Protein Expression Research Group (PERG) 2012 Study

Richard J Heath¹, Fei P Gao², Pamela Scott Adams³, Cynthia Kinsland⁴, James Bryson⁵, Bo Xu⁶, Thomas Neubert⁷.

¹St Jude Children's Research Hospital, ²University of Kansas, ³Trudeau Institute, ⁴Cornell University, ⁵Bristol-Myers Squibb Co., ⁶University of Texas Medical Branch, ⁷New York University
Insoluble Proteins in *E. coli*

- Many overexpressed proteins in *E. coli* (up to 50%) form insoluble inclusion bodies.
- Inclusion bodies contain primarily unfolded ectopic protein.
- If biologically active protein is required, inclusion bodies are often deemed undesirable.
- However, they can be a ready source of relatively pure protein.
The PERG 2012 Study

• Goal:
  • To get protein labs to consider refolding proteins as a viable option

• Outline:
  • Protein that expresses as inclusion body
  • Simple, validated methods for extraction, solubilization and refolding
  • Simple assay for refolded protein
General Methods for Protein Refolding

• Chromatographic
  – Solvent exchange by size exclusion
  – On-column refolding
  – Immobilized-chaperone assisted refolding

• Non-Chromatographic
  – Rapid Dilution
  – Dialysis
Structure of ATP Synthase

- $\delta$-Subunit
- $\gamma$-Subunit
- $\alpha$-Subunits
- $\beta$-Subunit
- $\varepsilon$-Subunit
Two views of $F_1$ ($\gamma$ subunit red)  PDB file 1COW
The Chloroplast ATP Synthase γ-subunit-GFP Fusion Protein

Regulatory domain
where GFP was inserted
Sequence of the Chloroplast ATP Synthase

$\gamma$-subunit-GFP Fusion Protein

ANLRELDRIGSVKNTQKITEAMKLVAAAAKVRRAQEAVVNGRPFS
ETLVEVLYNMNEQLQTEDVDVPLTKIRTVKKVALMVVTGDRGLCG
GFNNMLLLKKAESRIAEKLKLGVODYTIISIGKKGNTYFIRRPEIPVDRY
FDGNTNLPTAKEAQAIADDVFSLFVSEEVDKVEMLYTTFVSLVKSVP
VIHTLLPLSPKGEICDINGKCVDAEDELFRKTTKEGKSKEELFTGV
VPILVELDGDVNGHKFSVSGEGEGDATYGKLTKFICTTGKLPMVPW
PTLVTFFAYGLQCFARYPDHMKQHDFFKSAEMPQGYVQERTIFFKD
DGNYKTRAEVKFEGDTLVRIELKGIYFKEDGNILGHKLEYNYNSH
NVYIBMADKQKNGIKVNFIRHNIEDGSGVQLADHYQQNTPIGDGP
VLLPDNHYLSTQSALSKDPSHQPFEKRDHMVLLEFVTAAGITHG
MDELYKLTVERMIKTETPAFSPILEFEQDPAREQLDDALLPLYLNSQIL
RALQESLASLAARMTAMSNDTNDNANEELKKTLTTINYNRARQAKIT
GEILEIVAGANSCV
Protocol for expression and inclusion body isolation

• Express \( \gamma \)-ATP synthase-GFP fusion protein in BL21(DE3)
  – LB medium,
  – 3 hr induction @ 37°C
  – Harvest cell pellet

• Isolate and solubilize inclusion bodies
  – Suspend cells in Tris buffer and sonicate briefly
  – Centrifuge and resuspend pellet in Tris buffer
  – Repeat 3 times
Protocol for solubilization and refolding

• Solubilize and refold the fusion protein
  – Resuspend pellet in Tris buffer with 8M urea or 6 M guanidine-HCl
  – Centrifuge to remove debris
  – Dialyze against 500 vols of Buffer 1 (4 M urea)
  – Dialyze against 500 vols of Buffer 2 (2 M urea)
  – Centrifuge to remove debris.
  – Protein is now in solution (which should be green!)

• Measure absorbance & fluorescence of refolded protein
  – Major absorption peak at a wavelength of 395 nm and a minor one at 475 nm
  – Excitation wavelength 395 nm with fluorescence emission peak is at 509 nm
Fluorescence Emission Spectrum of Refolded $\gamma$-ATPS-GFP Fusion Protein
Fluorescence Spectrum of MBCF $\gamma$-ATPS-GFP Fusion Protein

395 Excitation/Absorbance peak
No emission peak?
Amino acid sequence of EGFP cloned in pTIGS1 at COBRE-PPG:

10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260
MELHIVRRLQK VCPRRQKQ QQLAAEAGSA AARRKEDIK ASGKQKAKA
CQKICLQKQ QQLAAEAGSA AARRKEDIK ASGKQKAKA
CQKICLQKQ QQLAAEAGSA AARRKEDIK ASGKQKAKA
CQKICLQKQ QQLAAEAGSA AARRKEDIK ASGKQKAKA
CQKICLQKQ QQLAAEAGSA AARRKEDIK ASGKQKAKA

Number of amino acids: 264

Molecular weight: 29854.6

Theoretical pI: 5.10

Extinction coefficient: EGFP has an extinction coefficient (denoted ε) of 55,500 M⁻¹cm⁻¹. The fluorescence quantum yield (QY) of EGFP is 0.90. The relative brightness, expressed as εQY, is 33,000 M⁻¹cm⁻¹.

Major excitation peak: 488 nm

Major emission peak: 509 nm
EGFP runs as a dimer and fluoresces after refolding.
Revised folding study

Provided by P Gao

• Folded protein  EGFP Standard
• Inclusion body prep
• Plasmid DNA for 6 His-TEV-EGFP
Protocol for solubilization and refolding

• Solubilize and refold the fusion protein
  – Resuspend pellet in Tris buffer with 8M urea at ~1 mg/ml
  – Centrifuge to remove debris
  – Dialyze against 500 vols of Buffer 1 (4 M urea)
  – Dialyze against 500 vols of Buffer 2 (2 M urea)
  – Centrifuge to remove debris.

• Measure absorbance & fluorescence of refolded protein
  – Major absorption peak at a wavelength of 488 nm
  – Excitation wavelength 488 nm with fluorescence emission peak at 509 nm
Fluorescence Spectrum of MBCF GFP vs Std EGFP

Std EGFP

MBCF EGFP

Very green

Slight green tinge, but no emission peak?

CONCENTRATE!
Fluorescence Emission Spectrum of Refolded MBCF EGFP vs Std EGFP

MBCF EGFP

Std EGFP

Graph showing emission spectra for MBCF EGFP and Std EGFP.
Conclusions

• Synthetic EGFP construct is a good tool for a folding study.
• Should be “foldable” by non expert protein personnel.
• Participants can participate at 2 different levels
  – Folding only
  – Protein Expression and folding
What Next?

- Ask Phillip Gao for the plasmid!
- gao@ku.edu
- Send a copy of you absorbance and/or fluorescent spectrum back once you have refolded the protein
- Results to be presented next year
One-step Batch Affinity Chromatography: Protein Refolding for Dummies

**Major equipment:** one column, two hands.

**Method:**

- Incubate with Ni$^{2+}$ resin.
- Wash / refold: on column, gravitation flow.
- Elute protein. Collect, especially the front fraction.

12% SDS PAGE of Rv0008c, a membrane protein from TB
When the refolding work:

18 TB membrane protein spectra were obtained of which more than 90% of the expected amide $^{15}$N to $^1$H correlation resonances were observed.

2 were completely assigned.

Lesson 1: Protein production in NOT the bottleneck for Membrane Protein Structural Genomics.
Protein Expression Research Group (PERG):
We focus on recombinant protein expression and purification.

Help needed for upcoming events:
1. Protein refolding study: participation.
2. Recruiting new members: recommendation.
3. Organizing a workshop on production of recombinant protein: input.