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Characterization on the aggregation of self-aggregating green fluorescent protein variant

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ABSTRACT

s-DL4 is a variant of green fluorescent protein (GFP), exclusively deposited *in vivo* into active inclusion bodies (IBs). In this study, we demonstrated that s-DL4 is a self-aggregating molecule by performing structural analysis of s-DL4 IBs and studying *in vivo*/*in vitro* aggregating properties of the molecule. Fourier transform infrared analysis of IBs revealed that there were native GFP structures and intermolecular interactions between the protein molecules. s-DL4 was always deposited into insoluble intracellular IB aggregates, regardless of the protein expression rate. The active s-DL4 IBs dissolved in urea solution were aggregated and precipitated when the urea was removed by dialysis.
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Introduction

Production of heterologous proteins in bacterial cells often results in the formation of inclusion bodies (IBs), which are pseudospherical particles, ranging from nanometers to micrometers in diameter. In general, IBs represent misfolded and inactive protein deposits, which have been considered to be waste by-products of protein expression. However, this viewpoint is changing because IBs are protein particles which have some advantages over conventional synthetic polymer-based particles. IB protein particles composed of natural amino acids and peptide bonds are very biocompatiable, and therefore they are of great interest in biomedical research. For instance, they can be used as novel materials for stimulating mammalian cell proliferation in tissue engineering studies and as vehicles for therapeutic proteins in advanced cell therapy [1–4]. They can be ecofriendly produced by conventional recombinant DNA technology and fermentation technology. In addition, protein engineering tools allow us to fabricate their physical and chemical properties very simply. Recently, it was reported that even the activity of a protein can be retained in IB particles by protein engineering [5–8].

Green fluorescent protein belongs to a family of fluorescent proteins that are functionally active when the β-barrel structure, composed of 11 β-stands and a single central helix, is properly folded. A few studies have demonstrated that active GFP IBs could be obtained by fusing GFP and peptide or protein sequences with self-aggregation/assembly properties [9–11]. It has been proposed that such active GFP IBs can be good fluorescent particles or precursors for the preparation of biomaterials.

We were recently able to obtain a GFP variant exclusively deposited to intracellular active IBs [12,13]. The mutant s-DL4 devoid of an exposed loop sequence (191-GPVLLP-196) was discovered in the process of studying the effects of loop deletion mutations on GFP folding and activity [12]. The mutant was expressed in insoluble form with fluorescent activity when produced in *Escherichia coli*, indicating the possibility of preparing fluorescent protein particles on a large scale. Their morphologies and sizes were characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS) [13]. The study was the first example to demonstrate that active IBs can be generated by engineering an intrinsic protein sequence without tagging any peptide or protein sequences showing self-aggregation/assembly properties.

In our previous studies, it was demonstrated that spectral properties, quantum yield, refolding rate, and specific fluorescent activity of s-DL4 were similar to those of its native form, and s-DL4 IBs were found to be composed of active s-DL4 proteins. Considering these results, it was assumed that s-DL4 was properly folded and that folded s-DL4 molecules might have aggregated in the cell. However, the studies focused on the demonstration of

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intracellular formation of active IBs, and therefore, the possibility that some artifacts may have caused intracellular aggregation could not be ruled out.

In this study, the hypothesis that s-DL4 has intrinsic self-aggregating properties, which leads to the formation of active intracellular aggregates, was confirmed by characterizing s-DL4 IBs using several approaches. First, the protein structure and intermolecular interactions in s-DL4 IBs were studied by Fourier transform infrared spectroscopy (FTIR). Second, *in vivo* aggregation of s-DL4 was tested under various expression conditions that are capable of inhibiting protein aggregation. Finally, s-DL4 molecules were dissociated from IBs and reaggregated to confirm their *in vitro* aggregation properties.

Materials and methods

Expression and purification of s-DL4 IBs

Gene variants encoding mutant (s-DL4), cloned in the pET30b vector, were expressed in *E. coli* BL21 (DE) and purified as previously described [13]. Briefly, IBs of DL4 were purified by collecting and washing the insoluble pellet.

Secondary structure analysis by FTIR

The insoluble pellet was purified by repeated washing using a wash buffer (50 mM Tris, 50 mM NaCl, 1% Triton X-100, 1 M urea, pH 8.0) and dried in a Speed-Vac system for 40 min prior to analysis to reduce water interference in the spectrum. The spectrum was recorded on an FT/IR 4000 spectrometer (Jasco) equipped with a TGS detector and analyzed after subtracting the background. Specifically, 128 scans were averaged at a resolution of 4 cm^{-1}, and all the processing procedures were performed so as to improve the quality of the spectrum in the amide I and II regions. The second-derivative spectrum was calculated by the Savitzky–Golay method (third polynomial, 13 smoothing points) using the spectra manager software (Jasco).

Effects of temperature and inducer concentration on mutant GFP expression

E. coli BL21 (DE) harboring the recombinant GFP was induced at different temperatures with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Alternatively, different concentrations of IPTG were used to induce the expression of mutant GFP for 5 h at 37 °C. Combined effects of temperature and IPTG concentration were also studied by inducing the expression at 15 °C with 0.1 mM IPTG. Soluble and insoluble fractions were prepared after the expression and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

In vitro *reaggregation of s-DL4*

IBs were purified from 25 ml of overexpressed culture, and the protein pellet was solubilized in 8 M urea (50 mM Tris, 200 mM NaCl, pH 8.0). The suspension was incubated at room temperature with shaking for 15 min and centrifuged at 10,000 rpm for 15 min to remove visible aggregates. The urea-solubilized supernatant was filtered through a 0.22-μm syringe filter to remove residual aggregates and then dialyzed overnight against 1× phosphate-buffered saline to demonstrate spontaneous self-assembly.

Determination of particle size by light scattering

The size distribution of IBs was studied by dynamic light scattering using a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation). Reaggregated protein particles were suspended in 50 mM Tris (pH 8.0) and sonicated for 1 min at room temperature. The samples were diluted 100-fold in the same buffer, and measurements were made at 25 °C with the dust filter turned on.

Results and discussion

Structural characterization of s-DL4 aggregates by FTIR

The assumption that s-DL4 IBs are formed by intermolecular interactions between properly folded, active s-DL4 molecules was based on our previous observation that s-DL4 is mostly composed of active proteins [12,13]. To demonstrate the assumption, the protein structure and intermolecular interactions in s-DL4 were investigated by FTIR. FTIR is a powerful tool for studying structural characteristics of aggregated proteins and has been extensively used to characterize protein structures and intermolecular interactions in several aggregated proteins, such as amyloid-like proteins and active IBs [14–16].

Fig. 1(a) shows the FTIR spectra of s-DL4. In general, the amide I absorption peak of FTIR is primarily due to the stretching and vibration of the carbonyl group of the peptide bond, whereas the amide II peak is mainly due to the N—H bending with some contribution from the C—N stretching vibration. The amide I region of the IR spectra acts as a sensitive marker for the protein secondary structure, and therefore, a band analysis of this region retrieves accurate information about the protein secondary structure. The amide band of s-DL4 was in the spectral region between 1600 cm^{-1} and 1700 cm^{-1}, showing almost the same pattern as the one previously reported for active GFP aggregates [17]. On the other hand, components of the amide I band, which belong to different secondary-structure elements, are sometimes overlapped. Therefore, second derivatives and Fourier deconvolution should be applied to extract the components. The spectra of the second derivative of the amide I region are presented in Fig. 1(b). Two prominent components were detected around 1666 cm^{-1} and 1694 cm^{-1}, indicating the presence of β-turns and β-sheet components of GFP, respectively [9,14]. The spectral band around 1630 cm^{-1} was owing to the overlap of β-sheet and cross β-sheet signals [18]. As GFPs contain 47% β-sheets, these results indicate that s-DL4 IBs include native GFP structures and suggest intermolecular cross β-sheet interactions.

s-DL4 is a GFP mutant in which the loop region is deleted. As discussed in our previous studies, the hypothesis regarding self-aggregating properties of the deletion mutant considers that the deletion slightly destabilizes parts of the GFP structure while maintaining its main structure and that there could be some interactions between the destabilized regions [12,13]. The FTIR results obtained in this study show the possibility that the destabilized regions are β-strands in GFP. The peaks observed in the s-DL4 IB spectra were almost the same as those of β-sheets, and their intermolecular interactions were similar to those detected in amyloid-like aggregates. Therefore, it can be presumed that the loop deletion in GFP destabilizes the β-strands of GFP, which may lead to intermolecular hydrogen bonding in s-DL4, similar to that in amyloid aggregations. This is simply a hypothesis, but the FTIR results reported in this study could be a starting point to understand the mechanism underlying s-DL4 self-aggregation.

Effects of expression rate on the formation of intracellular s-DL4 IBs

In general, formation of intracellular IBs is induced when a target protein is overproduced at a high expression rate. Under such conditions, unfolded and folded proteins are crowded in the cell, which may lead to non-specific aggregation of expressed

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