+0.01% polyethyleneimine (high MW)		21%	
	+0.1% polyethyleneimine (high MW)		51%	
	+1% polyethyleneimine (high MW)		64%	
LDH (rabbit muscle)	2 mg ml^{-1} in 20 mM potassium phosphate buffer (pH 7.5)	Freeze-drying	46%	Prestrelski et al., 1993a
	+1% PEG		46%	
	+10 mM mannitol		39%	
	+10 mM lactose		43%	
	+10 mM trehalose		39%	
	+1% PEG and 10 mM mannitol		81%	

Table 2 (*Continued*)

Proteins	Formulation compositions	Study conditions	Remaining Activity (or as stated)	References
	+1% PEG and 10 mM lactose		100%	
	+1% PEG and 10 mM trehalose		90%	
LDH (rabbit muscle)	50 μg ml^{-1} in 20 mM potassium phosphate buffer (pH 7.6)	Freeze-drying	12%	Ramos et al., 1997
	+500 mM KCl		0%	
	+500 mM trehalose		54%	
	+500 mM mannosylglycerate		85%	
PFK	2 mg ml^{-1} in 20 mM potassium phosphate buffer (pH 8.0)	Freeze-drying	0%	Prestrelski, et al., 1993a
	+1% PEG		20%	
	+10 mM mannitol		5%	
	+10 mM lactose		5%	
	+10 mM trehalose		0%	
	+1% PEG and 10 mM mannitol		30%	
	+1% PEG and 10 mM lactose		103%	
	+1% PEG and 10 mM trehalose		100%	
PFK	50 μg ml^{-1} in 10 mM potassium phosphate (pH 8.0) and 5 mM dithiothreitol	Freeze-thawing	0%	Carpenter and Crowe, 1989
	+150 mg ml^{-1} trehalose		~65%	
	+300 mg ml^{-1} trehalose		~90%	
PFK	25 μg ml^{-1} in 1 mM sodium borate (pH 7.8), 25 mM K_2SO_4, 2 mM $(NH_4)_2SO_4$, and 5 mM dithiothreitol	Freeze-drying	0%	Carpenter et al., 1986
	+1 mM $ZnSO_4$		<13%	
	+50 mM trehalose, sucrose, or maltose		<10%	
	+1 mM $ZnSO_4$ and 50 mM trehalose		~80%	
	+1 mM $ZnSO_4$ and 50 mM sucrose, or maltose		~60%	
	+1 mM $ZnSO_4$ and 50 mM glucose, glycerol or inositol		~85%	
PFK	40 μg ml^{-1} in 1 mM sodium borate (pH 7.8), 25 mM K_2SO_4, 2 mM $(NH_4)_2SO_4$ and 5 mM dithiothreitol	Freeze-drying	0%	Carpenter et al., 1987
	+$ZnSO_4$ (up to 1 mM)		0%	
	+400 mM trehalose or maltose		~80%	
	+400 mM sucrose		~60%	
	+400 mM glucose or galactose		0%	
	+0.9 mM $ZnSO_4$ and 100 mM trehalose or maltose		80%	
	+0.9 mM $ZnSO_4$ and 100 mM glucose		90%	

Table 2 (*Continued*)

Proteins	Formulation compositions	Study conditions	Remaining Activity (or as stated)	References
	+0.9 mM ZnSO4 and 100 mM galactose		85%	
PFK	50 μg ml^{-1} in 10 mM potassium phosphate (pH 8.0) and 5 mM dithiothreitol	Freeze-drying	0%	Carpenter and Crowe, 1989
	+150 mg ml^{-1} trehalose		~45%	
	+300 mg ml^{-1} trehalose		~20%	
PFK	150 μg ml^{-1} in 10 mM potassium phosphate (pH 8.0) and 5 mM dithiothreitol	Freeze-drying	0%	Carpenter and Crowe, 1989
	+50 mg ml^{-1} myo-inositol	Freeze-drying	~20%	
	+100 mg ml^{-1} myo-inositol	Freeze-drying	~5%	

much more effective than sucrose in stabilizing lyophilized restriction enzyme PstI during storage at 37°C (Colaco et al., 1992). Nevertheless, the relative effect of the two disaccharides in stabilizing solid proteins depends on many factors, such as the relative concentration of the disaccharides and the storage temperature at which stability studies are conducted. Duddu and Dal Monte (1997) demonstrated that the aggregation rate for a lyophilized monoclonal antibody (2.5 mg ml^{-1}) was about the same in the presence of either 31.3 mg ml^{-1} sucrose or trehalose during storage at 5 or 40°C for 2 months. However, at 60°C the aggregation percentage (6.0%) in sucrose formulation was significantly higher than that (1.1%) in trehalose formulation, apparently because the storage temperature was higher than the glass transition temperature of the sucrose formulation (59°C by DSC) but lower than that of the trehalose formulation (80°C by DSC). Both sucrose and trehalose have been shown to stabilize lyophilized rFXIII and *Humicola lanuginosa* lipase to a similar level during storage at 40°C for 3 months, but sucrose was much less effective at 60°C due to significant crystallization (Kreilgaard et al., 1998a, 1999). It has been shown that the tendency of crystallization of sucrose is higher than that of trehalose during storage at temperatures above T_g (Hatley, 1997). Partly because of the difference in crystallization tendency, the glucose/trehalose (1:10, w/w) formulation preserved the activity of G6PDH more effectively than the glucose/sucrose (1:10, w/w) formulation at storage temperatures between 33 and 90.5°C (Sun and Davidson, 1998).

The level of protein stabilization afforded by sugars/polyols during long-term storage varies significantly. In certain cases, they destabilize proteins, particularly at high concentrations. A frequent cause of destabilization is crystallization of these excipients during storage. Mannitol at 100 mM does not improve the storage stability of lyophilized rFXIII at 40 or 60°C due to mannitol crystallization (Kreilgaard et al., 1998a). A co-spray-dried formulation containing recombinant humanized anti-IgE monoclonal antibody and 10% (or 20%) mannitol was stable during storage at 5 or 30°C, but the antibody in 30% mannitol formulation exhibited a drastic decrease in stability during storage due to mannitol crystallization (Costantino et al., 1998a). Costantino et al. (1998b) demonstrated that mannitol, sorbitol, or methyl α-D-mannopyranoside inhibited aggregation of lyophilized rhGH maximally at an excipient:rhGH molar ratio of 131:1 during storage at 50°C for 4 weeks. However, at higher ratios of 300:1 and 1000:1, rhGH showed increased formation of insoluble aggregates, apparently due to crystallization of these excipients. In contrast, disaccharides (lactose, trehalose, and cellobiose) were more effective and stabilized the protein continually at ratios of 131:1 and above. In a recent study, Tzannis and Prestrelski (1999a) demonstrated that the thermal stability (T_m) of trypsinogen in a spray-dried sucrose formulation was highest at a mass ratio (sucrose:trypsinogen) of 1:1 and varying the mass ratio in either direction decreased the T_m.

Sugars/polyols may inhibit chemical degradations in solid protein formulations. Sucrose at 10% significantly decreased deamidation of lyophilized rhIL-1ra at 50°C (Chang et al., 1996a). Covalent dimerization of lyophilized insulin could be significantly suppressed in the presence of trehalose (5 mg ml^{-1}) at 35°C (Strickley and Anderson, 1997).

5.3.3. Polymers

Many polymers can increase the long-term stability of lyophilized proteins. Dextran, CMC, DEAE-dextran, and PEG have been shown to reduce aggregation of lyophilized BSA significantly during storage at 37°C (Liu et al., 1990). HP-β-CD was found to stabilize a mouse monoclonal antibody during storage at 56°C (Ressing et al., 1992), to inhibit moisture-induced aggregation of solid insulin (Katakam and Banga, 1995), to stabilize IL-2 against aggregation during storage at 5°C (Hora et al., 1992b), and to inhibit the dimerization of TNF during storage at 37°C (Hora et al., 1992a). Dextran 40 at 10% increased the activity of lyophilized elastase at 20 mg ml^{-1} (in 10 mM sodium acetate, pH 5.0) from 33 to 82% during storage for 2 weeks at 40°C and 79% RH (Chang et al., 1993). Dextran (162 kD) at 3.5 and 5% (w/v) improved the storage stability of

lyophilized rFXIII and *Humicola lanuginosa* lipase, respectively, at 40 or 60°C (Kreilgaard et al., 1998a, 1999). Both PVPs and maltodextrin stabilized lyophilized invertase during incubation at 90°C (Schebor et al., 1996; Cardona et al., 1997). Polyethyleneimine was shown to increase the storage stability of lyophilized LDH in a concentration-dependent manner at 36°C (Table 2).

However, polymers may not always stabilize solid proteins and, in certain cases, have adverse effect, as discussed in Section 3.1. For example, neither insulin nor dextran could stabilize lyophilized restriction enzyme PstI during storage at 37°C (Colaco et al., 1992). Inclusion of dextran 40 in a lyophilized IL-6 formulation containing sucrose significantly increased protein aggregation during storage at 40°C for 9 months (Lueckel et al., 1998b). The destabilization can be attributed to a failure of inflexible dextran molecules to interact with the protein effectively by hydrogen bonding. Apparently for the same reason, the activity of lyophilized BO in a dextran formulation decreased faster than that in a PVA formulation during storage at 70°C (Nakai et al., 1998).

5.3.4. Salts

Salts have been shown to stabilize proteins in a few cases. Liu et al. (1990) found that NaCl or sodium phosphate could significantly inhibit aggregation of lyophilized BSA (in water) on incubation at 37°C. rHA co-lyophilized with NaCl at a NaCl:protein weight ratio of 1:6 did not aggregate upon incubation at 37°C and 96% RH for 4 days, while the protein without NaCl lost over 80% solubility in just one day under the same conditions (Costantino et al., 1995b). NaCl at an excipient:protein weight ratio of 1:5 was also able to reduce aggregation of lyophilized TT during storage at 37°C and 86% RH for 6 days (Schwendeman et al., 1995).

5.3.5. Surfactants

Although surfactants may be effective protein stabilizers during lyophilization, they seem to be incapable of stabilizing proteins effectively during long-term storage, based on a limited number of studies. Bush et al. (1998) demonstrated that Tween 80 could inhibit aggregation of FIX during freezing and thawing, but did not provide enough protection for the lyophilized product during storage. Inclusion of 0.002% Tween 20 in a lyophilized rFXIII formulation did not improve its storage stability at 40 and 60°C (Kreilgaard et al., 1998a). Although Tween 80 at 0.05 or 0.1% could inhibit spray-drying inactivation of LDH, it actually destabilized the dried protein during storage at 25, 40 and 60°C (Adler and Lee, 1999).

5.3.6. Miscellaneous compounds

A combination of 2.0% arginine and 2.3% carnitine significantly decreased aggregation of lyophilized IL-2 during storage at 37°C for 4 weeks (Hora et al., 1992b). Combined use of phenylalanine, arginine, and a mineral acid inhibited aggregation of vacuum-dried rhG–CSF or LDH during storage at 40°C (Mattern et al., 1999). Several excipients, such as D-glucaric acid and D-gluconic acid, have been shown to inhibit aggregation of lyophilized albumin during storage (Costantino et al., 1995b).

Calcium ions has been shown to protect solid rhDNase significantly against aggregation during storage at 40°C (Chen et al., 1999).

5.4. Formulation of solid protein pharmaceuticals

Although significant progress has been made in the past decade in protein formulation, there is still no single pathway to follow in formulating a solid protein product. In most cases, solid protein products have been developed on a trial-and-error basis.

To achieve successful formulation of solid protein products by lyophilization, one or more of the following formulation excipients may be needed: a buffering agent(s), a bulking agent(s), a protein stabilizer(s), and an antimicrobial agent(s), although stable proteins, such as recombinant human tPA, may be lyophilized without any of the above agents (Overcashier et al., 1997).

5.4.1. Selection of a formulation pH

Lyophilization starts with preparation of a protein solution. Many proteins in solution are stable only in a narrow pH range (see Section 2.2). In addition, pH can strongly affect the solu-

bility of certain proteins, such as rIFN-β-1b (Betaseron®), a hydrophobic protein (Lin et al., 1996). Therefore, an optimum pH is needed to keep a protein stable and soluble in solution.

The solution pH has also been shown to affect stability of a protein during freezing and/or freeze-drying. Freeze-thawing ovalbumin at neutral pH did not cause denaturation, but induced significant structural changes at pH 1.9 (Koseki et al., 1990). Freezing human growth hormone (hGH) at pH 7.4 resulted in the formation of more insoluble aggregates than at pH 7.8 (Eckhardt et al., 1991). Lyophilization of IL-2 at pH 7 induced significant irreversible protein unfolding and aggregation while at pH below 5, the protein remained essentially native (Prestrelski et al., 1995). Thus, the solution pH must be optimal to minimize protein denaturation during lyophilization.

On top of these effects, the formulation pH may have significant impact on long-term stability of solid protein pharmaceuticals, as discussed in Section 5.2. Therefore, the formulation pH should be optimal to allow maximum long-term stability for lyophilized proteins.

To meet all these requirements in different stages, the formulation pH needs to be carefully chosen. Proper selection of a solution pH is the first step toward stabilization of solid protein pharmaceuticals. Very often, the most stable pH for proteins in solution does not offer the best stability in solid state. This is because the inactivation mechanisms of proteins may well be different in the two different states, as reported for bovine pancreatic RNase A (Townsend and DeLuca, 1990). In such cases, a balanced pH must be used.

5.4.2. Selection of a buffering agent(s)

Many buffering agents covering a wide pH range are available for selection in formulating solid proteins. These agents include acetate, citrate, glycine, histidine, phosphate, Tris, etc. A buffering agent, that also stabilizes a protein, is preferable, such as histidine for freeze-dried FVIII SQ (Österberg et al., 1997). For pH-sensitive proteins, sodium phosphate should be avoided because the selective crystallization of Na_2HPO_4 can cause a significant pH drop during freezing, denaturing proteins (see Section 2.2). Instead, potassium phosphate, citrate, histidine, and Tris can be used due to their minimal changes in pH during freezing (Franks, 1990; Carpenter et al., 1997). In addition, decreasing the buffer concentration can also mitigate the pH shift (Pikal, 1999).

Since the effect of different buffering agents on long-term stability of lyophilized proteins is usually unpredictable (see Section 5.2), selection of a buffering agent(s) can only rely on stability studies. In addition, the selection of a proper buffer concentration is also important, as the buffer concentration not only affects the storage stability of lyophilized proteins (see Section 5.2) but also plays a critical role in stabilizing proteins during lyophilization. For example, mannitol could not protect β-galactosidase (2 μg ml^{-1}) in water during lyophilization due to mannitol crystallization (Izutsu et al., 1993). In the presence of 10 mM sodium phosphate buffer (pH 7.4), crystallization of mannitol at 50 mM was inhibited, and about 95% of the enzyme activity was protected. Increasing the concentration of sodium phosphate buffer (pH 7.4) to 200 mM completely inhibited the crystallization of mannitol (500 mM) in β-galactosidase (2 μg ml^{-1}) solution during lyophilization (Izutsu et al., 1993).

5.4.3. Selection of a bulking agent(s)

A crystallizing bulking agent(s) is usually needed in a solid protein formulation to have one or more of the following functions: to provide mechanical support of the final cake, to improve product elegance, to improve formulation dissolution, and to prevent product collapse and blow-out. A bulking agent(s) should have enough solubility, compatibility with the protein, no or minimal toxicity, and high eutectic temperature, allowing efficient freeze-drying.

Different bulking agents may affect stability of solid proteins to different degrees. Therefore, careful selection of a suitable bulking agent may be necessary. For example, both the degradation and aggregation rates of lyophilized IL-1ra (pH 6.5) during storage at 8, 30 or 50°C have been shown to be different in three bulking agents:

mannitol, glycine or alanine (Chang et al., 1996c). Among these three agents, glycine was apparently best in protecting protein stability.

Two bulking agents are frequently used: glycine and mannitol. Glycine has several advantages, including non-toxicity, high solubility, and high eutectic temperature (Akers et al., 1995). Although both free or salt form of glycine can be used, it has been found that neutral glycine crystallizes rapidly, whereas glycine hydrochloride crystallizes slowly, even at the same pH (Akers et al., 1995). As a bulking agent, mannitol may stabilize certain proteins. It has been used both as a bulking agent and as a stabilizer in a lyophilized formulation of transforming growth factor-β_1 (TGF-β_1) (Gombotz et al., 1996).

Most amino acids are potential bulking agents as they easily crystallize out (Mattern et al., 1999). However, formation of acid salts reduces their tendency to crystallize (Mattern et al., 1999). Another crystalline agent, NaCl, is not preferable as a bulking agent due to its low eutectic and glass transition temperature (Carpenter et al., 1997). It was, however, used as a bulking agent in a lyophilized factor VIII SQ formulation because it solubilized the protein (Österberg et al., 1997).

An amorphous excipient(s) in a protein formulation may inhibit crystallization of the bulking agent(s), thus affecting protein stability. Increasing the relative amount of sucrose in a mixture of sucrose and glycine gradually inhibited crystallization of glycine, and at a weight ratio of 1.4:1 (glycine:sucrose), crystallization of glycine was not detectable (Lueckel et al., 1998a). In a different study, crystallization of glycine was not observable under microscope when its concentration was below 29% in the same mixture during freeze-drying; partial crystallization was observed at 43%, but a good lyophilized cake was obtained only at a glycine concentration of about 50% or higher (Kasraian et al., 1998). Similarly, mannitol did not crystallize in the presence of a second non-crystallizing component, such as sucrose, lactose, maltose, or trehalose until the mannitol concentration was over about 30% (w/w) (Kim et al., 1998). Because of this effect, lyophilized rFIX formulation containing 2% (0.26 M) glycine and 1% sucrose (as a stabilizer) had both excellent cake appearance and protein stability, but that containing 1.7% (0.23 M) glycine and 2% sucrose only offered reasonable protein stability with crumbly cake appearance (Bush et al., 1998). Therefore, proper selection of a suitable bulking agent(s) and its relative amount is critical.

5.4.4. Selection of a stabilizer(s)

A solid protein product should be stable during storage at least above 0°C, and preferably under room conditions. To achieve this goal, a protein stabilizer(s) is usually needed to protect a protein during lyophilization and/or long-term storage. Based on the stabilization mechanisms discussed in Section 5.3, a stabilizer(s) for a solid protein product should be at least partially amorphous and able to replace water, forming intimate hydrogen bonds with the protein. Formation of an amorphous glassy state is considered to be a prerequisite, not a guarantee, for protein stability (Pikal et al., 1991; Skrabanja et al., 1994). Since a disordered amorphous material has a lower energy barrier for dissolution than a structured crystalline solid, use of an amorphous stabilizer(s) can also lead to faster dissolution of a solid protein product (Miller et al., 1997).

The widely used stabilizers in solid protein products are sugars. Most sugars do not crystallize under normal operating conditions (Chang and Randall, 1992). Reducing sugars are generally not preferable due to the possibility of reacting with dried proteins via the Maillard reaction. However, reducing sugars can be used if they are better stabilizers. For example, dextrose, a reducing sugar, has been shown to inhibit moisture-induced aggregation of bovine insulin significantly at 1:1 protein:excipient ratio, while trehalose did not stabilize the protein to a significant level (Katakam and Banga, 1995).

Among sugars, sucrose seems to be the most commonly selected. Recently, trehalose started to gain attention due to several advantages, as discussed in Section 3.1. Their relative effect in stabilizing solid proteins for long-term storage is protein-dependent (also see Section 5.3). Here are some more examples. Sucrose was shown to be more effective than trehalose in inhibiting chemical degradation of lyophilized IL-1ra during stor-

age at 8, 30 and 50°C (Chang et al., 1996c), while trehalose at 30 mg ml^{-1} in lyophilized IL-6 formulation inhibited IL-6 aggregation more effectively than sucrose during storage at both 25 and 40°C for 9 months (Lueckel et al., 1998b). Two possible reasons have been offered why trehalose was more effective, (1) trehalose formulation had a higher glass transition temperature; and (2) sucrose could be hydrolyzed to form two reducing sugars for the Maillard reaction. However, the protein molecular mobility in a trehalose formulation was shown to be higher than that in a sucrose formulation at certain temperatures, which might make trehalose a less effective stabilizer than sucrose depending on the protein, degradation mechanisms, and storage conditions (Miller et al., 1997).

Certain salts can be used to stabilize solid proteins (also see Section 3.1). However, the presence of uncrystallized salts in a freeze-concentrate usually depresses T'_g, so the salt content in protein formulations should be kept to a minimum (Franks, 1990). Several factors may affect the extent of salt crystallization, including the nature of salt, salt concentration, and cooling rate. Chang and Randall (1992) have classified salts into three types based on their glass-forming tendency at a given cooling rate and subsequent thermal history, (1) crystallizing salts such as maleic acid, Na_2HPO_4, Na_2SO_4, Na_2CO_3, KCl, and $(NH_4)_2SO_4$; (2) partially crystallizing (doubly unstable glass) salts such as NaCl, $NaHCO_3$, K_2HPO_4, KH_2PO_4, $CaCl_2$, $MgCl_2$, glycine, and β-alanine; and (3) glass-forming salts such as NaH_2PO_4, sodium/potassium citrate, citric acid, histidine, and sodium/potassium acetate. Since glass-forming excipients can inhibit salt crystallization, salts can be potential protein stabilizers in the presence of other amorphous excipients (Hatley and Franks, 1991).

Some polymers can be chosen as protein stabilizers in solid state, as they can increase T_g of protein formulations. On the other hand, they may not be as effective as sugars due to inefficient hydrogen bonding with proteins (Schebor et al., 1996; Cardona et al., 1997; Kreilgaard et al., 1998a). An alternative is to use both polymers and sugars together to achieve enhanced protein stabilization. While polymers increase the T_g of a protein formulation and inhibit crystallization of other excipients, sugars can form intimate hydrogen bonds with proteins. This strategy, however, has not been very effective at least for some proteins. Inclusion of dextran 40 in a lyophilized IL-6 formulation containing sucrose significantly increased protein aggregation during storage at 40°C for 9 months (Lueckel et al., 1998b).

Proteins, like other polymers, have high T_gs. Therefore, increasing protein:excipient ratio increases T_g of protein formulations. Increasing the content of a lyophilized monoclonal antibody from 2.5 to 25 mg ml^{-1} in sucrose (or trehalose at 31.3 mg ml^{-1}) formulation increased the T_g of the formulation from 59 (or 80) to 89 (or 100)°C by DSC (Duddu and Dal Monte, 1997). Increasing the relative content of rbSt to 50% in a sucrose/rbSt mixture gradually increased the T_g from 74°C for pure sucrose to 96°C for the mixture (Sarciaux and Hageman, 1997). Therefore, the relative protein concentration in a formulation should be kept relatively high to prevent collapse. More importantly, a high protein:excipient ratio strongly inhibits excipient crystallization (also see Section 5.3). For example, the lyophilized rhDNase–mannitol formulations were partially crystalline when the relative protein quantity was 17% (w/w) or less, but became amorphous at 80% or more (Chan et al., 1999). At a higher protein:sucrose ratio, lyophilized *Humicola lanuginosa* lipase was shown to be more stable during storage at 60°C, due to effective inhibition of sucrose crystallization by the protein (Kreilgaard et al., 1999). In a more detailed study, the T_{cry} of sucrose at 4.4% water content was $\sim$70°C and increased to 120°C when 20% rbSt was included (Sarciaux and Hageman, 1997). On the other hand, increasing the protein concentration too high may eventually destabilize a protein due to insufficient quantity of a stabilizer, as has been reported for rhIL-1ra (Chang et al., 1996c).

The presence of a bulking agent may affect properties of an amorphous excipient. The T'_g of a mixture of sucrose and glycine (or lysine–HCl) decreased with increasing amino acid:sucrose ratio (Lueckel et al., 1998a). Addition of glycine (up to 71%, w/w) in a sucrose solution decreased

linearly the T'_g of the mixture from -32 to -52°C (Kasraian et al., 1998). When glycine concentration was over 86%, no T'_g was observed. Similarly, increasing mannitol concentrations up to 30% decreased T'_g of sugars, such as sucrose, lactose, maltose, and trehalose (Kim et al., 1998). To keep T'_g at a level high enough for efficient lyophilization, the relative quantity of a bulking agent should be properly chosen and optimized.

5.4.5. Selection of other excipients

Other formulation excipients, such as antimicrobial agents or solubilizers may be used depending on the protein. Both Tween 80 and SDS (more effective) facilitate solubilization of rIFN-β-1b (Betaseron®) (Lin et al., 1996). SDS has also been used to solubilize IL-2 in Proleukin® (Physicians' Desk Reference, 1999).

5.4.6. Overall consideration of formulation excipients

Generally, the total quantity of solid in protein formulations is between 2 and 10%. While a solid content lower than 2% may not form a strong cake, higher amount of solid ($>10\%$) may be difficult to process (Hatley et al., 1996; Carpenter et al., 1997; Jennings, 1999; Willemer, 1999). In addition, higher solid content may affect reconstitution of lyophilized formulations. Breen et al. (1998) demonstrated that lyophilized cakes prepared from 110 mg ml^{-1} bulk took approximately 60 min to reconstitute as compared with less than 5 min for those from dilute bulks. SEM analysis showed that cakes lyophilized from highly concentrated bulks had smaller pores with thicker walls than those from more dilute bulks.

The relative quantity of different excipients is also important because their physical properties are mutually affected. Excipients that provide more than one function in protein formulations should be selected first, such as sugars, which may be used as both cryoprotectants and lyoprotectants. High levels of buffers or salts should be avoided because this may lead to potential pH changes during freezing and likely depression in T'_g and T_g of dried formulations (Pikal, 1990b).

To expedite selection of formulation excipients, two screening methods have been used: measurement of T_g of a lyophilized formulation in the presence of different excipients or determination of IR spectrum of a formulation in comparison to that of a reference (see Section 2.3). Both methods should be used with caution, as these parameters may not always reflect changes in protein activity or stability.

Recent investigations suggest that combined use of sucrose as a protein stabilizer and glycine as a bulking agent may be a good starting point when formulating a solid protein product. The combination of these two excipients has been used successfully in formulating several proteins. The recently developed albumin-free formulation for recombinant factor VIII (rFVIII) contains sucrose, glycine, histidine, $CaCl_2$, and NaCl (Nayar, 1998). Lyophilized rFIX formulation contains sucrose, glycine, histidine, and polysorbate 80 (Bush et al., 1998). Chang et al. (1996c) developed a stable lyophilized IL-1ra formulation, which contains sucrose, glycine, and sodium citrate. This stable IL-1ra formulation was also able to stand a single-step freeze-drying cycle (Chang and Fischer, 1995).

5.4.7. Selection of containers and stoppers

In close relation to selection of formulation excipients, a compatible container should be used. Type I borosilicate glass (treated or untreated) is usually the material of choice for containers due to its strong chemical resistance and low level of leachables. Nevertheless, proteins can be absorbed to glass surfaces to different extents (Gombotz et al., 1996). Since the loss of protein activity from glass surface absorption and surface-induced denaturation is protein-dependent, containers need to be evaluated on a case-by-case basis (Burke et al., 1992).

The volume of a solution for bolus intravenous injection generally does not exceed 10 ml. Therefore, the size of containers for these applications is usually smaller than 20 ml, assuming the volume of the solution to be lyophilized is ideally between about 20 and 50% of the container volume. A relatively small filling volume is preferable for an efficient freeze-drying cycle and a higher protein concentration often results in a lower loss of activity due to surface absorption and stability

during freeze-drying. The lyophilized product can be reconstituted with different volumes of water or solution based on the protein solubility and stability.

Container stoppers may significantly affect long-term stability of solid protein pharmaceuticals. This effect is often due to a stopper-induced increase in moisture content of lyophilized formulations during storage. The percent change in moisture content depends on both hygroscopicity and quantity of the protein formulation in the container. Also, oxygen permeation through stoppers or stopper leakage may increase the partial pressure of oxygen in a product vial and cause potential oxidation of proteins during storage (Wang et al., 1997).

Three stopper-related processes can lead to an increase in residual moisture of protein formulations, (1) transfer of moisture from stoppers to the formulation; (2) diffusion or transmission of moisture through the stopper; and (3) microleaks in the stopper-vial seal (House and Mariner, 1996). The first process is usually dominant (Ford and Dawson, 1994). Stoppers absorb moisture during routine steam sterilization and sterilized stoppers can transfer some of its moisture back to the protein product (DeGrazio and Flynn, 1992; Corveleyn et al., 1997). In fact, the steam autoclaving process is responsible for the majority of water driven into elastomeric stoppers. Chang et al. (1996c) demonstrated that two out of three lots of siliconized stoppers showed transfer of moisture to the lyophilized rhIL-1ra formulation, resulting in a significant increase in moisture content during stability studies. Storage of a lyophilized IL-6 formulation at 25 or 40°C for 9 months led to an increase in moisture content from 0.5 to 2% (Lueckel et al., 1998b). The increase in moisture content can cause a significant drop in T_g. It has been shown that moisture transfer from the stopper dropped the T_g of a sucrose-formulated rFXIII formulation by 11°C after storage at 40°C for 3 months (Kreilgaard et al., 1998a) and that of a vacuum-dried LDH formulation by 35°C after storage at 60°C for 26 weeks (Mattern et al., 1999).

There are many types of stopper materials available. Butyl or halobutyl rubber is often the choice for solid protein formulations because they have relatively low moisture absorption and vapor transmission rates (DeGrazio and Flynn, 1992). Among halobutyl stoppers, bromobutyl stoppers have been shown to be more resistant than chlorobutyl stoppers to moisture absorption during storage and steam sterilization (Corveleyn et al., 1997). To minimize the moisturizing effect, stoppers can be autoclaved at 121°C for 30 min and immediately dried in an oven at 135°C for 5 h before use. On the other hand, overdried stoppers may absorb moisture from the lyophilized formulation and dropped the moisture content of the formulation during storage (Corveleyn et al., 1997). The integrity of stoppers can be tested under high humidity by monitoring the change in moisture content of the protein formulation. Therefore, stoppers should be carefully chosen based on their compatibility with the protein formulation, resistance to formulation pH, excipients and sterilization, moisture/vapor transfer property, and resealability.

5.4.8. Stability testing of final formulations

Selection of the final protein formulation can only be based on stability studies. A variety of formulation parameters in stability studies can be used to compare different formulations, including protein activity, cake physical attributes (shape, color and texture), particulate formation, moisture content, and reconstitution (or rehydratability). Another parameter is the volume of protein formulation preparation, which should not change much after lyophilization and during storage. Although minor shrinkage in volume may be seen, especially at low solute concentrations (Daukas and Trappler, 1998), a significant drop in volume may be an indication of formulation meltback or collapse.

To expedite selection of the final protein formulation, accelerated stability studies are frequently conducted. These stressed stability conditions include high temperature, high humidity, or intensive lighting. One key issue in conducting accelerated stability studies is whether and how well the data obtained at high temperatures can be extrapolated to those under real-time conditions. Often, protein stability results obtained at

high temperatures do not reflect or predict what happens under real-time conditions, due to the complexity of multiple protein degradation pathways at different temperatures. For example, Sun et al. (1998) found that the temperature dependence of G6PDH inactivation in carbohydrate (glucose or sucrose) glassy matrix deviated from Arrhenius relationship between 8 and 93°C. On the other hand, if the multiple degradation processes in proteins can be described separately, or the rate-limiting degradation step does not change within a certain temperature range, prediction of protein stability based on accelerated stability studies is very optimistic. The loss of lactase activity in a lyophilized PVP formulation followed Arrhenius behavior well in a temperature range between 37 and 70°C (Mazzobre et al., 1997). Yoshioka et al. (1994) examined the stability of six protein preparations, including α-chymotrypsin troche, α-chymotrypsin tablet, bromelain tablet A and B, kallikrein capsule, and β-galactosidase powder at elevated temperatures between 40 and 70°C (50 or 75% RH). They found that all of them had complex kinetics, but the inactivation rate exhibited approximately linear Arrhenius relationship. Nevertheless, for proteins with unknown degradation pathways, real-time stability testing has to be conducted for selection of the final protein formulation.

6. Summary

Lyophilization (freeze-drying) is the most common process for making solid protein pharmaceuticals. However, this process can generate a variety of stresses, and denature proteins to various degrees. These stresses include low temperature, formation of dendritic ice crystals, increase in ionic strength, pH changes, phase separation, and removal of the protein hydration shell. Structural changes in proteins during lyophilization can be either reversible or irreversible depending on the protein, and can be conveniently monitored by IR. Proteins sensitive to freezing and/or drying stresses can be stabilized by selection of proper cryo- and/or lyoprotectants. The commonly used cryoprotectants include sugars/polyols, non-aqueous solvents, polymers, protein itself, surfactants, and amino acids. These cryoprotectants can also be used as potential lyoprotectants except non-aqueous solvents. These stabilizers may protect proteins by one or more of the following mechanisms: preferential interaction, replacement of water, formation of a glass, hydrogen bonding, and steric hindrance.

The design of a lyophilization cycle may have significant impact on protein stability during and after lyophilization. Lyphilization cycle-related stresses include freezing rate and temperature, thermal treatment condition, drying rate and temperature, and the final moisture content. Therefore, efforts should be made to design a cycle, which is robust and efficient, and has minimal adverse effects on protein stability. To design such a cycle, protein formulations need thorough characterization and all critical temperatures need to be determined as discussed in Section 4.1.

Solid protein pharmaceuticals may experience a variety of instabilities. Major instabilities include aggregation, deamidation, oxidation, the Maillard reaction, hydrolysis, and disulfide bond formation/exchange. Many factors affect these instabilities, including storage temperature, glass transition temperature of the formulation, residual moisture content, formulation 'pH', crystallization of amorphous excipients, and presence of destabilizing excipients or contaminants. These instabilities may be minimized by proper selection of formulation pH, residual moisture content, and more importantly, formulation stabilizers, such as sugars/polyols, polymers, salts, and surfactants.

Formulation of solid protein pharmaceuticals may require not only suitable stabilizers but also other excipients such as bulking, buffering, and antimicrobial agents. Since the physical properties of these agents are mutually affected, the relative quantity of these agents in a protein formulation is critical and should be determined based on sound experimentation and appropriate stability studies. A proposed protein formulation for initial development trial is 2% glycine, 1% sucrose, and 20 mM buffering agent controlling the formulation pH. To expedite selection of formulation excipients, two screening methods may be used with caution: comparison of T_{gs} or IR spectra of

formulations in the presence of different excipients. However, selection of the final protein formulation requires real-time stability studies.

In summary, development of a lyophilized protein product usually takes an enormous amount of time, labor, and effort, simply because there is no single, short, and mature pathway to follow in formulating such a product, and many experiments are done on a trial-and-error basis. This trend will continue until a breakthrough is achieved in understanding the basic behavior of proteins and their stabilization.

7. Abbreviations

aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
BGG	bovine γ-globulin
BO	bilirubin oxidase
BPTI	bovine pancreatic trypsin inhibitor
BSA	bovine serum albumin
CHAPS	3-[(3-cholamidepropyl) -dimethylammonio] -1-propanesulfate
CMC	carboxymethyl cellulose
Cyt c	cytochrome c
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DSC	differential scanning calorimetry
G-CSF	granulocyte colony-stimulating factor
GDH	glutamate dehydrogenase
G6PDH	glucose-6-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony stimulating factor
HEC	hydroxyethyl cellulose
hIGF-I	human insulin-like growth factor I
HLL	*Humicola lanuginosa* lipase
HP-β-CD	hydroxypropyl-β-cyclodextrin
HPMC	hydroxypropyl methylcellulose
HSA	human serum albumin
IFN-β	interferon-β
IFN-γ	interferon-γ
IL-1α	interleukin-1α
IL-1β	interleukin-1β
IL-2	interleukin-2
IR	infrared spectroscopy
LDH	lactate dehydrogenase
LMW-UK	low molecular weight urokinase
Mb	myoglobin
MDH	maleate dehydrogenase
Met-hGH	methionyl human growth hormone
MS	mass spectroscopy
NMR	nuclear magnetic resonance spectroscopy
PEG	polyethylene glycol
PFK	phosphofructokinase
PVA	polyvinylalcohol
PVP	polyvinylpyrrolidone
rbSt	recombinant bovine somatotropin
rconIFN	recombinant consensus α-interferon
rFIX	recombinant factor IX
rFXIII	recombinant factor XIII
rHA	recombinant human albumin
rhCNTF	recombinant human ciliary neurotrophic factor
rhIL-1ra	recombinant human interleukin-1 receptor antagonist
rhKGF	recombinant human keratinocyte growth factor
rhMCSF	recombinant human macrophage colony-stimulating factor
RNase A	ribonuclease A
SDS	sodium dodecyl sulfate
TGF	transforming growth factor
TNFbp	tumor necrosis factor binding protein
tPA	tissue plasminogen activator
TT	tetanus toxoid

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