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Disassembly and trimer formation of E2 protein cage: the effects of C-terminus, salt, and protonation state

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Abstract

E2 protein cages, which consist of the assembled 60 subunits, were simulated at two levels of protonation to mimic their electrostatic properties at pH 4 and 7. Starting with the initial configuration of the assembled 60-mer, either the truncation of C-terminus or the protonation state of pH 4 induces the disassembly of 60-mer, leading to the formation of trimers. Hydrodynamic radii (R_h) of E2 monomer, trimer, and 60-mer are calculated from diffusivities, which agree well with experimental values. R_h become smaller for the disassembled E2, confirming the formation of clusters smaller than 60-mer. Anionic Asp419 and Glu421 of C-terminus interact with Lys240 of a neighboring trimer, while hydrophobic Leu424 and Met425 of C-terminus interact with Pro313~Ala318 of a neighboring trimer. In particular, when anionic residues of E2 60-mer are protonated (neutralized), the assembled 60-mer can be still stabilized by hydrophobic interactions of C-terminus. These indicate that the stability of E2 60-mer is attributed to both electrostatic and hydrophobic inter-trimer interactions of C-terminus. These findings support the experimental interpretation regarding the formation of trimer as an intermediate between E2 monomer and 60-mer, and help explain the key role of C-terminus for electrostatic and hydrophobic inter-trimer interactions.

Keywords: molecular dynamics simulations, protein self-assembly, caged protein, drug delivery

1. Introduction

Caged proteins, which have hollow structures composed of self-assembled protein subunits, have shown great potential for therapeutic applications such as gene and drug transporters,[1-4] because their size distribution, surface functionality, conformation, and stability can be easily controlled by genetic engineering.[5-12; 3] A number of protein subunits self-assemble to a spherical cage that can encapsulate drug molecules and transport them to specific cancer cells.[13; 14] Then, the encapsulated drugs can be released from a caged protein through biodegradation, diffusion, or pH change.[13; 14] These caged proteins are typically derived from viruses, enzymes, the ferritin superfamily, and heat shock proteins.[4; 2] In particular, E2 (dihydrolipoamide acetyltransferase) subunits of the pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus* self-assemble to a spherical caged protein of 60-mer that can retain the folding structure at temperatures up to ~85 °C,[15-18] and thus they have been widely studied. To increase its efficiency of drug delivery, the assembly mechanism and structure of E2 protein cage need to be understood, which has motivated many experimental and simulation studies.

The effects of protein modification and solvent condition on the structural stability of E2 protein cage have been investigated using various experimental techniques such as circular dichroism (CD), differential scanning calorimetry (DSC), dynamic light scattering (DLS) and transmission electron microscopy (TEM). The Wang group showed that the mutated E2 protein cages are highly thermostable and have the inner cavity to encapsulate molecules [18]. In particular, they observed that E2 protein cage with truncation of N-terminus retains the assembled structure

of 60-mer at pH 7 but irreversibly dissociates at pH 5, which respectively correspond to pH conditions of cell surface and lysosome, indicating potential applications for therapeutic delivery.[19] They also found that this pH dependence is modulated by ionic strength, repulsive histidine interactions, and electrostatic interactions,[20] which were tested for drug molecules such as doxorubicin[8; 21] and CpG.[22; 23] Peng et al. showed that the truncation of α -helix of C-terminus promotes the transition between monomer and trimer, but not the formation of the assembled 60-mer, implying that C-terminus is important for the self-assembly process from trimer to 60-mer.[24] Iron-binding peptides were incorporated to the interior surface of E2 protein cage for iron mineralization,[25] and antibody fragments were conjugated to the surface of E2 protein cage,[26] showing successful modification of inner and exterior surfaces of E2 protein cage. Recently, α -helix of E2 C-terminus was replaced with GALA peptide, showing that the cage disassembles at pH 7 but self-assembles at pH 4, indicating the inversed pH dependence.[27] These experiments have shown the structural change of E2 protein cage under various protein modifications and solvent conditions, although no direct observation of E2 conformation was provided because of the limited resolution of experimental techniques.

To support and complement experimental observations at nearly atomistic resolution, molecular dynamics (MD) simulations have been performed. Our group showed that monomers of E2 wild-type have inter-trimer interactions, while those with the truncation of C-terminus disperse, in agreement with experiments.[24] In particular, we found that anionic Asp419 (D419) and Glu421 (E421) of E2 monomer interact with cationic Lys233 (K233), Arg238 (R238), and Lys240 (K240) of a neighboring E2 monomer, indicating that these electrostatic inter-trimer interactions are important for the assembly of 60-mer.[24] C-terminal group of E2 monomer was

replaced with GALA peptide, showing that GALA peptide retains α -helical structure at pH 4 but predominantly becomes a random coil at pH 7, which supports the experimental observation of the inversed pH dependence.[27] Recently, Hezaveh et al. simulated E2 and E3BP (E3-binding protein) monomers, showing that C-terminals are important for the stability of the assembled structure of E2 and E3BP monomers.[28; 29] Although these simulations have revealed inter-trimer interactions of E2 C-terminus, the whole E2 protein cage of 60-mer, which has a size of ~25 nm, has not been simulated, and hence the transition between trimer and 60-mer has not yet been directly observed through computation.

In this work, we therefore perform all-atom MD simulations of the whole E2 protein cage of 60-mer with explicit water and ions. Starting with the initial configuration of experimentally observed E2 protein cage (60-mer) [15], E2 protein cage of 60-mer is either retained or disassembled to trimers, to an extent dependent on the presence of C-terminus, different levels of protonation, and ionic strength, which favorably compare with experiments. Hydrodynamic radii of E2 monomer, trimer, and 60-mer are calculated from diffusivities, which also agree with experimental measurement. In particular, specific amino acids of C-terminus are found to be important for electrostatic and hydrophobic inter-trimer interactions, which helps explain the experimental observations regarding the formation of trimer as an intermediate between monomer and 60-mer, as well as supports the role of C-terminus for the assembly of 60-mer.

2. Methods

All simulations and analyses were performed using the GROMACS2016.4 simulation

package [30-32] with the GROMOS 54a7 force field (FF) [33-35]. The structure and coordinates of E2 protein cage were downloaded from the Protein Data Bank (PDB code: 1B5S) [15]. Since the downloaded structure includes only backbones, appropriate coordinates of side chains of E2 protein cage and its mutated (or truncated) ones were generated using the Swiss-Pdb viewer.[36] All Asp, Glu, and His residues were protonated to represent electrostatic properties at pH 4, while only D419 and E421 were protonated for specific mutated one, as described in Table 1.

Table 1. List of simulations

No. of monomers	Name	pH	NaCl (mM)	Protonation or truncation
1	E2-mono	7	-	-
3	E2-tri	7	-	-
60	E2	7	-	
	E2-ΔC9	7	-	Truncation of C-terminus (419-425)
	E2-P2	7	-	Protonation of Asp419 and Glu421
	E2-500	7	500	-
	E2-4	4	-	Protonation of all Asp, Glu, and His
	E2-500-4	4	500	Protonation of all Asp, Glu, and His

A single E2 protein cage of 60-mers was solvated with ~800,000 SPC waters[37] in a periodic box of size 30 nm/size, and 120~2460 counterions (Cl^-) were added to establish electro-neutrality (Figure 1). Also, 8130 Na^+Cl^- ions were additionally added to generate the salt concentration of 500 mM, as shown in Table 1. A temperature of 298 K and a pressure of 1 bar were maintained by using a velocity-rescale thermostat[38] and Parrinello-Rahman barostat[39] in the NPT ensemble. A real space cutoff of 1.2 nm was applied for Lennard-Jones and electrostatic forces with the inclusion of particle mesh Ewald for long-range electrostatics.[40] The LINCS

algorithm was used to constrain the bond lengths.[41; 42] Simulations were carried out for 100 ns with a time step of 2 fs on computer facilities supported by the National Institute of Supercomputing and Networking/Korea Institute of Science and Technology Information with supercomputing resources including technical support (KSC-2018-C3-004). The last 20 ns trajectories were used for analyses.

Figure 1. Snapshots at the beginning (left; 0 ns) and the end (columns 2-6; 100 ns) of simulations. Black lines represent E2 proteins, while 500 mM NaCl ions are shown as blue and red dots. For clarity, water and counterions are omitted. Note that side views show only one cross section of the system and cannot capture all E2 proteins. The images were created using Visual Molecular Dynamics [43].

3. Results and Discussion

3.1. Effects of C-terminus, pH, and salt on the disassembly of E2 protein cage

A single E2 protein cage, which consists of 60 subunits, was simulated with explicit water for 100 ns. E2 protein was modified by either truncation of C-terminus or protonation of Asp, Glu,

and His residues to mimic electrostatic properties at pH 4. Simulated systems are named in Table 1. “E2-mono” and “E2-tri” represent E2 monomer and trimer, respectively. For E2 60-mer, “E2” indicates the unmodified E2 protein cage, which is followed by “- Δ C9”, “-P2”, and “-500”, respectively corresponding to the truncation of C-terminus, the protonation of D419 and E421, and the inclusion of 500 mM NaCl. “-4” was also attached at the end of names, indicating E2 cage with protonated Asp, Glu and His residues (pH 4). For example, “E2-500-4” designates E2 60-mer with 500 mM NaCl at the protonation level of pH 4.

Figure 1 shows initial and final snapshots for simulations of E2 protein cage composed of initially assembled 60 subunits, as observed in experiments [15]. E2, E2-P2, E2-500, and E2-500-4 retain their assembled structures for whole simulation time, while E2- Δ C9 and E2-4 become disassembled, indicating the effect of C-terminus and the protonation state. Also, E2-4 becomes disassembled, but E2-4-500 keeps the assembled structure, indicating the dependence on salt ions. Secondary structures of E2 and E2- Δ C9 were calculated using the DSSP program,[44] which show that fractions of α -helix, β -sheet, and random coil compare favorably with experimental values, although the α -helix fraction of E2 is slightly higher for experiment (Table 2). Secondary structures of E2 and E2- Δ C9 do not significantly differ for both simulation and experiment, indicating that the disassembly of E2 cage is not modulated by its secondary structure.

Table 2. Fractions of secondary structures of E2 and E2- Δ C9. Errors for simulation values are within 0.01.

	E2		E2- Δ C9	
	Simulation	Experiment[24]	Simulation	Experiment[24]
α -helix	0.33	0.39	0.32	0.32-0.33
β -sheet	0.23	0.19	0.23	0.19-0.20
Random coil	0.24	0.24	0.25	0.23-0.24

To quantify the conformation of E2 protein cage, mass density profiles (kg/m^3) of E2 proteins and water were calculated. Figure 2 shows that proteins and water are regularly positioned for E2, E2-P2, E2-500, and E2-500-4, indicating the symmetrical structure of the assembled 60-mer. However, mass densities for E2- Δ C9 and E2-4 are irregularly distributed in the simulate box, indicating the disassembled conformation. To further quantify the extent of disassembly, we calculated the number of “clusters” and the average number of E2 monomers per cluster as a function of time, where a “cluster” is either a complex of any size or a single monomer. Here, if the distance between any atoms of different molecules is less than 0.2 nm, those molecules are considered to be a cluster. Other criteria with a larger distance up to 0.4 nm produced similar qualitative trends, confirming that the analysis does not significantly depend on the distance criteria. In Figure 3, E2, E2-P2, E2-500, and E2-500-4 show that the number of cluster and the average number of E2 monomers per cluster are respectively ~ 1 and ~ 60 for whole simulation time, while those values for E2- Δ C9 and E2-4 significantly fluctuate. These results show that the assembled 60-mer structure is retained for E2, E2-P2, E2-500, and E2-500-4, but not for E2- Δ C9 and E2-4, indicating that C-terminus, ionic strength, and protonation state (or pH) significantly influence the stability of the assembled E2 60-mer.

Figure 2. Mass density profiles of E2 proteins and water.

Figure 3. Number of “clusters”, where a “cluster” is an aggregate of any size, including single E2 monomers (top), and average number of aggregated E2 monomers per cluster (bottom) as a function of time. Note that for E2, E2-P2, E2-500, and E2-500-4, 60 subunits remain assembled as a protein cage for whole simulation time, and thus their plots for the number of cluster (~ 1) and average number of E2 monomers per cluster (~ 60) are overlapped.

3.2. Disassembly and trimer formation of E2 60-mer: diffusivity and hydrodynamics

To quantify the disassembled structure of E2- Δ C9 and E2-4, we calculated distributions for the number of E2 monomers per cluster. In Figure 4, E2, E2-P2, E2-500, and E2-500-4 show a high peak only for the value of 60, indicating the assembled structure of 60-mer. For E2- Δ C9 and E2-4, the numbers of E2 monomers per cluster are distributed in the broad range of 60 or less, indicating the formation of clusters smaller than 60-mer. In particular, E2- Δ C9 and E2-4 show a high peak for the value of 3, indicating the formation of trimers, which supports Peng et al.'s experimental suggestion regarding the role of trimer as an intermediate between monomer and 60-mer.[24]

Figure 4. Distributions of the number of E2 monomers per cluster.

This decreased cluster size was also confirmed by calculating diffusion coefficients, which were obtained from the slopes of the mean-square displacements (MSD) of all E2-protein atoms versus time (D_{PBC}) and then correcting D_{PBC} for finite size effects using the formula $D = D_{PBC} + k_b T \xi / 6\pi\eta L$ [45], where k_b is Boltzmann's constant, T (298 K) is temperature, L (~30 nm) is the equilibrated cubic-box length, $\xi = 2.837297$, and η is the solution viscosity. Since the viscosity increases with higher solute concentration, the solution viscosity was corrected using the Einstein formula $\eta = \eta_w(1 + 2.5\phi)$ [46], where ϕ (~0.09) is the volume fraction of the solutes, and η_w is the viscosity of pure water (taken to be 0.5 cP at 298 K for SPC water) [47], leading to $\eta = 0.6125$. Thus, the final diffusion constant is given by $D = \left[D_{PBC} + \frac{k_b T \xi}{6\pi \times 0.005(1+2.5\phi)L} \right]$. Using this diffusion coefficient, we calculated the hydrodynamic radius (R_h) from the Stokes-Einstein equation for a sphere with no-slip boundary conditions, $R_h = k_b T / 6\pi\eta D$. [48] For comparison, E2 monomer and trimer were also simulated for 100 ns, and their D and R_h were calculated. In Table 3, D values are 23.0, 13.7, and $4.4 \times 10^{-7} \text{ cm}^2\text{s}^{-1}$ for E2 monomer, trimer, and 60-mer, respectively yielding the R_h values of $1.9 (\pm 0.1)$, $3.2 (\pm 0.2)$, and $10.0 (\pm 0.1)$ nm, which favorably compare with the experimental radii of 4 nm (trimer) and 12.5 nm (60-mer) measured by DLS [24]. Table 3 also shows that R_h nearly equal radii of gyration (R_g), apparently because E2 protein cage is symmetrically spherical with large inner cavity. Figure 5 compares D and R_h for different E2 systems, showing much higher D and lower R_h for E2- $\Delta C9$ and E2-4 than for others, indicating that E2 protein cages disassemble and thus yield smaller clusters. Note that E2- $\Delta C9$ and E2-4 disassemble to trimers as well as larger ones (Figure 4), and hence their calculated R_h are larger

than that of trimer. Also, it cannot be ruled out that the disassembly of E2 protein cage might eventually occur in the E2 system that do not show it for the simulation described here. Whether or not the results here can show a complete disassembly of E2 60-mer, simulations clearly show the formation of trimers disassembled from E2 60-mer with the truncation of C-terminus or low pH, which supports experiments that measured the size and molecular weight of clustered particles and suggested the formation of trimers as an intermediate between monomer and 60-mer.[24]

Table 3. Diffusion coefficients (D), hydrodynamic radii (R_h), and radii of gyration (R_g) of E2 monomer, trimer, and 60-mer.

		Diffusivity ($10^{-7} \text{ cm}^2 \text{ s}^{-1}$)		Radius (nm)	
		D_{PBC}	D	R_h	R_g
Monomer	E2-mono	12.0 ± 0.1	23.0	1.9 ± 0.1	1.8 ± 0.1
Trimer	E2-tri	4.5 ± 0.8	13.7	3.2 ± 0.2	2.6 ± 0.1
60-mer	E2	1.0 ± 0.1	4.4	10.0 ± 0.1	9.8 ± 0.1
	E2- ΔC9	2.7 ± 0.3	6.1	7.2 ± 0.3	-
	E2-P2	1.1 ± 0.1	4.5	9.8 ± 0.2	9.7 ± 0.1
	E2-500	1.6 ± 0.3	5.0	8.8 ± 0.6	9.7 ± 0.1
	E2-4	3.1 ± 0.1	6.4	6.8 ± 0.1	-
	E2-500-4	1.7 ± 0.1	5.1	8.6 ± 0.1	10.0 ± 0.1

Figure 5. Diffusion coefficients (D) and hydrodynamic radii (R_h).

3.3. Electrostatic and hydrophobic inter-trimer interactions

As discussed above, simulations show that truncation of C-terminus or low pH can induce the disassembly of E2 60-mer, leading to the formation of trimers, which indicates that the stability of E2 protein cage is modulated by inter-trimer interactions. Previous simulations and experiments showed that anionic residues (D419 and E421) and hydrophobic residues (L422, L423, L424, and M425) of C-terminus interact with the neighboring trimer,[24] although the strengths of those electrostatic and hydrophobic interactions have not been systematically studied. To resolve this,

we calculated radial distribution functions (RDFs) between those specific charged or hydrophobic residues, defined as $g_{AB}(r) = \frac{\langle \rho_B(r) \rangle}{\langle \rho_B \rangle_{local}}$, where $\langle \rho_B(r) \rangle$ is the density of particles B at a distance r around particles A , and $\langle \rho_B \rangle_{local}$ is the density of particles B averaged over all spheres around particles A with radius r_{max} (half of the box length). Figure 6 shows RDFs of K233, R238, K239, and K240 with respect to D419 and E421 of C-terminus for the system E2. RDF peaks of D419 and E421 are much higher for K240 than for other cationic residues, indicating that anionic D419 and E421 of C-terminus interact electrostatically with cationic K240 of the neighboring trimer rather than with other cationic residues, as visualized in Figure 6. Recall that E2-4 disassembles to trimers, but E2-4-500 retains the assembled 60-mer structure. This implies that salt ions weaken repulsive interactions between protonated (cationic) His residues of E2-4, again confirming the dependence on electrostatic inter-trimer interactions. To understand hydrophobic inter-trimer interactions, we also calculated RDFs between L422~M425 residues of C-terminus and P313~A318 residues of the neighboring trimer, showing much higher peaks for L424 and M425 than for L422 and L423 (Figure 7), which indicates that L424 and M425 of C-terminus are important for hydrophobic inter-trimer interactions.

Figure 6. Radial distribution functions (RDFs) of cationic K233, R238, K239, and K240 with respect to anionic D419 and E421 for the system E2. Monomers of different trimers are distinguished as yellow and green ribbons. Side chains of anionic and cationic residues are shown as thick red and blue lines, respectively.

Figure 7. RDFs between hydrophobic 313~318 and 422~425 residues for the system E2. Colors of proteins are the same as those in Figure 6.

Since both anionic D419/E421 and hydrophobic L424/M425 of C-terminus are important for the inter-trimer interaction, we further calculated RDFs between D419/E421 and K240, and those between L424/M425 and 313PIFALA318 for different E2 systems. Figure 8 shows that RDF peaks of the D419/E421-K240 interaction are much higher for E2 and E2-500 than for E2-P2, E2-4, and E2-500-4, as expected, since D419 and E421 of E2-P2, E2-4, and E2-500-4 are protonated (neutralized), leading to weaker charge interactions. Note that E2- Δ C9, which does not include C-terminus and thus cannot have both electrostatic and hydrophobic interactions of C-terminus, is disassembled to trimers, while E2-P2 (with protonated (neutralized) D419 and E421) retains the assembled structure, implying that the assembled structure of E2 60-mer can be stabilized by

hydrophobic inter-trimer interactions of C-terminus. This is confirmed by calculating RDFs between L424/M425 and P313~A318, showing high RDF peaks regardless of different E2 systems (Figure 8, bottom), indicating strong hydrophobic interactions of C-terminus. These indicate that the stability of E2 60-mer is attributed to both electrostatic and hydrophobic interactions of C-terminus, which helps understand experimental observations regarding the key role of electrostatic and hydrophobic residues for inter-trimer interactions.

Figure 8. RDFs between charged D419/E421 and K240 (top), and those between hydrophobic L424/M425 and 313PIFALA318.

Experimentally, the trimer formation has been interpreted by measuring the size and molecular weight of clustered E2 particles, although the trimer has not been directly observed [24]. This structural transition of E2 may be modulated by electrostatic and hydrophobic inter-trimer interactions of C-terminus [24], which has been supported by all-atom MD simulations. However, previous all-atom MD simulations have been performed with only a few monomers due to limitations of system size and time scale. In this work, we simulate the whole E2 60-mer and its transition to the trimer, which has been experimentally interpreted but not directly observed from experiments and previous simulations. Our simulations capture the formation of trimers disassembled from E2 60-mer with C-terminus truncated or at pH 4, as well as show that electrostatic and hydrophobic inter-trimer interactions are modulated by specific amino acids of C-terminus. In particular, when anionic residues of E2 60-mer are protonated, the assembled 60-mer can be still stabilized by hydrophobic interactions of C-terminus, indicating that the stability of 60-mer is modulated by both electrostatic and hydrophobic interactions, which supports the experimental observation and suggestion regarding the trimer formation and inter-trimer interactions. Note that these effects of C-terminus and pH may differ in other protein cages, and thus our results should be applied to specific protein cages with caution. It would obviously be interesting to investigate various protein cages under different solvent conditions, which we hope to report on elsewhere.

4. Conclusions

We performed MD simulations of E2 protein cages composed of the assembled 60

subunits. E2 cage, which was initially composed of the assembled 60 subunits with a spherical shape, retains its assembled structure, while the truncation of C-terminus or the protonation state of pH 4 yields the disassembly and trimer formation of E2 60-mer. Hydrodynamic radii of E2 monomer, trimer, and 60-mer are calculated from their diffusivities, in agreement with experimental values of trimer and 60-mer. Hydrodynamic radii become smaller when E2 60-mer disassembles, confirming the formation of small clusters. To understand the key amino acids that influence the stability of E2 60-mer, interactions of specific residues of C-terminus were analyzed, showing that anionic D419 and E421 of C-terminus interact with K240 of a neighboring trimer, while hydrophobic L424 and M425 of C-terminus interact with P313~A318 of a neighboring trimer. This indicates that the stability of E2 60-mer can be modulated by electrostatic and hydrophobic inter-trimer interactions. In particular, when anionic residues of E2 60-mer are protonated (neutralized), the E2 60-mer still retains the assembled structure, indicating the importance of hydrophobic interaction of C-terminus for the stability of E2 60-mer. These results support the experimental observation of trimer formation as an intermediate between monomer and 60-mer, and help explain the roles of specific amino acids of C-terminus for electrostatic and hydrophobic inter-trimer interactions.

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