

A Molecular Dynamics Study of β -Cyclodextrin Inclusion of Glucagon During Simulated Freeze-Drying and Reconstitution [†]

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Abstract

Freeze-drying is a critical process for ensuring the long-term stability of therapeutic polypeptides. This study used all-atom molecular dynamics simulations to investigate the interactions between glucagon and β -cyclodextrin (β -CD) during a multistage process designed to mimic freeze-drying and reconstitution. Glucagon is a highly flexible 29-amino-acid peptide hormone with an inherently unstable secondary structure, and the molecular-level basis of excipient-mediated protection remains unclear. Simulations were performed using the OPLS-2005 force field and the SPC water model. The protocol included room-temperature equilibration at 300 K, cooling to 233 K, four consecutive drying stages represented by the progressive removal of water molecules, and subsequent reconstitution. Structural changes were evaluated using backbone root-mean-square deviation, radius of gyration, residue-level flexibility, dihedral-angle distributions and peptide–excipient interactions. Through specific inclusion complexation, β -CD achieved superior preservation of the local C-terminal α -helix and a functionally relevant conformation associated with glucagon receptor binding, although protection came at the expense of overall conformational stability. At the end of the fourth drying stage, the glucagon C-terminus was located inside the hydrophobic cavity of β -CD. Five hydrogen bonds were also observed between glucagon and β -CD. Local stabilisation within β -CD did not extend to the peptide as a whole. Following reconstitution, the backbone RMSD increased to approximately 6–7 Å, showing that β -CD helped preserve the C-terminal helix but did not maintain the initial global conformation. These findings provide an atomistic explanation of how β -CD may selectively protect structural regions of glucagon through supramolecular host–guest interactions during freeze-drying-related stresses.

Keywords: glucagon; β -cyclodextrin; molecular dynamics; freeze-drying; peptide stability



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1. Introduction

Freeze-drying (FD) serves as a key formulation technology to enhance long-term stability. The process involves freezing the aqueous drug solution, followed by sublimation of the ice under vacuum to remove the majority of water, thereby converting the drug into a more stable solid powder [1]. However, the freeze-drying process can introduce physical and chemical instabilities to the drug molecules.

Glucagon is a peptide hormone secreted by pancreatic A-cells, clinically used via subcutaneous or intramuscular injection for the emergency treatment of hypoglycaemia. In its crystalline state, glucagon's secondary structure is entirely α -helical. In solution, however, its conformation is highly unstable, existing in a dynamic equilibrium between α -helical and random coil structures [2]. This intrinsic flexibility makes it highly prone to aggregation under various concentration, pH, and temperature conditions, accompanied by a structural transition to more stable anti-parallel β -sheets [3]. Crucially, research has shown that the preservation of the C-terminal α -helical conformation enhances its receptor binding potency, which is essential for its bioactivity and long-term stability [4]. The binding of glucagon to its receptor is a two-domain binding model, where the C-terminal first binds to the extracellular domain, and this interaction facilitates the binding of the N-terminal to the transmembrane domain to activate the receptor [5].

As potential alternatives to polysorbate surfactants, cyclodextrins (CDs) have garnered attention due to their unique molecular structure and stabilisation mechanisms. Their most central and unique mechanism is inclusion complexation. CDs possess a hydrophobic inner cavity and a hydrophilic outer surface, enabling them to specifically encapsulate exposed aromatic amino acid residues on the protein surface, forming inclusion complexes that inhibit protein aggregation [6]. In addition, CDs also exhibit surfactant-like behaviour by preferentially adsorbing to interfaces and competing with the protein, thereby reducing interface-induced denaturation [7].

For example, MD simulations were used to investigate the effects of excipients on protein stability during freeze-drying and successfully simulated key protective mechanisms such as "preferential exclusion" and "water replacement" [8]. Furthermore, other studies have successfully implemented full-cycle simulations covering the freezing, drying, and reconstitution stages, utilising a coarse-grained model to reveal key damage and repair mechanisms of protein during the FD process [9]. Together, these precedents establish MD simulation as a mature and validated tool for investigating protein–excipient interactions under freeze-drying conditions. However, the majority of this research has focused on well-structured proteins. In contrast, the protective mechanisms for small therapeutic peptides like glucagon, which are often conformationally flexible and lack a persistent, well-defined tertiary structure in solution, remain to be fully explored under freeze-drying stresses.

2. Materials and Methods

2.1. Initial Structure and Simulation Setup

Glucagon is a single-chain peptide containing 29 residues. The initial structure of glucagon was obtained from the RCSB Protein Data Bank (PDB ID: 6PHI), which was determined by X-ray diffraction. This structure, which modelled 28 of 29 residues, was completed by adding the missing N-terminal histidine residue using the Schrödinger Maestro software (2026-3).

All simulations were performed with Desmond (Schrödinger Release 2024-4; Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, USA; Maestro–Desmond Interoperability Tools, Schrödinger, LLC, New York, NY, USA), using the OPLS-2005 force field. Each system was prepared by centering the glucagon peptide in a solvated cubic box (15 Å minimum distance to edge) and adding 0.005 M NaCl and neutralising counterions. The β -CD formulation comprised one glucagon molecule, six glycine (buffer) molecules and six β -CD molecules at pH 3, following the experimental freeze-drying formulations [10]. Hereafter, the glucagon + glycine + β -CD formulation is referred to as the β -CD system. The RESPA integrator was used with a time step of 2.0 fs for bonded interactions. A cutoff radius of 9.0 Å was applied to short-range non-bonded interactions. For reproducibility, initial velocities were generated using a randomised custom seed of 2007.

2.2. Simulated Freeze-Drying and Reconstitution Cycle

Room Temperature (RT) Equilibration: The system was simulated for 100 ns at 300 K (NPT) to achieve a stable, solvated state, with the trajectory recorded every 25 ps. Freezing (F): A 31 ns protocol cooled the system from 300 K to 233 K (NPT), with the trajectory saved every 10 ps. Drying (1D-4D): This was performed in four consecutive steps (1D, 2D, 3D, 4D), each lasting 20 ns at 233 K (NVT), with the trajectory saved every 20 ps. Water molecules beyond 15 Å, 10 Å, 5 Å, and 3 Å from the glucagon and other molecules in the system were removed. Reconstitution (REC): The dried system was rehydrated with SPC water models in a cubic box with 20 Å between the box edge and glucagon. The stage was simulated for 200 ns at 300 K (NPT), with the trajectory saved every 50 ps. Extended Reconstitution (REC_E): To ensure thorough equilibration and conformational sampling, reconstitution simulation was extended for an additional 300 ns under the same conditions, with the trajectory saved every 100 ps. The simulation protocol is summarised in Table 1.

Table 1. The protocol of full-cycle freeze-drying and reconstitution simulation.

Stage	Duration	Ensemble	Temperature	Key Condition
RT	100 ns	NPT	300 K	Full solvation
F	31 ns	NPT	300 → 233 K	Freezing
1D-4D	4 × 20 ns	NVT	233 K	Progressive water removal: 15, 10, 5 and 3 Å
REC	200 ns	NPT	300 K	Full re-solvation
REC_E	300 ns	NPT	300 K	Extended re-solvation

2.3. Structural and Interaction Analysis

To assess the overall conformational changes and stability of glucagon, the root-mean-square deviation (RMSD) of the backbone C α atoms was calculated using the RMSD plot in Schrödinger Maestro. To measure the changes in the volume and compactness of glucagon, the radius of gyration (Rg) was calculated using the Rg plot in Schrödinger Maestro. To investigate the flexibility of individual amino acid residues, the root-mean-square fluctuation (RMSF) of each residue's C α atom was calculated using the RMSF plot in Schrödinger Maestro.

The conformation of proteins can be described by their dihedral angles, phi (ϕ) and psi (ψ). To evaluate the stability of the C-terminal α -helical structure during a long-term reconstitution simulation, dihedral angle analysis was also performed on residues 15–29 from the REC_E stage. To investigate the intermolecular interactions between glucagon and excipients (β -CD), hydrogen bond analysis was conducted throughout the full-cycle FD process using the hydrogen bond interaction count tool in Schrödinger Maestro. A hydrogen bond was considered to exist if the following criteria were met: (i) the distance between the donor and acceptor atoms was less than 3.5 Å, and (ii) the hydrogen-donor-acceptor angle was less than or equal to 30°.

3. Results

3.1. Global Conformational Behaviour

At room temperature, the β -CD system remained conformationally stable. Its RMSD stabilised at ~8 Å, while its Rg was maintained at ~11 Å, preserving a more extended, native-like conformation.

The β -CD system underwent a significant conformational rearrangement during the 1D stage, after which its Rg slowly decreased, suggesting it guides the peptide to adapt to the anhydrous environment. Notably, the stabilising effect of β -CD observed at RT did not persist after the freeze-drying process.

Ultimately, the reconstituted glucagon conformation exhibited high diversity and heterogeneity, with elevated RMSD and Rg values, indicating poor overall stability.

3.2. C-Terminal Secondary-Structure Preservation

The results show that β -CD preserved more α -helical segments throughout the simulation, retaining not only the central helix but also an additional short helix near the C-terminus. This suggests that β -CD may have a unique advantage in maintaining specific secondary structure segments. The β -CD system preserved the α -helical structure, showing a densely populated cluster in the α -region.

To further investigate the long-term changes in the secondary structure of glucagon, a dihedral angle analysis of the C-terminal residues (15–29) was performed on the REC_E stage, as shown in Figure 1. The β -CD system remained stable, with its dihedral angles tightly clustered in the α -helical and bridge regions.

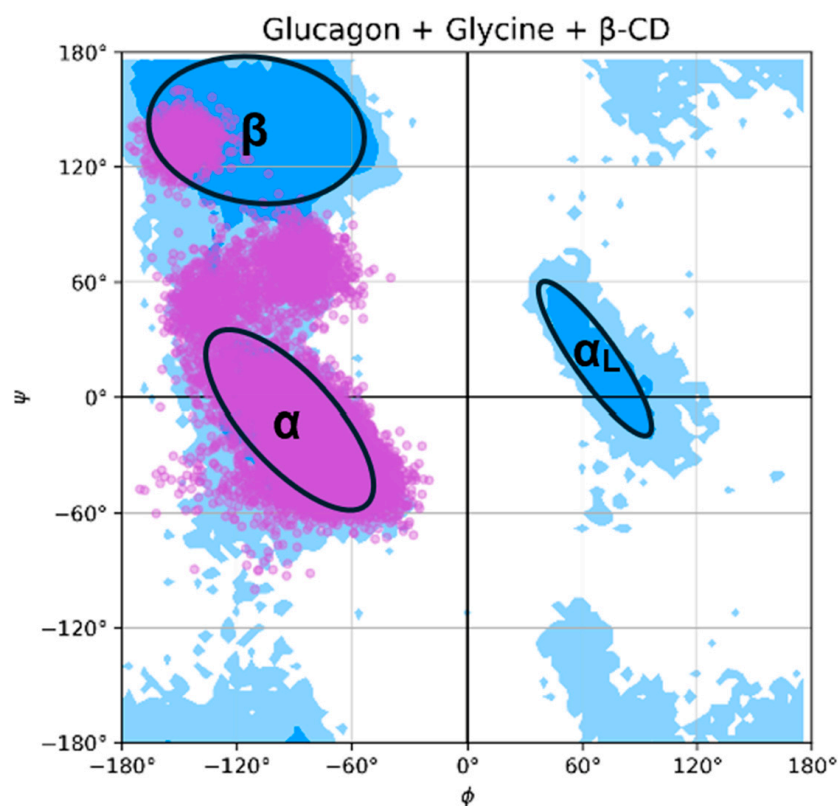


Figure 1. Ramachandran plot for glucagon residues 15–29 in the β -CD formulation (glucagon + glycine buffer + β -CD) during extended reconstitution (REC_E). The points represent the final 1001 trajectory frames. The background regions indicate sterically allowed conformations, with the darker blue areas corresponding to the most favoured canonical secondary-structure regions.

3.3. β -CD–Glucagon Interactions During Drying

At the end of the 4D simulation, five hydrogen bonds were formed between β -CD and glucagon, four at the N-terminus and one at the C-terminus, though two of these were unfavourable (Figure 2). Furthermore, the C-terminal of glucagon bound to the hydrophobic inner cavity of β -CD, preserving part of its α -helical structure.

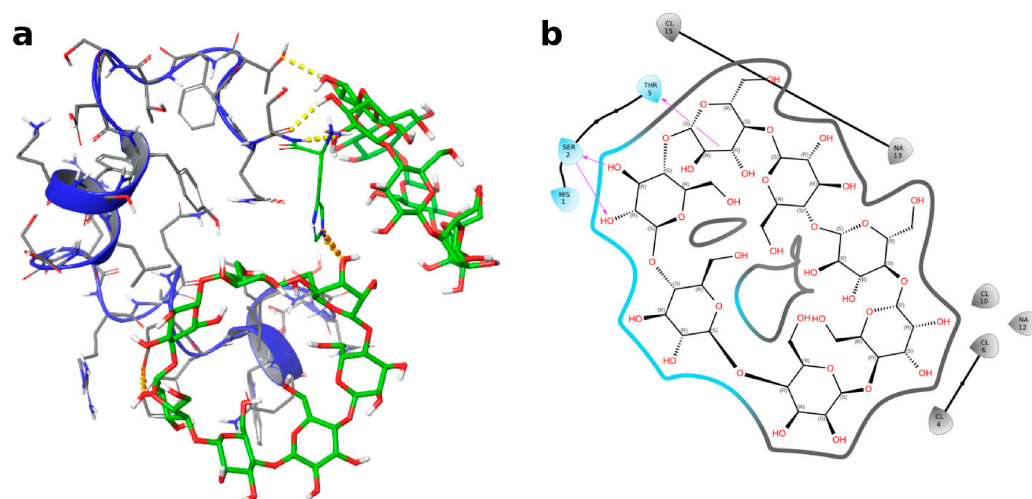


Figure 2. Interactions between β -cyclodextrin (β -CD) and glucagon derived from the final frame of the fourth drying (4D) simulation. **(a)** 3D representation of the binding site between β -CD (stick model) and glucagon (blue ribbon, residues involved in interactions are depicted as sticks). Hydrogen bonds are shown as dashed lines, where yellow and orange lines represent favourable and unfavourable interactions, respectively. **(b)** 2D ligand interaction diagram of the specific residues involved in the interaction. Hydrogen bonds are indicated as purple arrows.

4. Discussion

During the simulation, β -CD demonstrated a highly specific stabilisation mechanism, the core of which involves its hydrophobic inner cavity interacting with multiple aromatic amino acids in the glucagon peptide (Phe6, Tyr10, Tyr13, Phe22, Trp25) to form stable inclusion complexes [6,11]. This is consistent with observations in Figure 2, where β -CD's hydrophobic binding to the C-terminal of the peptide helped preserve parts of its secondary structure, providing a direct, dynamic confirmation of this well-established mechanism.

Although β -CD exerts a strong protective effect at room temperature, the limitations of its mechanism become apparent under the harsh conditions of freeze-drying. At this stage, the primary challenge for the protein shifts from “preventing hydrophobic aggregation” to “finding an effective substitute for water molecules.” The simulation results indicate that β -CD's specific inclusion mechanism is not as effective as before. During the initial drying (1D stage), the peptide underwent a drastic conformational rearrangement to adapt to the removal of water, which was confirmed by sharp fluctuations in RMSD, Rg, and hydrogen bond counts. Ultimately, the reconstituted glucagon conformation exhibited high diversity and heterogeneity, with elevated RMSD and Rg values, indicating poor overall stability.

While the molecular dynamics simulation approach employed in this study provides a powerful tool for understanding the intermolecular interactions between excipients and glucagon, it also possesses certain inherent limitations. The most significant of these is the vast discrepancy between the simulation timescale and that of the physical process. Moreover, to approximate drying computationally, we employed a staged removal of water, which is a useful proxy but does not capture the complex heat and mass transfer dynamics of the actual process. Furthermore, discrepancies exist between the simulation system setup and real experimental conditions. Our simulation box contained only a single glucagon molecule, allowing for detailed analysis of intramolecular changes and conformational tendencies. Therefore, it is essential to perform multiple independent replicate simulations for each condition to ensure that observed phenomena are statistically significant and reproducible, rather than random fluctuations.

5. Conclusions

The research reveals that the effective structural stabilisation of glucagon is not straightforward, involving a critical balance between maintaining global conformational stability and preserving a specific, functionally important α -helical structure. By specific inclusion complexation, β -cyclodextrin preserves the local α -helical structure within the C-terminal region, a conformation associated with the peptide's receptor binding and long-term stability. However, this targeted protection comes at the cost of global conformational stability. These findings provide molecular insights into the structure–function consequences of peptide–excipient interactions, offering a theoretical basis for the rational design of effective freeze-drying formulations for peptide drugs.

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Abbreviations

The following abbreviations are used in this manuscript:

β -CD	β -cyclodextrin
FD	Freeze-drying
MD	Molecular dynamics
RMSD	Root-mean-square deviation
Rg	Radius of gyration
RMSF	Root-mean-square fluctuation
RT	Room temperature
F	Freezing stage
1D–4D	First to fourth drying stages
REC	Reconstitution stage
REC_E	Extended reconstitution stage
NPT	Constant number of particles, pressure and temperature
NVT	Constant number of particles, volume and temperature
SPC	Single-point-charge water model
PDB	Protein Data Bank

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