disulphide linkages—one intrachain between cysteine (Cys) A6 and A11 and two interchain linkages between Cys A7 and B7 and Cys A20 and B19, respectively. The secondary structure of human insulin comprises two α helices in the A chain (A2–A8 and A13–A20) and a single α-helix (B9–B19) and a β strand (B21–B30). While the A chain and the B9–B19 helix form a rigid structural unit, the B25–B30 and the B1–B8 regions are flexible. Endogenous insulin is synthesized after tryptic cleavage of the C-peptide from proinsulin (Fig. 1a). Individual synthesized monomers of insulin, combine to form a dimer, and two such dimers with two zinc ions form a tetramer which is eventually assembled and stored as hexameric complex with two zinc ions and a calcium ion. (Fig. 1b) [12, 38]. The hexameric assembly of insulin–zinc complex and its allosteric regulation is key to biophysical properties of insulin in liquid pharmaceutical preparations. It is well understood now that insulin undergoes phenolic ligand- and zinc-mediated conformational change between the T_6–T_3R_3 and the R_6 states, wherein the T and R conformations, respectively, represent the tense and relaxed structure of the monomers in a hexameric assembly [32, 33, 38, 41–43]. Liquid pharmaceutical preparations of insulin normally containing phenol and/or metacresol as preservatives are effectively stabilized such that the insulin hexamer exists in the R_6 conformation and has optimal chemical stability (Insulin's chemical stability and the underlying instability mechanisms are discussed in subsequent section). It is the underlying knowledge on the physiological hexameric assembly of insulin in complex with zinc that forms the basis for using zinc-based bulk crystallization as a final recovery and polishing step in industrial insulin manufacture process.

Potential Routes of Degradation: Stabilization Approaches Insulin undergoes chemical modifications primarily at the Asn A21 and Asn B3 sites with the respective desamido species (Asp) formed under different pH conditions. The Asp A21 species is formed under acidic conditions, whereas the Asp B3 species is formed under neutral conditions [6–8]. Besides the deamidation pathway, insulin also undergoes covalent dimerization. Following a common pathway as the deamidation reaction, covalent dimerization primarily occurs via covalent linkage of the cyclic intermediate at A21 position with the A or B chain N-terminal from another insulin molecule as described in the scheme in Fig. 2 [45].

Factors influencing the formation of desamido species and covalent dimers at low pH (typically in the range of pH 2–5), and also during lyophilization (particularly at low pH), have been well characterized [14, 15, 44, 45]. Pikal and Rigsbee evaluated the stability of amorphous insulin prepared by freeze-drying of the supernate from a zinc–insulin crystal suspension, the pH of which was previously adjusted to 7.1. Their comprehensive storage stability evaluation (at high temperatures) using size-exclusion chromatography and reverse-phase chromatography suggested that the amorphous form of insulin was far more stable than the corresponding crystals of insulin. The degradation pathways for insulins as evaluated by chromatographic assays suggested that the A21 site was involved in instability mechanisms. The secondary structures of both types of insulins were further evaluated by differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR); the findings suggested that both forms of insulin retained a higher-order structure.