FlAsH-LABELING OF TETRA-CYS-MODIFIED PROTEINS IN E. COLI

CONTACTS

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INTRODUCTION:

We have taken advantage of an experimental system that we designed to observe the *in vivo* stability and aggregation of a protein of interest [1]. We can use any cellular protein, modified to incorporate a Cys-Cys-Gly-Pro-Cys-Cys sequence that can bind the biarsenical fluoroscein derivative, 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FlAsH) [2]. The tetra-Cys motif can be incorporated at either N- or C-terminus of a protein and use it to monitor the localization of the protein. Using CRABP as a model protein, we have demonstrated that the *in vivo* FlAsH signal reports on whether tetra-Cys CRABP is native or not when incorporated in a flexible omega-loop within the protein sequence. Note that this method can be used to label only of cytosolic proteins or the cytoplsmaic parts of membrane proteins; it is not amenable to label in the oxidizing environment of the periplasm.

MATERIALS

MEDIA

- LB medium per litre:
  - Tryptone 10 g
  - Yeast Extract 5 g
  - NaCl 5 g
  - For solid media add 14 g Agar per litre
  - For selective media add the following concentrations of antibiotics: ampicillin (100 mg/ml stock solution in dd H_2O), kanamycin (25 mg/ml stock solution in dd H_2O), chloramphenicol (25 mg/ml in EtOH). Ampicillin and kanamycin are sterile filtered through 0.2 µm filter pore size. All antibiotics are aliquoted in 500 µL and kept at -20 °C.
    All antibiotics are provided by Sigma-Aldrich Company Ltd. (Germany).

- M9 minimal medium: 200 mL M9 salts without NaCl, 2 mL of 1 M $MgSO_4$, 0.1 mL 1M $CaCl_2$, 20 mL 20% glucose, add water to 1 L.
  - M9 salts without NaCl: 64 g/L of Na_2HPO4 7H2O, 15 g/L KH_2PO_4, 5 g/L NH_4Cl, add ddH_2O to 1L; autoclaved and stored at room temperature
  - 1 M $MgSO_4$: $MgSO_4$ dissolved in ddH_2O and autoclaved
  - 1M $CaCl_2$: $CaCl_2$ dissolved in ddH_2O and autoclaved
  - 20% glucose: dissolved in ddH_2O and autoclaved; Note, autoclave glucose solution separately.

REAGENTS AND STOCK SOLUTIONS:

- 1000x stock of IPTG: 400 mM dissolved in ddH_2O and sterile filtered through 0.2 μm filter pore size
- 6M NaCl dissolved in ddH_20 and sterile filtered through 0.2 μm filter pore size
- 1000x lysozyme stock: 50 mg/mL lysozyme dissolved in ddH_2O
- 9 M urea in 10 mM Tris.HCl buffer pH 7.5; determine urea concentration by measuring the refractive index

PROCEDURE:

LABELING:

1. Freshly transform E.coli competent cells with 1 μL of the plasmid bearing the protein with tetra-Cys sequence. Transformation protocol: 45 min on ice, heat shock at 42 °C for 45 sec, 2 min on ice, and transfer into 1 mL pre-warmed LB medium and culture for 1 hr at 37 °C at 900 rpm. Plate on a LB-agar plate with corresponding antibiotic (here 100 μg/ml ampicillin)
2. A single colony is inoculated in 5 mL LB medium and grown over night at 30 °C, 220 rpm constant shaking. This and all subsequent growths are in the presence of 100 μg/ml ampicillin or the corresponding antibiotics the plasmid bears.
3. On the next day, gently spin down the cells at 2000 rpm in a table top centrifuge and resuspend them in fresh LB medium containing the antibiotic (here 100 μg/ml ampicillin). Grow the cells at 37 °C in 50 mL LB medium containing ampicillin at 220 rpm till OD600 ~ 0.5.
4. Gentle permeabilization of the cells with lysozyme: Place the culture tubes on ice and add lysozyme to 50 ng/mL end concentration. Incubate on ice for 10 min, spin down at 300 rpm in a table top centrifuge and resuspend in an equal volume of fresh, modified M9 minimal medium, and add FlAsH dye along with EDT to suppress the labeling of endogenous cysteine pairs. In the usual protocol, cell aliquots of 1 ml were 0.2 μM FlAsH-EDT_2 and 1 μM EDT. The volume of the aliquots can be adjusted depending on the needs for subsequent experiments.
5. After one generation, at OD600=1.0, induce protein synthesis with 0.4 mM IPTG.

Your negative controls are:

1. Labeled cells with no DNA (plasmid) added.
2. Non-labeled cells with DNA (plasmid) added.

MEASURING THE PROTEIN STABILITY IN-CELL:

1. To monitor the thermodynamic stability in vivo, 2 hours after induction with IPTG add to the medium urea from a sterile stock to various final concentrations (not higher than 3 M end concentration).
2. Adjust the volumes of all samples to equal amounts with a sterile modified M9 medium. Incubate at 37 °C for at least 75 min to insure equilibration (but no longer than 120 min).
3. Subject the bulk cell suspensions to fluorescence measurements at 530 nm (excitation 500 nm). From each point subtract a blank of FlAsH-labeled cells expressing the plasmid with a protein with no tetra-Cys motif, treated with the corresponding urea concentration in modified M9 medium.

References

1. Ignatova, Z. and L.M. Gierasch (2004) Monitoring protein stability and aggregation in vivo by real-time fluorescent labeling. *Proc. Natl. Acad. Sci. USA*, **101:** 523-528.
2. Adams, S.R., et al. (2002) New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications. *J Am Chem Soc.* **124**: 6063-6076.