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# Folding and unfolding of a non-fluorescent mutant of green fluorescent protein

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**Abstract**

Green fluorescent protein (GFP), from the Pacific jellyfish *A. victoria*, has numerous uses in biotechnology and cell and molecular biology as a protein marker because of its specific chromophore, which is spontaneously created after proper protein folding. After formation, the chromophore is very stable and it remains intact during protein unfolding, meaning that the GFP unfolding process is not the reverse of the original folding reaction; i.e., the principles of microscopic reversibility do not apply. We have generated the mutant S65T/G67A-GFP, which is unable to efficiently form the cyclic chromophore, with the goal of investigating the folding, unfolding and competing aggregation of GFP under fully reversible conditions. Our studies have been performed in the presence of guanidinium hydrochloride (GdnHCl). The GFP conformation was monitored using intrinsic tryptophan fluorescence, and fluorescence of 1,1′-bis(4-anilino-5-naphthalenesulphonic acid) (bis-ANS). Light scattering was used to follow GFP aggregation. We conclude from these fluorescence measurements that S65T/G67A-GFP folding is largely reversible. During equilibrium folding, the first step is the formation of a molten globule, prone to aggregation.

## 1. Introduction

There is still very little known concerning the general rules that govern $\beta$-sheet formation. In contrast to $\alpha$-helical proteins, the formation of a $\beta$-sheet structure requires the creation of an interaction between amino acids that are far away in the sequence of the polypeptide chain [1]. Alternatively, this more complex topological search problem can also lead to a misfolded $\beta$-sheet structure, such as the amyloid structures connected to the neurodegenerative diseases like Alzheimer's, Huntington's or prion diseases [2]. On average, proteins with mainly $\beta$-sheet structure are more prone to aggregation than $\alpha$-helical proteins [3]. Yet $\beta$-sheet proteins have

persisted through evolution. In light of this, it is important to understand the forces that drive the formation of the native $\beta$-sheet structure, and likewise, the forces that govern the formation of misfolded, potentially toxic, $\beta$-sheet aggregates.

One representative all-$\beta$-sheet protein is green fluorescent protein (GFP) from the pacific jellyfish *A. victoria*. GFP is well known for its biotechnological relevance as a protein marker [4] because of its intrinsic chromophore, which is spontaneously created after proper protein folding. Chromophore biosynthesis involves residues Ser65, Thr66, and Gly67 in the wild-type GFP [5]. For efficient chromophore formation, close proximity of the backbone atoms of amino acids 65 and 67 is required, and this is achieved via the conserved glycine residue at position 67 (any other amino acid imposes too much steric hindrance) [6]. After formation, the chromophore is very stable and it remains intact during protein denaturation [7], meaning that the GFP unfolding process is not the reverse of the original folding reaction; i.e., the principles of microscopic reversibility do not apply. GFP chromophore fluorescence is quenched upon protein denaturation, presumably by the interaction of the chromophore with molecular oxygen [8], or by exposing the chromophore to the aqueous solvent [6].

The all-$\beta$ structure of GFP suggests it should be an excellent model to investigate the formation of the $\beta$-sheet structure, but the presence of the chromophore may alter the unfolding and refolding properties. Because of this, we have generated the mutant S65T/G67A-GFP, which is unable to form the cyclic chromophore, with the goal of investigating the folding, unfolding, and competing aggregation of GFP under fully reversible conditions. Our studies have been performed in the presence of guanidinium hydrochloride (GdnHCl). The GFP conformation was monitored using intrinsic tryptophan fluorescence, 1,1′-bis(4-anilino-5-naphthalenesulphonic acid) (bis-ANS) fluorescence, and light scattering.

## 2. Materials and methods

### *2.1. Construction of the mutant S65T/G67A-GFP and protein preparation*

A plasmid encoding S65T-GFP with a polyhistidine tag was a gift from Roger Tsien. The mutant S65T/G67A-GFP was prepared using a Stratagene QuikChange Mutagenesis Kit according to the manufacturer's direction. S65T/G67A-GFP was overexpressed in *E. coli* strain BL21(DE3). The protein was recovered from inclusion bodies by dissociating in 6 M GdnHCl. The unfolded S65T/G67A-GFP with the polyhistidine tag was purified by metal affinity chromatography. All buffers used during purification contained GdnHCl with 6 M concentration. The protein was stored in the unfolded state at 4 °C in 50 mM phosphate buffer pH 8 with 6 M GdnHCl and 300 mM NaCl. Folding was initiated by diluting S65T/G67A-GFP into buffers with decreasing concentration of GdnHCl. For the unfolding experiments the first step was folding of the protein into the buffer without GdnHCl. The created aggregates were removed by centrifugation and the unfolding was measured by diluting S65T/G67A-GFP into buffers with increasing concentration of GdnHCl. All buffers consisted of a 50 mM mixture of $Na_2HPO_4$ and $NaH_2PO_4$ pH 8, and 300 mM NaCl. As GFP contains two cysteine residues (reduced), 1 mM DTT was included in all buffers to prevent the formation of intra- or intermolecular disulfide bonds.

### *2.2. Chemicals*

All chemicals were of guaranteed reagent grade. Guanidinium hydrochloride (GdnHCl), NaCl, dithiothreitol (DTT) and buffer salts were purchased from Roth, and 1,1′-bis(4-anilino-5-naphthalenesulphonic acid) (bis-ANS) was from Molecular Probes. All solutions were prepared using deionized and filtered water from a Millipore water purification system.

### *2.3. Spectroscopic measurements*

All spectrometric measurements were performed at 20 °C in 50 mM phosphate buffer pH 8.0, 300 mM NaCl, 1 mM DTT and GdnHCl concentration ranging from 0 to 6 M. The concentration of protein was 1 $\mu$M.

Absorbance measurements were performed on a Uvikon spectrophotometer (Kontron, Austria). Protein concentration was determined using a molar extinction coefficient of $\varepsilon_{280\ \mathrm{nm}} = 22\,140\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$ calculated from the amino acids sequence, which is identical (within error) to the value presented by Tsien ($\varepsilon_{280\ \mathrm{nm}} = 22\,000\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$) for the wild-type GFP [6].

Steady-state S65T/G67A-GFP fluorescence data were collected on an LS-50 Perkin–Elmer spectrofluorimeter with excitation at 280 nm, and emission at 340 nm. The fluorescence intensity was measured 15 min after adding the protein to the buffer. The quartz cuvette has path length 4 mm for excitation and 10 mm for emission. The spectral bandwidth was 2.5 nm for excitation and emission.

The population of the intermediate state was followed by bis-ANS fluorescence. The fluorescence emission intensity (excitation at 420 nm, and emission at 490 nm) was measured 10 min after adding the protein to the buffer, and again after the removal of aggregates.

Protein aggregation was monitored by light scattering at 640 nm, (with the excitation and emission wavelengths at 640 nm, chosen after [9]), 10 min after adding the protein to the buffer, and again after the removal of aggregates. Under these conditions, fluorescence of amino acids (Phe, Tyr and Trp) is not observed.

The removal of aggregates was performed by 30 min of centrifugation at 11 500$g$. The light scattering of a sample without bis-ANS, after centrifugation, was equal to the scattering of the buffer. In the case where the light scattering of a mixture of native and aggregated protein was measured, the aggregates were not removed from the sample containing folded and aggregated protein created during folding. Alternatively, aggregates were added to samples of native protein.

The corresponding buffer spectrum was subtracted from each protein spectrum. Each point represents the average from three independent measurements. Errors were calculated as standard deviations from the mean.

GFP folding was initiated by diluting the protein from high to low concentration of GdnHCl. Unfolding was performed by increasing the concentration of GdnHCl.

All data analysis was performed with commercially available software Microcal Origin version 6 [10].

## 3. Results and discussion

### *3.1. Tryptophan fluorescence as a probe of GFP folding and unfolding*

GFP contains only one tryptophan (and eleven tyrosines) but, as with other Trp-containing proteins [11], tryptophan fluorescence dominates the emission spectrum upon excitation at 280 or 295 nm, and is sensitive to the environment of the Trp chromophore, in contrast to Tyr fluorescence, which is not particularly sensitive to the surrounding environment. We collected the fluorescence emission intensity at 340 nm after excitation at either 280 or 295 nm. In both cases, a similar trend of fluorescence emission changes as a function of [GdnHCl] was observed, but the absolute intensity was higher for excitation at 280 nm. As excitation at 280 nm results in more efficient total protein fluorescence excitation, this wavelength was used for all measurements.

During folding, S65T/G67A-GFP remains unfolded at concentrations down to 3 M GdnHCl. At lower [GdnHCl], the fluorescence emission increases, reaching a maximum at

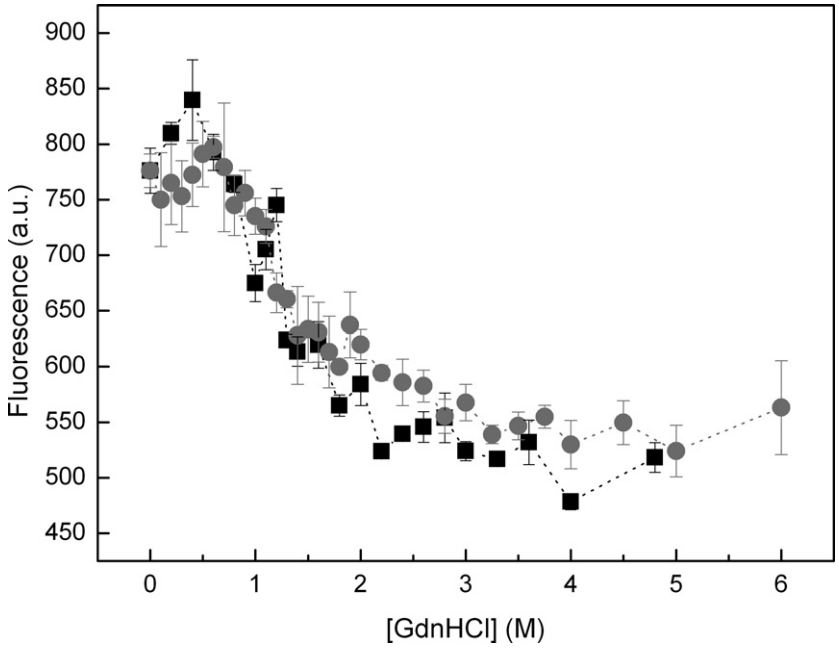


**Figure 1.** Changes in fluorescence emission intensity for excitation wavelength 280 nm and emission measured at 340 nm during folding (grey circles) and unfolding (black squares) of S65T/G67A-GFP as a function of denaturant concentration. Measurements were conducted at 20 °C in 50 mM phosphate buffer pH 8.0 with 300 mM NaCl and 1 mM DTT after 10 min of equilibration.

0.6 M GdnHCl, consistent with the burial of the Trp residue in a hydrophobic environment (figure 1). From 0.1–0.3 M GdnHCl, the emission intensity decreased slightly; at 0 M GdnHCl, the intensity is ∼97% of the maximal intensity.

During unfolding at low concentrations of GdnHCl, the fluorescence intensity increased slightly, reaching a maximum at 0.4 M GdnHCl. At higher GdnHCl concentrations, the fluorescence decreases dramatically, reaching a plateau at concentrations higher than 3 M. The midpoint of the folding/unfolding transition is 1.5 ± 0.2 M GdnHCl. The similarity of the folding and unfolding titrations indicates that the folding of this mutant is largely reversible (figure 1). Nevertheless, the nonlinear dependence of the fluorescence emission intensity at low [GdnHCl] means that it is difficult to accurately calculate a free energy for GFP folding using standard techniques [12].

### *3.2. Probing exposed hydrophobic surface area using bis-ANS*

Bis-ANS is a polarity-sensitive fluorescent probe that is minimally fluorescent in a polar environment, such as aqueous solution, but its fluorescence emission intensity increases in nonpolar environments. In the common protein folding intermediate termed a molten globule, the hydrophobic side chains of amino acids can create nonpolar surfaces accessible to external agents and can effect binding interactions with hydrophobic probes, such as bis-ANS. The increase in fluorescence emission intensity upon bis-ANS binding has been used to estimate the population of folding intermediates during protein folding reactions [13, 14].

The fluorescence emission of bis-ANS as a function of the folded state of GFP is shown in figure 2. The fluorescence was measured before and after removing aggregates

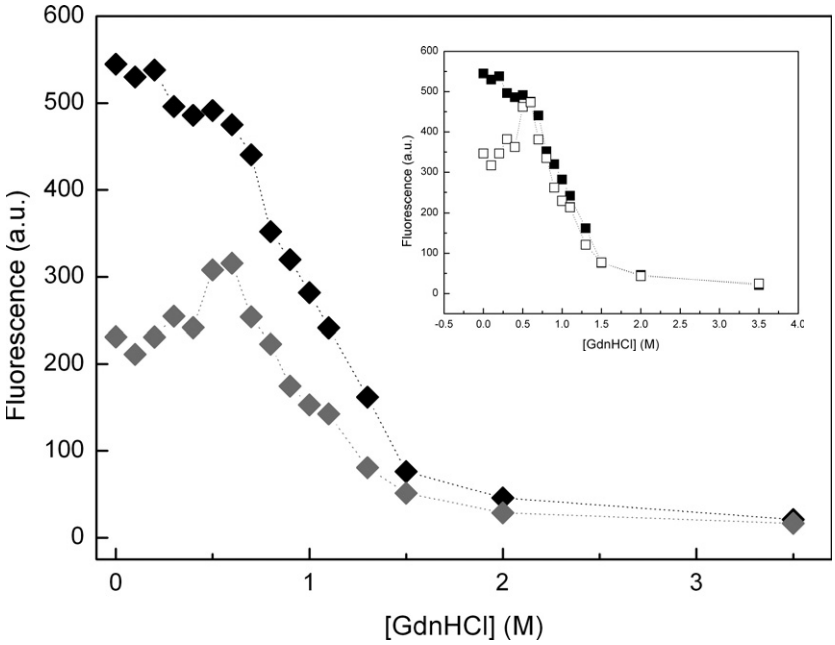


**Figure 2.** Binding of bis-ANS to S65T/G67A-GFP monitored by fluorescence emission intensity at 490 nm (excitation at 420 nm) at various concentration of GdnHCl before (black diamonds) and after (grey diamonds) removing aggregates by centrifugation at 11 500*g*. Data were collected at 20 °C in 50 mM phosphate buffer pH 8.0 with 300 mM NaCl and 1 mM DTT. Inset (obtained by multiplication of fluorescence of bis-ANS after centrifugation by constant value and compared with the fluorescence before centrifugation): fluorescence before (filled squares) and after (open squares) removing aggregates.

by centrifugation. At GdnHCl concentrations down to 2.0 M, the protein remains unfolded and no bis-ANS fluorescence was observed. At lower concentrations of denaturant, before removing aggregates, the fluorescence of bis-ANS increases, indicating the presence of an exposed hydrophobic surface area in the partially folded and aggregated protein.

Bis-ANS fluorescence was measured again after the removal of aggregates by centrifugation. From 3.5–0.6 M GdnHCl, bis-ANS fluorescence intensity after centrifugation increases uniformly (figure 2). This is clearly seen in the inset, which presents the multiplication of the data for all concentrations of denaturant after centrifugation (figure 2) by a constant value. In the range 3.5–0.6 M GdnHCl the fluorescence changes in the same way as the fluorescence before centrifugation. In the range 0.5–0 M GdnHCl a decrease in fluorescence is observed, caused by the removing of the aggregates. After centrifugation, there is a significant maximum of bis-ANS fluorescence intensity at GdnHCl concentrations between 0.5 and 0.6 M. This result suggests that a maximal concentration of molten globule-like intermediate occurs at these concentrations of GdnHCl.

At lower concentrations of GdnHCl, the bis-ANS fluorescence decreases, though there is a slight increase at 0 M GdnHCl. The persistent bis-ANS fluorescence intensity at GdnHCl concentrations below the threshold for S65T/G67A-GFP folding, even after the removal of aggregated material by centrifugation (see section 3.3, below), indicates that the solution contains, in addition to folded S65T/G67A-GFP, small soluble yet misfolded conformations, possibly as small oligomeric structures.

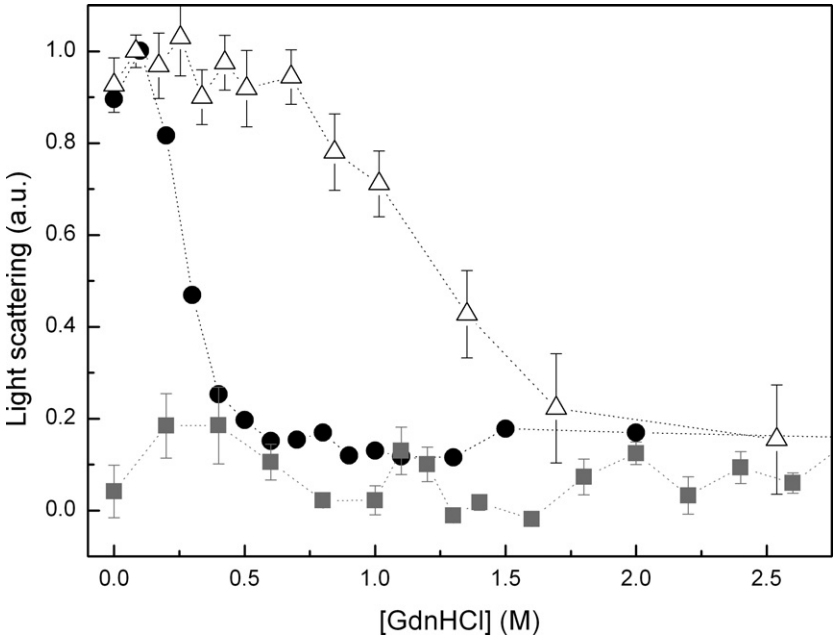


**Figure 3.** Light scattering of S65T/G67A-GFP at 640 nm as a function of denaturant concentration during unfolding of native protein without aggregates (grey squares), unfolding the native protein in the presence of aggregates (open triangles) and during folding of S65T/G67A-GFP (black circles). Data were collected at 20 °C in 50 mM phosphate buffer pH 8.0 with 300 mM NaCl and 1 mM DTT.

### *3.3. Light scattering measurements of GFP aggregates*

Substantial light scattering was observed during the folding of S65T/G67A-GFP to residual concentration of GdnHCl (figure 3). Light scattering was low at GdnHCl concentration down to 0.6 M, but increased dramatically at lower concentrations of GdnHCl, reaching a maximum at 0.2 M GdnHCl. These results suggest that, at intermediate concentrations of GdnHCl, GFP populates an intermediate conformation that is particularly prone to aggregation. Unfolding native (without aggregates) GFP in increasing concentration of denaturant did not result in increased light scattering, indicating that aggregation does not occur during GFP unfolding (figure 3). The intensity of scattering was constant as a function of GdnHCl concentration, showing that scattering is not sensitive to GFP conformational changes during unfolding.

Observation of the light scattering during unfolding was also performed on a mixture of folded and aggregated GFP. At low concentrations of GdnHCl, light scattering was high, decreasing only above 0.9 M GdnHCl, suggesting that higher concentrations of GdnHCl are required to dissociate and solubilize S65T/G67A-GFP aggregates.

## 4. Conclusions

As measured by tryptophan fluorescence, S65T/G67A-GFP folding is largely reversible. Upon dilution from 6 M GdnHCl, S65T/G67A-GFP remains unfolded down to 3 M GdnHCl. Between 0.6 and 3 M GdnHCl, a partially folded intermediate is formed. This intermediate binds bis-ANS, indicating that it resembles a classical 'molten globule'-type folding intermediate. The existence of intermediate is typical for many $\beta$-sheet

proteins [15, 16]. However, the complicated shape of the folding curve, especially pronounced below 0.6 M concentration of GdnHCl, is not a typical phenomenon observed during folding of other $\beta$-sheet proteins.

The persistent bis-ANS fluorescence intensity at GdnHCl residual concentrations after the removal of aggregates indicates that the solution contains a mixture of folded S65T/G67A-GFP and small misfolded conformations, which are not removed by centrifugation.

During folding, the increasing of light scattering is delayed in comparison with tryptophan and bis-ANS fluorescence. It is minimal at/above 0.6 M GdnHCl, but increases dramatically at lower concentrations of GdnHCl. These results suggest that, at equilibrium, upon gradual removal of GdnHCl, GFP first forms a molten globule structure, which next can choose a way either to an aggregation or to the creation of the native structure. These aggregates are present in solution during the next steps of folding. According to the fluorescence of bis-ANS it is not possible to remove aggregates by centrifugation.

During unfolding of the native protein, aggregation is not observed. In the case of a mixture of aggregated and folded protein, probably two processes are observed: unfolding of the native protein and dissociation of aggregates clearly seen at concentrations of GdnHCl higher than 0.9 M.

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