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Investigating the Influence of Ionic Concentrations and Subunit Interactions on the Self-Assembly of E2 Protein

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Abstract. Understanding of self-assembly mechanism of virus-like protein cage is important in controlling release of molecular cargo for applications in drug delivery. E2 core protein is composed of 60 subunits which self-assemble into a hollow 25-nm porous protein cage. Due to its virus like dodecahedral structure without infectious capacity, we are interested in its potential application as nanocapsule in drug delivery. In our study, extrinsic and intrinsic factors that influence self-assembly were evaluated. Extrinsic factors, such as salts and denaturants, were introduced into E2 protein solution. The hydrodynamic diameter of the E2 core protein was used to monitor its disassembly or aggregation. We found that the protein size increased proportionally with the salt concentration while the size decreased as the denaturant concentration increased. To assess intrinsic factors that influence E2 self-assembly, we identified some key amino acids at interfaces of subunits and performed site-directed mutagenesis on them. Characterizations of each mutant for size and secondary structure contents were performed. We found that mutations at the inner surface have no apparent effects on both protein sizes and secondary structures. The mutations at intra-trimer interfaces changed the secondary structure contents but the protein sizes remained stable.

Keywords: Nanocage, Protein cage, Intersubunit, Intrsubunit
PACS: 87.14.E-

INTRODUCTION

Over the years, nanoparticles have been developed to enable effective drug delivery. Drug delivery using nanocarriers takes advantages of their small sizes which allow penetration through smaller capillaries and subsequently the passive accumulation at tumor sites. The drug of interest can be adsorbed on, attached to, or entrapped/encapsulated in the nanoparticles. There are different types of nanoparticles, such as nanospheres, micelles, polymersomes, dendrimers, nanocapsules, liposomes, protein cages, peptide nanotubes, viral-based platforms, and DNA nanostructures, which serve as nanocarriers in drug delivery [1]. Protein caged nanoparticles have several advantages over competitive polymer-based synthetic delivery systems such as micelles, dendrimers, and liposomes [2]. The protein cages which are homogenous in size can be used as templates to synthesize nanoparticles with narrow size distribution. The protein cages can also be used to encapsulate cargos such that they are sequestered from the external environment. The exterior surfaces of the protein cages can be modified by introducing functional groups to attach ligands which enable targeted drug delivery. Currently, various protein cages, such as virus capsids, ferritin, and heat shock proteins have drawn much attention for their potential applications in drug delivery.

The E2 protein cage, which is the dihydrolipoyl acetyltransferase component of pyruvate dehydrogenase from *Bacillus stearothermophilus*, has the potential to serve as a molecular carrier [3]. It consists of 60 E2 polypeptides that are arranged in 532 symmetry to create a pentagonal

dodecahedron with each vertex composed of three E2 chains and each face of five E2 chains [4]. Previous works have demonstrated that drug molecules can be encapsulated into the inner architecture of the E2 protein cage [3, 5]. In order to control the release of these drugs at target sites, understanding the self-assembly mechanism of E2 protein is required.

Salts are known to have a variety of effects on the structure and function of proteins such as the solubility and stability of proteins. The influence of anions and cations on protein solubility/stability tends to follow the Hofmeister series [6]. The order of ions in the series can be understood in terms of the effectiveness of ions in increasing the surface tension of the surrounding water; this will alter the stability of a protein by influencing the extent of its hydration. Salts at higher position of the Hofmeister series such as sulfate are good salting-out agents because they interact strongly with water; water molecules surrounding the salt ions are more structured relative to bulk water. In addition, electroselectivity effects can arise if there are appropriate ion binding sites in proteins. These effects are exploited in protein purification procedures and in many protocols for the growth of crystals for X-ray diffraction analysis [7].

The interactions between subunits provide important contributions to the protein quaternary structure. Virus capsid proteins are composed of multiple subunits that form dodecahedral structure. The N-terminus of the subunits are involved in forming a trimer structure in the assembly of virus capsids [8-10]. The removal of the N-terminus has been shown to effect the interactions between subunits and result in smaller capsid size [10]. The alteration of interaction at critical sites will alter the assembly behavior of the protein complex. The introduction of histidines at dimeric interfaces of N-methyl-D-aspartate (NMDA) receptors generates repulsive electrostatic interactions at

acidic pH and consequently affects the stability of the multimeric protein receptor. Similar characteristic is also observed in virus-like particles (VLP) such as the E2 protein in this investigation [11, 12].

In this work, the effects of ionic concentration on the assembly of E2 protein were investigated to understand the electronic shielding of the protein cage. In addition, potential key amino acids at the subunit interfaces were mutated to evaluate their effects on the assembly of E2 protein and further understand its assembly mechanism.

MATERIALS AND METHODS

Materials

E. coli strains DH5 α (Zymo Research) and BL21(DE3) (Stratagene) were used as host cells. Plasmid pET-11a containing wild-type E2 gene (pE2-WT), was generously provided by Prof. Szu-Wen Wang at UC Irvine. Ampicillin and ammonium sulfate were purchased from Sigma. Sodium chloride and potassium chloride were obtained from Merck. Tris was from Vivantis. Sodium phosphate was supplied by Affymetrix. LB was purchased from BD. Guanidine hydrochloride (GuHCl) and urea were from Sigma-Aldrich.

Construction of Mutant Genes

Using a molecular visualization program, PyMol [13], we visualized the crystallographic structure of E2 protein (PDB ID 1B5S) and identified some key amino acids at subunit interfaces that may play critical roles in the correct assembly of E2 subunits (Figure 1). Three prolines at position 377 (P377) located at the interfaces of three E2 subunits (intra-trimer interface) formed a trimeric cluster. On the inner surface of the protein cage, alanine 351

(A351) might mediate the interactions between any two subunit. In order to evaluate the roles of these potential key amino acids in the E2 protein assembly, we performed site-directed mutagenesis on these critical amino acids. The principle of performing mutation is to introduce a new amino acid with alternative physicochemical properties to replace the original one. We incorporated either a large hydrophobic amino acid (phenylalanine, F) and a large hydrophilic amino acid (histidine, H) to replace the original small, slightly hydrophobic with the smallest side chain amino acid (alanine, A) at site 351, or used histidine and alanine to replace the original relatively large, hydrophobic amino acid (proline, P) at site 377.

FIGURE 1. Molecular structure highlighting identified key amino acids and relevant protein domains. (A) Overview of the E2 protein and identified key amino acids: A351 in red spheres, and P377 in cyan spheres. (B) Top view of hypothesized trimer intermediate highlighting residues A351 (red) at the inner surface, P377 (cyan) at the intra-trimer interface.

To construct these mutants, the gene encoding E2-WT was PCR-amplified using *Pfu* DNA polymerase (Fermentas) with the following corresponding oligonucleotides: 5'- GC ACT ATT ACC AAC ATC GGC TCT CAT GGT GGT CAG TGG TTC ACC CC -3' for E2-A351H; and 5'- GC ACT ATT ACC AAC ATC GGC TCT TTT GGT GGT CAG TGG TTC ACC CC -3' for E2-A351F; and 5'- GGT ATT GGT CGT ATA GCC GAA

AAG CAT ATC GTT CGT GAC GGT GAA ATC -3' for E2-P377H; and 5'- GGT ATT GGT CGT ATA GCC GAA AAG GCG ATC GTT CGT GAC GGT GAA ATC -3' for E2-P377A. The sequences of the mutated genes were confirmed by DNA sequencing service from 1stBASE. The mutant expression plasmids were constructed by digesting the expression vector pET-11a and the mutated genes with NdeI and BamHI restriction endonucleases (Fermentas). Both vector and synthetic genes were purified with 0.8% agarose gel electrophoresis and ligated with T4 DNA ligase (Fermentas).

Protein Expression and Purification

E. coli strain BL21(DE3), transformed with the pE2-WT or mutant plasmid, was cultured in Luria-Bertani medium supplemented with ampicillin (100 mg/L) under constant shaking at 37 °C. Expression was induced by the addition of 1 mM IPTG when the culture reached an optical density of 0.6-0.8 at 600 nm. Cells were harvested 3 hours after induction by centrifugation and stored at -80 °C. Thawed cells were resuspended in 50 ml of Tris buffer (20 mM Tris, pH 8.7, filtered, containing 0.02 % NaN₃ and 5 mM EDTA) and lysed in a French pressure cell (Thermo Scientific). The insoluble fraction was removed by ultracentrifugation at 40,000 ×g for 1 hr. The resulting supernatant was subjected to heat treatment at 72 °C for 20 min. Ultracentrifugation (40,000 ×g, 1 hr) was performed to remove the denatured *E. coli* protein aggregates. The recovered supernatant was loaded onto an anion exchange column (HiPrep™ Q 16/10 Q FF; GE Healthcare) previously equilibrated with Tris buffer. Fractions containing E2 protein were pooled and concentrated using stirred ultrafiltration cell 8200 (Millipore) with membrane filtration of 100 kDa molecular-weight-cut-off (Millipore). The protein solution was stirred gently under pressure limit

of 10 psi. For long-term storage, proteins were kept at -80 °C. Protein concentrations were determined using a Micro BCA Protein Assay Kit (Pierce) with bovine serum albumin as a standard. SDS–PAGE analyses were run on 4–20 %Tris–HCl gels.

Stability Characterization of E2-WT

To investigate the effects of salts and denaturants on the self-assembly, the E2-WT was incubated in buffers containing respective salts and denaturants. The amount of protein used in each measurement was approximately 275 µg in a final volume of 1 ml. Two types of buffers, 20 mM Tris and 50 mM sodium phosphate, pH 7.4, were used in the salt effects investigation. Stock solutions of 6.0 M NaCl, 4.5 M KCl, and 4.0 M (NH₄)₂SO₄ in either of the two buffers were mixed with the E2-WT. To evaluate the effects of denaturants on self-assembly, stock solution of 6M GuHCl or 10 M urea was added to sodium phosphate solution, pH 7.4, containing E2-WT to the desired denaturant concentrations. The mixtures were incubated for one hour at room temperature prior to measurements. The hydrodynamic diameter was measured using dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern) technique with 5-minute equilibration at 25 °C. Each result was obtained by an average of 3 measurements.

Characterizations of Mutant Proteins

The concentrations of mutant proteins were determined by using a Micro BCA Protein Assay Kit (Pierce). SDS-PAGE and MALDI-TOF mass spectrometry were adopted to determine the subunit molecular mass of mutant proteins.

In a possible drug delivery pathway, the drug carriers will be internalized to target cell via endocytosis. The drug

carriers will experience physiological pH and acidic pH in succession. Therefore, pH-dependent disassembly of E2 protein is preferable for its potential applications. To investigate the pH-dependent assemblies of mutant proteins after substituting its key amino acids, we characterized the conformational changes of these mutant proteins at physiological pH of 7.4 and lysosomal pH of 5.0.

We used DLS to measure the hydrodynamic diameters of E2-WT and mutant proteins, E2-A351H, E2-A351F, E2-P377H, and E2-P377A, at both pH 7.4 and 5.0. The protein concentration for each measurement was about 400 µg/ml in sodium phosphate buffer with final volume of 1 ml. All protein samples were measured after 1 hour incubation in each buffer at the specified pH value. Results were an average of three measurements.

Far-UV circular dichroism (CD) technique was performed on a Chirascan™ Circular Dichroism Spectrometer to evaluate the secondary structure and determine the unfolding of WT and mutant proteins. The ellipticity as a function of wavelength for each protein at both pH 7.4 and 5.0 was obtained. WT and mutant protein samples with concentration of 1 mg/ml in sodium phosphate buffer (pH 7.4 and 5.0) were scanned from 180 nm to 260 nm at 25 °C in 0.2 mm path length quartz cell. Results were an average of three scans. E2-WT and correctly assembled truncated E2 protein at the physiological pH gave the characteristic CD profile of an α -helix rich protein with minima at 208 and 222 nm [3, 11].

RESULTS AND DISCUSSION

The E2-WT Size Increases as the Buffer Ionic Concentration Increases

The hydrodynamic diameter of E2-WT was measured with increasing concentration of salts (NaCl, KCl, or $(\text{NH}_4)_2\text{SO}_4$) dissolved in Tris or sodium phosphate buffer at pH 7.4. As the concentrations of $(\text{NH}_4)_2\text{SO}_4$ or NaCl increased, the hydrodynamic diameters of E2-WT in both Tris and sodium phosphate buffer increased. At the same salt concentration, the protein sizes increased more significantly with $(\text{NH}_4)_2\text{SO}_4$ than NaCl (Figure 2A and 2B). The larger protein size is consistent to the specific Hofmeister ion interactions. It was reported that when pH is higher than its isoelectric point (pI), the effects of salts on protein follow the Hofmeister series, whereas when pH is lower than the pI, the effects follow reversed Hofmeister series [14]. The calculated pI of E2 subunit is 7.14 while the experiments were conducted at pH 7.4. The stabilization of protein is partially dependent on the water-mediated interactions between peptide groups. Hofmeister ions are generally thought to exert their effects on protein stabilities indirectly by changing the hydrogen bonding properties of water [6]. The combination of salting-out effect on nonpolar groups and salting-in effect on peptide groups of Hofmeister ions contribute to the protein stability and cause corresponding conformational changes. According to the effectiveness order of various salts in increasing the surface tension of water on the Hofmeister series [6], $(\text{NH}_4)_2\text{SO}_4$ is at higher position than NaCl, which means that the E2 protein should have lesser solubility in $(\text{NH}_4)_2\text{SO}_4$ solution because of the more stabilized folded state of protein resulting from higher water surface tension. We observed consistent result as reflected by the higher hydrodynamic diameter in the presence of $(\text{NH}_4)_2\text{SO}_4$

compare to NaCl. Additionally, the electroselectivity effect, which implies that the sulphate group can bind more effectively to the charged group of protein than chloride ions [15], may also contribute to the phenomenon.

FIGURE 2. The effects of salts on hydrodynamic diameters of WT E2 protein. Salts were dissolved in two kinds of buffer (sodium phosphate buffer and Tris buffer). (A) $(\text{NH}_4)_2\text{SO}_4$; (B) NaCl; (C) KCl.

KCl seemed to have no effects on the E2 protein stabilities. The hydrodynamic diameter of E2 remained relatively constant in both sodium phosphate buffer and tris buffer with KCl concentration range between 0 M and 3.5 M (Figure 2C). Even though KCl has the same anion as NaCl, potassium ion has higher salting-out effect than sodium ion in the Hofmeister series. The smaller hydrodynamic diameter observed in the presence of KCl (Fig. 2B and 2C) may be due to the screening effect as the atomic radius of potassium ion (1.3 Å) is larger than that of sodium ion (0.97 Å) [16].

Figure 2 also showed that at the same salt concentration, the observed hydrodynamic diameters of E2-WT in the sodium phosphate buffer was consistently lower than in tris buffer and was more consistent to the crystal structure data as well as electron micrograph measurements [3]. In a long-

term incubation study, results showed that the E2 protein retains its original size longer when suspended in sodium phosphate buffer (data not shown).

The Size of E2-WT Decreases as the Concentration of Denaturants Increase

The size of E2-WT in different concentrations of GuHCl was measured. Figure 3A indicated that GuHCl had no effect on E2 protein size when its concentration was lower than 2.5 M. At GuHCl concentration higher than 2.5 M, the protein size decreased abruptly and reached 13 nm which might be the size of either individual subunit or stable intermediate composed of multiple subunits. The observation indicated that GuHCl at concentration above 2.5 M can denature the E2 protein completely.. The GuHCl concentration is higher than previously reported values where the E2 protein was observed to dissociate at 1.5 M GuHCl upon 1-hour incubation time at room temperature [4]. This may be due to the different analytical technique used to determine the denatured condition.

Urea was also adopted as denaturing agent. Compared to GuHCl denaturation, E2 protein presented distinct response to urea (Figure 3B). The hydrodynamic diameters of E2 protein increased slowly until the concentration of urea was 6.5 M. It was reported that to denature CPSMV (capsid protein of cowpea severe mosaic virus) completely, urea concentration as high as 8.0 M was required [17].

FIGURE 3. The effects of denaturants on the hydrodynamic diameters of WT E2 protein. (A) GuHCl; (B) urea.

E2 protein was observed to be more susceptible to GuHCl than urea. Previous study showed that GuHCl was expected to be twice as strong as urea in denaturing globular protein [21]. GuHCl is a salt that will ionize in aqueous solution. At lower concentration, it has masking capability so that it reduces or eliminates any stabilizing or destabilizing electrostatic interactions in the protein. The results that E2 protein stayed stable until the concentration of GuHCl reached 2.5 M in in agreement with the theory. At higher concentration, the binding of guanidine ions to the protein pushes the equilibrium toward unfolded state [22]. On the other hand, the denaturing action of urea was assumed to be mainly based on its ability to bind to the protein. Urea molecule is uncharged so it is not expected to affect the intermolecular and intramolecular electrostatic interactions within the protein. Thus, protein requires relatively high concentration of urea to influence its stability. Monera and Vecchio *et al.* attempted to mimic the electrostatic masking effect of GuHCl by conducting urea denaturation in the presence of NaCl or KCl. The decrease of urea concentration requirement to denature protein implied that ion binding significantly prompted denaturation [17, 23].

Mutant Proteins Show Correct Assemblies at Physiological pH

The mutant plasmids were successfully constructed and the mutant proteins were produced and purified. SDS-PAGE (Figure 5) and MALDI-TOF MS (not shown) results presented that the molecular masses were within 0.2 % of the theoretical mass of the corresponding E2 proteins. The hydrodynamic diameters of these mutant E2 proteins were measured by DLS. The data showed that the modified mutant proteins were comparable to that of the E2-WT protein (Table 1). The similar dimensions and molecular weights of these mutant proteins to those of the E2-WT protein indicated the correct assemblies of the mutant scaffolds. The hollow sphere architecture of the E2 scaffold remained stable after mutations were performed on its subunits. These correct assemblies with non-native functionalities would allow the studies of controlled release from the E2 scaffold. Dalmau *et al.* had previously demonstrated that the E2 scaffold with mutations in its hollow cavity had the capability to encapsulate guest drug and drug-like molecules [3, 5].

FIGURE 4. SDS-PAGE showed correct molecular mass of the mutant proteins. Lane1: E2-WT; lane 2: E2-A351H; lane 3: E2-A351F; Lane 4: E2-P377H; Lane 5: E2-P377A.

We aimed to evaluate the extent of effects of mutations on key amino acids on the stability of E2 protein. Previous studies demonstrated that E2-WT protein was insensitive to pH change [11, 24]. Our objective was to design a drug carrier that would release its cargo upon experiencing pH change from intercellular fluid (pH 7.4) to lysosomal environment (pH 5.0) during endocytosis. To this objective, we investigated the sensitivity of the modified E2 scaffold to pH change from 7.4 to 5.0. Electrostatic repulsions between adjacent protonated histidines at the key mutation sites at pH 5.0 were expected to trigger disassemblies of mutant E2 proteins.

The protein hydrodynamic sizes at pH 5.0 were measured using DLS and the results were summarized in Table 1. Hydrodynamic radius of 24-28 nm implied that the E2 protein assumed a correctly folded quaternary structure [3]. The two mutant proteins that had mutation at their inner surface (E2-A351H, E2-A351F) yielded nearly constant size values which were comparable to that of E2-WT scaffold, and demonstrated that no pH-dependent disassemblies were observed. The slight deviations from the expected hydrodynamic radius [25] might have resulted from the purities of the samples rather than size differences in the mutant proteins. The results indicated that mutations at common site on inner surface of the E2 scaffold did not change the stability and pH-sensitivity of E2 scaffold. The interactions on this site were not critical in maintaining the folded state of E2 protein. Besides, the limited exposed surface to the surrounding solvent here might prevent the protonation of the imidazole residue of histidine and further reduced the effect of mutation on this site to the overall protein stability.

E2-P377H and E2-P377A had mutations at the intratrimer interfaces. Three prolines at position 377 formed a cluster at the intersection of the trimeric structure (Figure 1B). Amino acid replacement at this site was

expected to alter the close interactions which were observed in E2-WT. However, at both pH 7.4 and 5.0, DLS data indicated that the hydrodynamic diameters of these mutant proteins were within the E2-WT size range (Table 1). The mutations seemed to have no apparent influences on protein stabilities upon pH change. The expected repulsive interactions among the three histidines at pH 5.0 did not show any consequences on protein size.

TABLE (1). Summary of particle sizes for WT and mutant E2 proteins at pH 7.4 and 5.0. Hydrodynamic diameters (in nm) of WT and mutant E2 proteins which have mutations at their key amino acids at pH 7.4 and 5.0 (50 mM sodium phosphate, 150 mM sodium chloride, 5 mM EDTA and 0.02% sodium azide)

	E2-WT	E2-A351H	E2-A351F	E2-P377H	E2-P377A
pH 7.4	25.0 ± 0.6	27.2 ± 0.9	28.0 ± 0.3	24.3 ± 0.2	25.2 ± 0.4
pH 5.0	25.0 ± 0.7	26.8 ± 0.7	27.6 ± 0.6	24.6 ± 0.4	25.4 ± 0.6

CD was adopted to further examine the secondary structure changes of mutant E2 proteins. Figure 6 showed ellipticity as a function of wavelength for E2-WT at pH 7.4 and pH 5.0. The spectra for E2-WT at both pH conditions show the characteristic profile of an α -helix-rich protein with minima at 208 and 222 nm, which is a standard profile for an intact, correctly folded and assembled protein complex. The spectra of the common site on inner surface mutated proteins (E2-A351H and E2-A351F) were almost identical to that of E2-WT at both pH 7.4 and 5.0 (Figure 7A and 7B), which further demonstrated that mutation at this site had no effect on protein folding and assembly.

FIGURE 5. Molar ellipticity vs. wavelength for E2-WT at pH 7.4 and 5.0 using far-UV CD.

FIGURE 6. Far-UV CD spectra for WT and mutant E2 proteins at pH 7.4 and 5.0. Molar ellipticity vs wavelength for (A) E2-A351H; (B) E2-A351F; (C) E2-P377H; (D) E2-P377A.

In contrast to A351, the CD profiles of the P377 mutated proteins were different. The spectra of E2-P377A mutant proteins presented similar spectra as E2-WT with two minima at 208 and 222 nm characteristic of α -helix-rich proteins at both pH 7.4 and 5.0 (Figure 7D). However, the spectra of E2-P377H presented a partial loss of 222 nm

minimum and a new slight 248 nm minimum, indicating the loss of α -helix structures (Figure 7C). The results implied the important role of P377 in folding and associating the E2 subunits even though the quaternary structure of E2 protein remained intact upon mutation.

CONCLUSIONS

In our study, we aimed to understand the self-assembly mechanisms of E2 protein of pyruvate dehydrogenase from *B. stearothermophilus*. Extrinsic factors such as salts and denaturants, and intrinsic factors of the interactions from key amino acids were considered.

Quantitative analyses were adopted to assess the extent of salts and denaturants influences on the assemblies of wild type E2 protein. The protein size increased proportionally to the NaCl and $(\text{NH}_4)_2\text{SO}_4$ concentrations while the size decreased as the GuHCl concentration increased. $(\text{NH}_4)_2\text{SO}_4$ had stronger effects on protein stability than NaCl, which was consistent with the Hofmeister series. When the GuHCl concentration is higher than 2.5 M, E2 protein complex disassociated into either individual subunits or stable intermediate composed of multiple subunits. The findings would aid us to further our studies in subsequent experiments where we plan to monitor the assembly of the E2 complex from individual subunit.

To examine the key amino acids involved in self-assembly, we targeted the amino acids at the inner surface and intratrimer interfaces of the E2 protein. We found that mutations at the inner surface have no apparent effect on both protein size and secondary structure. The mutations at intra-trimer interfaces changed the secondary structure contents but the protein sizes, and hence the quaternary structure remained stable due to the limited solvent access. This work will help us understand the self-assembly

behavior of virus-like caged proteins, and lay the groundwork for future projects on studying the incorporation of subunits in other macrostructure.

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