Third, Carpenter et al. (36) recently documented more rigorously that freezing-induced denaturation can play an important role in the overall damage to a protein during lyophilization. The impetus for this research was the observation that the disaccharide trehalose was effective at protecting labile enzymes, whereas the constituent monosaccharide, glucose, was not. For example, when PFK is freeze-dried in the presence of 0.2 to 0.4 M trehalose, over 60% of the initial activity is recovered after rehydration (21). In contrast, when similar amounts of glucose are used, the recovery is less than 5%. When considering only the effect of the sugar on the protein during dehydration, these results present a dilemma. This is because if hydrogen bonding of sugar to dried protein in place of water were all that was needed for stabilization, then mono- and disaccharides should provide similar protection. Glucose does not protect during freeze-drying because it provides minimal stabilization during freezing (based on freeze-thawing results), whereas similar concentrations of trehalose are effective at protecting the protein during both freezing and dehydration (21,61).

To examine the separate roles of protein damage and stabilization by freezing and dehydration, Carpenter et al. (36) developed a two-component system for stress-specific stabilization during lyophilization. In this stabilization scheme, polyethylene glycol (PEG) is used as a cryoprotectant and various carbohydrates can be used to protect during dehydration. PEG alone completely stabilizes either LDH or PFK during freeze-thawing. However, it provides little or no protection during dehydration because it crystallizes during lyophilization. When small amounts (e.g., 10–100 mM initial concentration) of trehalose or glucose are added, which alone at the concentrations tested are ineffective at protecting these enzymes during freeze-thawing or freeze-drying, excellent stabilization is noted during freeze-drying. Under conditions where cryoprotection is provided by PEG, glucose is almost as effective as trehalose in stabilizing dried enzymes (i.e., LDH and PFK).

In a complementary structural study of stress-specific stabilization using FTIR spectroscopy, Prestrelski et al. (9) found that the recovery of activity after rehydration correlated directly with the ability of the additives to preserve the native structure of the enzymes in the dried state. Full activity recovery and maintenance of essentially aqueous structure in dried samples were only noted when a combination of PEG and sugar was employed. On the basis of these results, Prestrelski et al. (9) have proposed a model of the conformational events during lyophilization and rehydration, which is shown in Figure 8. Briefly, this model proposes that to recover structure and function after rehydration, the native structure of labile proteins must be retained, both upon freezing and during subsequent dehydration. The appropriate cryoprotectant is required for the initial structural preservation, and a specific stabilizer against drying is needed for the terminal stress during lyophilization. In some instances (e.g., with disaccharides), a single additive can serve both protective functions.

More recently, there has been direct observation of protein structural perturbation in the frozen state using phosphorescence lifetime measurements (62). Reductions in this parameter indicated that freezing perturbed the tertiary structure (at a protein concentration of 3–5 μM) of azurin, ribonuclease, alcohol dehydrogenase, alkaline phosphatase, glyceraldehyde 3-phosphate dehydrogenase, and LDH. The cryoprotectants sucrose and glycerol were tested and found to inhibit the freezing-induced structural perturbations, with almost complete protection noted at a 1 M concentration.