238b One Step Mechanism for the Nucleation of Insulin Crystals

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Insulin is stored in the pancreatic B cells before its release into the blood stream in the form of rhombohedral crystals of Zn-insulin hexamers. It is believed that the crystals protect the insulin from proteolysis in the Golgi complex. A single crystal nucleates and grows in each B cell, probably to minimize the void space. The likely mechanism that underlies the nucleation of a single crystal involves a very low homogenous nucleation rate and a suitable heterogeneous nucleation center on the B cell wall. To elucidate the physicals principles involved in the nucleation of insulin crystals we have investigated the homogeneous nucleation of rhombohedral crystals of porcine insulin in the presence and absence of the co-solvent acetone. We found that the nucleation rates for insulin are lower by two orders of magnitude than for any other protein, and that without acetone, i.e., under conditions which closely resemble the physiological, the nucleation rate is about ten times lower that in the presence of acetone. Application of the nucleation theorem revealed that the nucleus size with acetone is also smaller, indicating significantly lower nucleation barrier that in the absence of the co-solvent. This inconsistency could be due to two independent reasons: (i) the nucleation theorem does not apply to insulin nucleation, i.e., it follows a two-step nucleation mechanism, recently shown for several protein and small molecule systems (1), or (ii) insulin crystallization is regulated by kinetics of growth of the near critical clusters, reflected in the pre-exponential factor for the nucleation rate, rather than by free energy barriers. If a two-step mechanism applies, the nucleation precursor could be mesoscopic metastable droplets of a dense liquid phase that are undetectable by optical microscopy. We characterized supersaturated insulin solutions by time-dependent dynamic light scattering (DLS) (2). We found that indeed dense liquid droplets of limited lifetime of ~ 100 nm size, i.e., the potential nucleation precursors, are present in acetone-containing and acetone –free solutions. However, these droplets appeared more frequently and were larger in the acetone-free solutions, where the nucleation rate is lower. These observations suggest that the droplets are not precursors for the formation of ordered solid nuclei. Along with differential refractometry, static light scattering (SLS) was used to determine insulin's second osmotic virial coefficient B2. We found that the B2's were consistently lower for the acetone-containing insulin solutions, indicating stronger net attraction between insulin molecules. Since both conditions are inductive of crystallization, we conclude that the higher values of B2 in acetone free solutions reflect the presence of a repulsive maximum at intermediate separations in the intermolecular interaction potential, likely due to water structuring around the insulin molecules (3). This conclusion agrees with a previous thermodynamic analyses, according to which acetone destroys the shell of structured water (4). Numerous theoretical and experimental works, including in our group, have shown that in systems with intermolecular potentials with humps mesoscopic metastable liquid clusters fare present (5). This correlation explains the higher frequency of metastable dense liquid droplets in acetone free solutions, evidence by DLS. The DLS and SLS results indicate that if a two-step mechanism involving a dense liquid precursor operates, a higher nucleation rate should be expected in acetone free solutions, contrary to the actual observations. We conclude that the nucleation of rhombohedral crystals of insulin follows a one step mechanism and the reason for the low nucleation rate is the slow kinetics of growth of the near critical clusters. This conclusion is further supported by the fact that in acetone-containing solutions, where the nucleation rate is higher, the rate of attachment of molecules to their respective growth sites in a crystal is also higher (6).

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