reduced from on the order of 20% water to very low levels, often less than 1%. This additional drying may be considered an additional stress. Indeed, the water substitute hypothesis (6) is based on the proposition that a significant thermodynamic destabilization occurs when the hydrogen bonding between protein and water is lost during the last stages of drying. The use of a "water substitute" as a lyoprotectant allows a hydrogen bonding interaction between protein and the water substitute, which thermodynamically stabilizes the native conformation and preserves activity.

During the early portions of freezing, the system is mostly aqueous and of low or moderate viscosity. During the last stages of freezing, and during both primary and secondary drying, the system is a glass or at least not much above the glass transition temperature. These differences are potentially important in the protein's response to a given thermodynamic stress. The timescales of the various stages of freeze-drying are also different. Relative to drying, freezing is relatively fast. Freezing is typically over within a few hours while drying often requires days. However, it must be noted that primary drying, or ice sublimation, constitutes the longest portion of the drying process. Although some water desorption does occur during primary drying, low water content in the solute phase is not achieved until all ice has been removed, and the process enters the secondary drying stage (25). During the early portion of secondary drying, the water content decreases quickly from roughly 10% to 20% to less than 5% within a few hours. Thus, assuming that it is during this drying period that the "drying stress" occurs, the timescale is roughly the same as the freezing timescale.

Since both the last part of freezing and the entire drying stages are normally carried out in a glassy or near glassy state, molecular mobility should be greatly restricted relative to the fluid state that prevails during early freezing, and the dynamic response to a thermodynamic stress will be significantly slower than for a fluid system, assuming the dynamic response depends on viscosity. If the dynamic response is sufficiently slow, thermodynamic *instability* will have no consequence as insufficient time is available for unfolding and subsequent degradation. Recent studies in our laboratories of the kinetics of protein unfolding in highly viscous aqueous systems demonstrate that the protein unfolding rate is strongly coupled to viscosity, at least for the two proteins studied, phosphoglycerate kinase and β-lactoglobulin, in high sucrose content systems. These data (Figs. 1 and 2) suggest that unfolding is on the timescale of months, even at temperatures 10°C to 20°C above the glass transition temperature. The fact that protein inactivation during drying does occur suggests that the mobility needed for inactivation is nearly completely decoupled from viscosity in these systems. Of course, if the mechanism for inactivation does not involve protein unfolding, we do not necessarily expect inactivation kinetics to track with unfolding kinetics.

STABILIZATION OF PROTEINS FOR FREEZE-DRYING: SELECTED EMPIRICAL OBSERVATIONS

In-Process Stability: Freeze-Thaw and Freeze-Dry Stability

While many proteins survive the freeze-drying process with little or no degradation, other proteins exhibit significant degradation and loss of activity during processing. Multimeric proteins seem particularly prone to degradation during freeze-drying (26–29). Degradation during the freeze-drying process may arise