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Protocol for Spontaneous and Chaperonin-assisted *in vitro* Refolding of a Slow-folding Mutant of GFP, sGFP

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[Abstract] Understanding the folding pathway of any protein is of utmost importance for deciphering the folding problems under adverse conditions. We can obtain important information about the folding pathway by monitoring the folding of any protein from its unfolded state. It is usually very difficult to monitor the folding process in real time as the process is generally very fast, and we need a suitable read out. In this protocol, we have solved this issue by using a protein that is non-fluorescent in its unfolded state but fluoresces in its native state after folding. The kinetics of refolding can be monitored by following the increase in fluorescence in real time. Previously, this was generally achieved by either monitoring a protein's enzymatic activity or measuring the tryptophan fluorescence, where the signal output depends on well-described enzymatic activity or the frequency of tryptophan residues present in the proteins, respectively. Here, we describe a simple and real-time assay to monitor the refolding of sGFP, a recently described slow-folding mutant of yeGFP (yeast enhanced GFP). We unfold this protein using chemical denaturant and refold in a suitable buffer, monitoring the increase in fluorescence over time. GFP is fluorescent only when correctly folded; thus, using this technique, we can measure the true rate of protein refolding by following the increase in fluorescence over time. Therefore, sGFP can be used as an ideal model to study the *in vitro* protein folding process. Accordingly, the effects of different conditions and molecules on the protein folding pathway can be efficiently studied using sGFP as a model protein.

Graphical abstract:

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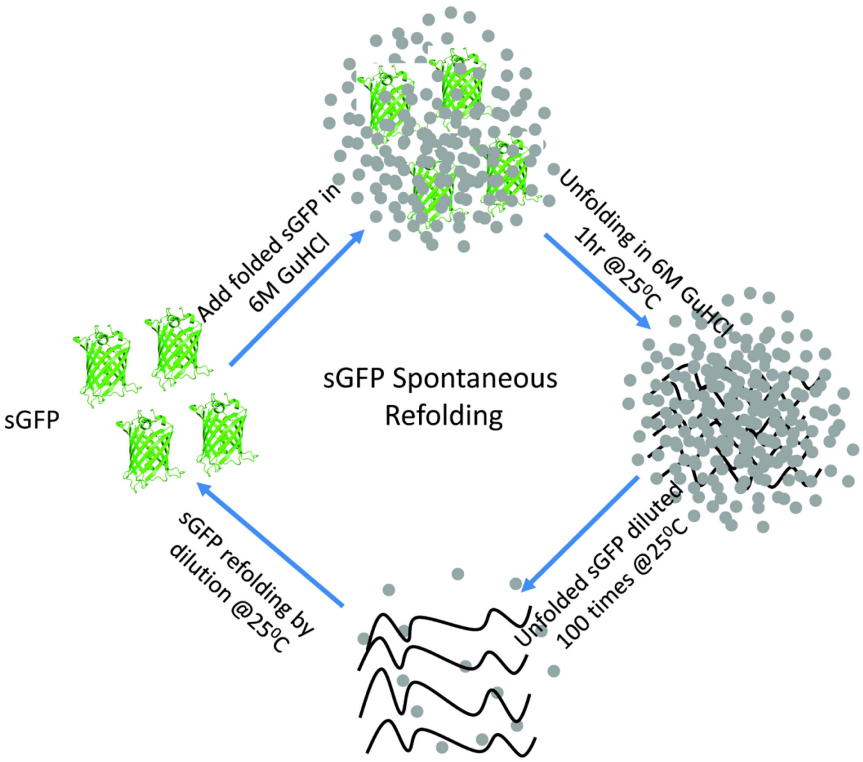

Schematic of the steps involved in the sGFP refolding pathway. Native sGFP is unfolded by chemical denaturation using 6 M GuHCl at 25°C for 1 hour and then refolded in refolding buffer by 100-fold dilution.

Keywords: Protein folding, *In vitro* folding, Refolding kinetics, Refolding rate, GroEL/ES-assisted folding, Chaperonin-assisted refolding

[Background] Deciphering the folding pathway of proteins is one of the most explored topics in biology to date. Once synthesized, a nascent polypeptide chain is folded into its native structure, which is essential for its biological function. These polypeptide chains, especially the unfolded chains of small single-domain proteins, often fold spontaneously without any active assistance from other proteins. In contrast, larger multi-domain proteins frequently require folding assistance from proteins, commonly known as molecular chaperones, to fold into their native structure. The process of protein folding from nascent chains to correctly folded native states is extremely fast on a biologically relevant time scale. Thus, in order to study the steps of the folding pathway, we need to monitor the folding process using a slow-folding protein in a cell-free system, which is achieved by mimicking the cellular environment *in vitro* using suitable buffers. In an *in vitro* experiment, we unfold the test protein using a suitable denaturant and then refold it in a folding-favorable environment. Currently, the available protocols for measuring refolding rates are either based on measuring the enzymatic activity of proteins after they are refolded or monitoring the intrinsic tryptophan fluorescence of the protein (Weissman *et al.*, 1994; Zahn *et al.*, 1994; Makio *et al.*, 1999; Doyle *et al.*, 2003; Taniguchi *et al.*, 2004; Kerner *et al.*, 2005; Sharma *et al.*, 2008; Malhotra *et al.*, 2014; Jain *et al.*, 2018). While measuring the refolding efficiency by estimating the enzymatic activity is one of the most suitable ways of checking the folded and active

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states of the protein, it largely depends on its well-known enzymatic activity. In the case of proteins without well-described enzymatic activity or activity that depends on the functional form of the protein, which may not form immediately after it is refolded, the estimation of refolding efficiency becomes difficult (Rospert *et al.*, 1993). Another way of evaluating *in vitro* refolding is by measuring the tryptophan fluorescence, which again is largely dependent on the number of tryptophan residues and their location within the native structure (Chakraborty *et al.*, 2010). We have devised an *in vitro* refolding assay of a recently identified *in vivo* substrate of the GroEL/ES chaperonin system (Sadat *et al.*, 2020). In this protocol, we describe the methodology to monitor the refolding of a slow-folding yeGFP mutant protein, hereafter referred to as sGFP, in real time. We unfold the sGFP protein using guanidine hydrochloride as a chemical denaturant and subsequently refold the unfolded protein in a suitable refolding buffer in the presence and absence of chaperones (Sadat *et al.*, 2020). The potential issues that are frequently encountered during *in vitro* refolding experiments are: (a) achieving complete unfolding of the protein in a reversible manner; (b) measuring the exact time taken by the unfolded protein to fold into its native form; and (c) accurately measuring the refolding rate, since most proteins fold very fast and this becomes a potential problem for measuring the *in vitro* refolding rate of a protein. Using sGFP successfully evades these potential problems in monitoring the refolding process in real time; therefore, our protocol can be a very useful guide for measuring the *in vitro* refolding rate of fluorescent proteins possessing similar characteristics to GFP.

Materials and Reagents

1. Tips: Sterile pipette tips (low-retention tips)
2. Falcon, 15-ml
3. Sterile microcentrifuge tubes (1.5-ml)
4. Wash bottle
5. Liquid discard beaker
6. Kimwipe tissue paper (Kimberly-Clark, catalog number: 34120)
7. Microcentrifuge tube rack
8. Falcon rack
9. Fluorescence cuvette (3.0 mm path length) (Hellma Analytics, catalog number: Z802336)
10. 0.22-µm filter (Millex, catalog number: SLGP099RS)
11. 10-ml syringe
12. MilliQ water (deionized water)
13. Methanol (100%) (HIMEDIA, catalog number: AS061-2.5L)
14. Guanidine hydrochloride (MP, catalog number: 105696)
15. Magnesium chloride (HIMEDIA MB, catalog number: 040-500G)
16. Potassium chloride (Sigma, catalog number: 793590)
17. Tris (ultra-pure) (MP, catalog number: 103133)
18. HCl (HMEDIA, catalog number: AS004-2.5L)

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19. ATP (Sigma, catalog number: A2383)
20. DTT (MP, catalog number: 100597)
21. Purified protein (sGFP)
22. Purified chaperones and co-chaperones (GroEL, GroES)
23. sGFP (Sadat *et al.*, 2020)
24. Buffer A (see Recipes)

Equipment

1. Pipettes: 2.5-µl, 10-µl, 200-µl, 1-ml
2. Centrifuge
3. Fluorescence spectrophotometer (Fluorolog 3 Spectrofluorometer, Jobin Yvon, Horiba)
4. Dry bath
5. pH meter

Procedure

A. sGFP spontaneous refolding

1. Protein Unfolding

   Add 2.5 µl sGFP (20 µM) to a microcentrifuge tube containing 7.5 µl 8 M GuHCl (final GuHCl concentration in the unfolding reaction is 6 M) in buffer A (see Recipes). Place the tube in a dry bath for 1 h at 25°C. This will lead to complete unfolding of the protein.

2. Protein Refolding

   a. Switch on the Fluorolog instrument (Fluorescence spectrofluorometer). Keep the instrument switched ON for 45 min prior to fluorescence measurement, so that the lamp is uniformly heated and fluctuations are minimal.

   b. Set the instrument to emission spectra mode. Keep the excitation wavelength at 350 nm and the emission spectra in the 365-430 nm range. After 45 min, add MilliQ H_2O to a quartz microcuvette and take a Raman Spectra. If you get an emission peak at 397 nm, the spectrofluorometer is working fine.

   c. Set the instrument to kinetic acquisition mode. Set the excitation wavelength to 480 nm and the emission wavelength to 515 nm. Set to anti-photobleaching mode. Set the collection interval to 20 s and the integration time to 0.7 s. The total time for the kinetics is set to 1,800 s. Set the temperature to 25°C. The slit-width is kept at 2 nm for excitation and 5 nm for emission.

   d. Clean a quartz cuvette (Hellma Analytics, 10 mm path length) with 100% methanol and then MilliQ water, and wipe using a kimwipe. Take 2 µl (200 nM final) completely unfolded protein, dilute 100-fold in Buffer A (198 µl), and mix thoroughly by pipetting (total reaction volume 200 µl) in the cuvette.

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e. Start the kinetics acquisition as soon as the mixing is finished (keep an equal delay in this step).

f. sGFP refolding typically follows a two-state pathway. The unfolded proteins are refolded in refolding buffer to their native state in a single step. In the refolding experiment, we monitor the increase in GFP fluorescence by exciting the refolding reaction at 480 nm and measuring the fluorescence emission at 515 nm. GFP fluoresces only after folding into its native state; therefore, GFP fluorescence is the readout for the amount of folded protein in our refolding experiment. The refolding kinetics trace obtained after the refolding reaction can be best fitted using nonlinear regression by a single exponential kinetics equation. The kinetics data are analyzed using the Origin software.

A mathematical model to fit the refolding kinetics:

The model for fitting the data obtained from refolding experiments is a mathematical equivalent of two-state folding kinetics. The one-phase exponential decay equation is as follows:

$$y = y_0 + A_1 e^{\frac{-x}{t_1}}$$

where y_0 is the offset, A_1 is the amplitude, and t_1 is the time constant. The apparent rate of the refolding reaction is obtained by taking the reciprocal of the time constant. A typical refolding kinetics plot is shown in Figure 1a.

B. Chaperonin-assisted sGFP refolding

1. Protein Unfolding

   Unfold sGFP as described earlier in Step A1.

2. Protein Refolding

   a, b, c follow steps A2a, b, c as described for spontaneous refolding.

   a. Take 2 µl (200 nM) completely unfolded protein and dilute 100-fold in Buffer A containing GroEL (400 nM tetradecamers of GroEL). Add GroES (800 nM heptamers of GroES), such that the ratio of substrate to GroEL toGroES is 1:2:4. The refolding is initiated by the addition of 2 mM ATP (final concentration); the total reaction volume is 200 µl. Mix the reaction thoroughly by pipetting slowly.

   b. Start the kinetics acquisition as soon as the mixing is finished (keep an equal delay in this step).

      A representative refolding kinetics plot is shown in Figure 1a. Figure 1b, 1c, 1d explains the various steps involved in the data analysis for calculating the refolding rate. 1/t1 gives the refolding rate/second.

   c. Same as Step A2f.

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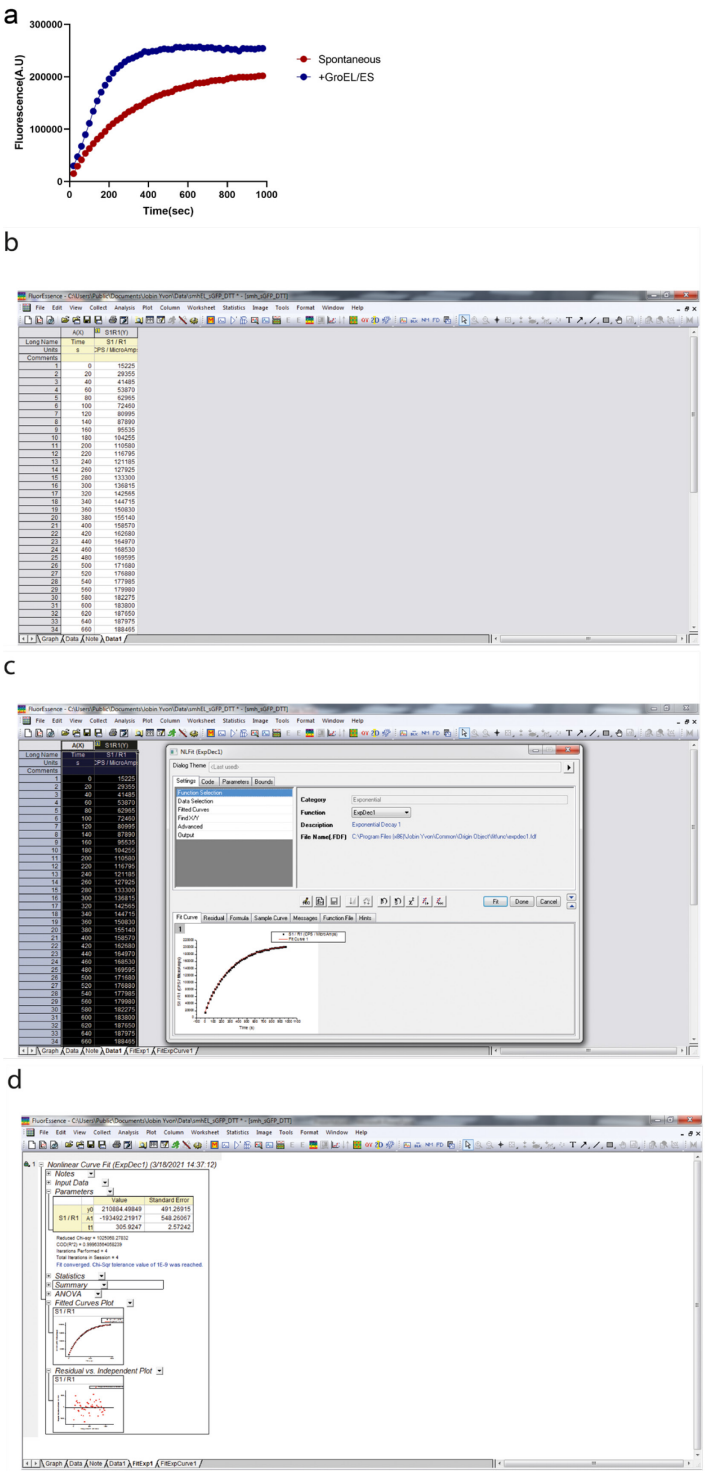

Figure 1. sGFP refolding plot. a. Plot showing the kinetics of sGFP refolding as measured by an increase in GFP fluorescence. sGFP was unfolded in 6 M GuHCl in Buffer A at 25°C for 1 h. Subsequently, unfolded sGFP was refolded by 100-fold dilution in Buffer A. **b.** The output screen after refolding. **c.** This screen appears by clicking on analysis after selecting the columns for time and fluorescence. **d.** This screen appears after the fitting by selecting 'expdec1' in the function section.

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Notes

While this method can be used for monitoring the refolding of any fluorescent protein, we must keep in mind that only those fluorescent proteins that start to fluoresce as soon as they are folded are exclusively suitable for measuring refolding kinetics using the described protocol. As described, sGFP fluoresces as soon as it is folded into its native state; hence, we actually measure the rate of increase in sGFP fluorescence, which corresponds to the refolding rate of the protein. Therefore, this is an indirect method of calculating the refolding rate of sGFP. We assume that there are no such limitations in using this method for measuring the refolding rate of other fluorescent model proteins by similar spontaneous or chaperonin-assisted *in vitro* refolding assays. This protocol can be easily modified, especially the instrument settings (slit-width, excitation-emission wavelengths, total time of kinetic measurements), time of unfolding, concentrations of GuHCl, and other parameters depending on the properties of the model fluorescent protein used. In the case of chaperone-assisted refolding experiments, the molar ratio of substrate to chaperones in the refolding buffer must be optimized for the individual chaperone/co-chaperones used for the refolding assay.

Recipes

1. Buffer A
   20 mM Tris pH 7.4
   20 mM KCl
   5 mM $MgCl_2$
   2 mM DTT
   Filtered using a 0.22-µm filter and a 10-ml syringe

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Competing interests

The authors declare no competing interests.

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