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A Method for Direct Measurement of Protein Stability In Vivo

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Abstract

The stability of proteins is tuned by evolution to enable them to perform their cellular functions for the success of an organism. Yet, most of the arsenal of biophysical techniques at our disposal to characterize the thermodynamic stability of proteins is limited to in vitro samples. We describe an approach that we have developed to observe a protein directly in a cell and to monitor a fluorescence signal that reports the unfolding transition of the protein, yielding quantitatively interpretable stability data in vivo. The method is based on incorporation of structurally nonperturbing, specific binding motifs for a bis-arsenical fluorescein derivative in sites that result in dye fluorescence differences between the folded and unfolded states of the protein under study. This fluorescence labeling approach makes possible the determination of thermodynamic stability by direct urea titration in *Escherichia coli* cells. The specific case study we describe was carried out on the predominantly β-sheet intracellular lipid-binding protein, cellular retinoic acid-binding protein (CRABP), expressed in *E. coli*.

Keywords

Protein stability; in-cell urea titration; FlAsH labeling; fluorescence; microscopy; CRABP

1. Introduction

Optimal thermodynamic stability of proteins is crucial to their physiological functions and activities. Reduced protein stability can be detrimental, leading to misfolding pathologies such as the neurodegenerative diseases – Alzheimer's, Parkinson's, and other amyloid diseases (1, 2). Conversely, overly stable proteins may lose the ability to respond to allosteric modulators. Therefore, a molecular understanding of a protein requires analysis of its thermodynamic stability. A wide array of informative and sophisticated in vitro approaches yields quantitative descriptions of stability under a given set of experimental conditions. For many studies, these stabilities have been assumed to be applicable also in vivo. The crowded cellular environment, however, will modulate conformational flexibility of a protein and adds complexity to folding and unfolding pathways (3,4). The thermodynamic stability of a protein is very likely to be altered by macromolecular crowding, which in turn will influence both folding and aggregation reactions. Additionally, molecular chaperones interact with a large fraction of the cellular proteome, as do many small ligands that are present in a cell (5); these interactions, along with changes in the oxidative potential due to responses to oxidative stress (6), can influence the thermodynamic stability of a protein in the cellular environment. Therefore, it is highly desirable to develop methods to measure protein stability directly in cells.

Technical challenges make the goal of measuring protein stability in cells extremely difficult, and only a few groups have reported in vivo stabilities. Oas and colleagues applied amide

hydrogen exchange detected by MALDI mass spectrometry in a pioneering study to provide the first direct measurements of in vivo protein stability in the *Escherichia coli* cytoplasm (7). Their results showed the thermodynamic stability of the small monomeric δ repressor in the cell to be the same as in the test tube. However, in this method cells are lysed prior to mass spectrometry measurement. Consequently, this approach cannot readily be used to explore directly how different physiological states alter thermodynamic stabilities in the cell. Similarly, the pulse proteolysis approach recently introduced by Marqusee and coworkers (8), which is based on selective digestion of unfolded proteins in equilibrium mixtures of folded and unfolded proteins, requires conversion of intact cells into lysates. For maximum versatility, a method to measure protein stability directly in the cell, during different physiological situations, would be advantageous.

A variety very of qualitative in vivo approaches have been designed that monitor folding and solubility (which is generally reliant on proper folding) of expressed proteins. For example, one strategy is based on the necessity for correct folding for successful structural complementation of a reporter protein (9). Here, the reporter protein is split into two parts that must recombine to function, providing an efficient way of screening for folding-competent and soluble mutants. Another approach is based on reading out the efficiency of fluorescence resonance energy transfer (FRET) between an N-terminal blue fluorescent protein and a C-terminal green fluorescent protein (10). FRET efficiency will be enhanced when the fusion protein folds to the compact native state. Fusion of a protein of interest to chloramphenicol acetyltransferase was used to identify well-folded mutants on the assumption that only these would be soluble and confer resistance to chloramphenicol (11). However, all of these approaches are limited in their ability to provide a quantitative measure of protein stability.

We have developed a fluorescence-based approach to determine protein stability in vivo (12, 13) using a well-behaved model system – the 136-amino-acid cellular retinoic acid-binding protein (CRABP) (14). CRABP is visualized in the context of all macromolecules present in the cell using the membrane-permeable bis-arsenical fluorescein-based dye ‘FlAsH’ (15). This fluorescent dye ligates to a genetically engineered tetracysteine motif (Cys-Cys-Xxx-Yyy-Cys-Cys); the extremely rare occurrence of this motif in the cellular proteome ensures high specificity of labeling (15). By engineering the specific tetracysteine sequence (here Cys-Cys-Gly-Pro-Cys-Cys) into the internal Ω-loop of CRABP (incorporating the native Gly-Pro present in this loop), we created a tetra-Cys CRABP variant that binds FlAsH and yields a fluorescent emission intensity sensitive to the conformational state of the protein, with the denatured ensemble hyperfluorescent compared to the native state (13). FlAsH fluorescence can therefore be used to follow the transition from native to unfolded CRABP during unfolding by chemical denaturant. This approach enables determination of the free energy of unfolding in vivo; FlAsH fluorescence can be used as a direct read-out to monitor the urea-induced unfolding of tetra-Cys CRABP directly in the cell. The complexity of the cellular environment demands cautious interpretation of the thermodynamic data obtained, as many cellular components may be perturbed by the urea treatment. Nonetheless, direct observations in cells will provide new insights. Fulfilling a requirement for its use in these measurements, tetra-Cys CRABP is soluble and indistinguishable in structure and function from its native counterpart whether FlAsH-labeled or unlabeled (13). The FlAsH labeling approach has also been applied to mutants of CRABP in order to explore the effects of specific residue substitutions: for example, mutation of the helix-terminating residue Pro39 to Ala was known to retard the folding and unfolding of CRABP (16), and P39A tetra-Cys CRABP shows a high tendency to form aggregates in vitro (17). By incorporating the tetra-Cys motif into P39A CRABP, we could follow formation of aggregates in real time in vivo (13).

The sensitivity of the FlAsH quantum yield to the conformational state of the protein is the premise for the application of this approach to directly measure in vivo stability. Application

of this strategy to other proteins necessitates a careful structure-directed choice of a sequence from the target protein, so that incorporation of the tetra-Cys motifis tolerated without structural perturbation, and also that the FlAsH quantum yieldis sensitive to the folding of the protein host. In addition to the overall structural constraints each protein provides for incorporation of the tetra-Cys sequence, the geometric properties of the binding sites are crucial to the FlAsH fluorescence characteristics (B. Krishnan and L. Gierasch, manuscript in preparation). Although the successful design of a FlAsH-binding tetracysteine tag into a given protein might require multiple trials, a clear advantage provided by this system is the direct read-out of stability in intact cells.

2. Materials

1. DIFCO™ Luria Bertani (LB) medium (BD Biosciences, San Jose, CA) (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.5)
2. Ampicillin (Sigma, St. Louis, MO) is dissolved in water at 100 mg/mL, sterile-filtered (0.2 μm cut-off), and stored in 1 mL aliquots at –20°C. It is added to the culture medium to a final concentration of 100 μg/mL.
3. Isopropyl-β-D-thiogalactoside (IPTG) (Gold Bio-Technology, St. Louis, MO) is dissolved in water at 400 mM, sterile-filtered (0.2 μm cut-off), and stored in 1 mL aliquots at –20°C. It is added to the culture medium at the time of induction in the appropriate amount for a final concentration of 0.4 mM.
4. Stock solution of lysozyme (500 mg/mL) (ICN Biomedicals, Irvine, CA) and DNase (100 mg/mL) (Roche Diagnostics, Indianapolis, IN) are stored in 500 μL aliquots at –20°C.
5. FlAsH-EDT_2 (marketed as Lumio™ by Invitrogen, Carlsbad, CA) in its commercial stock concentration of 2 mM was stored at –20°C. Ethanedithiol (Sigma) is aliquoted in DMSO to 10 mM and is diluted fresh prior to each experiment.
6. Urea stock solution (9 M in 10 mM Tris HCl buffer, pH 7.5) (MP Biomedicals, Solon, OH) is freshly made before use, and sterile-filtered (0.2 μm cut-off).
7. 3x SDS-ioading buffer contains 187.5 mM Tris HCl, pH 6.8, 6% (w/w) SDS, 30% glycerol, and 0.03% (w/v) bromophenol blue, and is stored at room temperature.

3. Methods

This article describes an approach that we recendy developed to determine in vivo protein stability using a high-copy-number plasmid for the expression of the protein of interest in *E. coli* BL21(DE3) cells. Our results show that other *E. coli* mutant strains WG710 and WG708 (18) give comparable and reproducible results to the *E. coli* BL21(DE3) cells (19,20). This provides evidence for the general applicability of the procedure to any *E. coli* strain. To obtain reliable and reproducible results it is important to start the urea titrations at a time point after induction when a sufficient protein amount is already synthesized in the cell. The use of high-copynumber plasmids yields adequate protein as soon as one hour after induction. One might consider using low-copy-plasmids as well; ultimately, the starting point of the urea titration should be established in any particular case depending on the rate and yield of protein biosynthesis for the plasmid used.

3.1. In-Cell Urea Titration

1. Tetra-Cys CRABP or P39A tetra-Cys CRABP (Note 1) is cloned into the pET16b (Amp^R) plasmid under the T7 promoter, and the resulting plasmids are transformed

into *E. coli* BL21(DE3) cells, carrying the DE3 lysogen for high-level expression of T7 polymerase. Only freshly transformed cells should be used for expression.

2. A small volume culture is inoculated with a single colony in LB medium containing 100 μg/mL ampicillin and grown overnight at 30°C with constant shaking at 200 rpm. The lower temperature retards the growth of bacteria and provides a more viable culture (with fewer dead cells).

3. Sterile harvested cells (2060 X *g*, 15 min, 4°C) are carefully resuspended in fresh medium and brought up to 25× the original volume in fresh sterile LB medium containing 100 μg/mL ampicillin. This culture is grown at 37°C until the OD_{600} = 0.5 (Note 2) and then treated with lysozyme (final concentration 50 ng/mL) for 10 min on ice. While the inner membrane is freely permeable to the FlAsH dye (15), lysozyme enhances the permeability of the outer membrane of the bacterial host cell to FlAsH. This gentle lysozyme pretreatment has no deleterious effect on the viability and growth of the cells (13). After removal of the lysozyme-containing medium by centrifugation (2060 × *g*, 15 min, 4°C), the cell pellet is resuspended in the same amount of fresh sterile LB medium containing 100 μg/mL ampicillin along with FlAsH dye and EDT (Note 3). EDT suppresses the labeling of endogenous cysteine pairs (15) and ensures that the FlAsH-fluorescence signal is a result of specific ligation to the tetracysteine motifs. In the usual protocol, cell aliquots of 1 mL are labeled with 0.2 μM FlAsH-EDT_2 and 1 μM EDT. The volume of the aliquots can be adjusted depending on the needs of the subsequent experiments. FlAsH and EDT remain in the medium during the entire time of cultivation.

4. After one generation, at OD_{600} = 1.0, protein synthesis is induced by adding IPTG to 0.4 mM. To establish the behavior of the protein under study, a time course of fluorescence increase for the cell culture is obtained: every 15 min, 150-μl aliquots are withdrawn and subjected to fluorescence measurements in bulk at 530 nm (excitation 500 nm). The temperature of the cuvette holder is maintained at 37°C. Fluorescence of cells with a plasmid bearing the wild-type CRABP without the tetra-Cys motif and labeled with FlAsH, is used as a blank, and the value is subtracted from each point. The observed increase in fluorescence reports on a combination of increased numbers of cells, increased concentration of expressed protein, and any onset of protein aggregation (Note 4). In experiments with any new protein, one should ensure that the fluorescence results only from labeled protein of interest residing within the cells; therefore, the following controls are advisable to demonstrate that the fluorescence signal is due to intracellular species: *(1)* Spin down 150-μl aliquots at 1503 X *g* for 5 min and check the fluorescence of the supernatant at 530 nm. *(2)* Rinse cells once with 50 mM Hepes, pH 7.5, and resuspend in the same

[1]This protocol can be adapted to any protein provided that a successful design of a FlAsH-binding motif that is sensitive to global conformational changes can be achieved. In addition, the engineered tetra-Cys sequence should not perturb the structural integrity and thermodynamic stability of the protein host. Moreover, the system can be used to compare the destabilizing effect of mutations before a time-costly and potentially low yield purification is undertaken.

[2]LB absorbs at 600 nm (OD_{600} is between 0.07 and 0.09), and fresh LB is used as a blank in the OD measurements.

[3]The EDT stock solution (10 mM in DMSO) is best stored at –20°C and is freshly diluted prior to each labeling experiment. EDT generates an extremely unpleasant thiol smell, and the initial aliquoting and addition to the culture needs to be handled exclusively under the hood. Containers with tight-fitting lids (i.e., falcon tubes, Eppendorf tubes, or Schott bottles) should be used instead of common flasks or culture tubes for cultivation of the cells. The used pipette tips or other plastic materials should be collected in a tightly closed container (preferably stored under the hood) and then discarded according to safety regulations.

[4]Different fluorescence patterns are observed upon induction of soluble and aggregation-prone proteins; the signal of a soluble protein reports on the steady increase of its amount during protein synthesis, while that for an aggregation-prone variant may show an altered signal reporting on aggregate formation. In die case of FlAsH-labeled tetra-Cys P39A CRABP, aggregates are hyperfluorescent, leading to a pronounced upswing in fluorescence of bulk cell samples upon initiation of aggregation (13). Fluorescence microscopy images confirm these results: Whereas the fluorescence is spread uniformly throughout the cytoplasm in cells expressing the soluble tetra-Cys CRABP, in cells expressing aggregation-prone P39A tetra-Cys CRABP hyperfluorescent aggregates are observed near the poles (Fig. 7.3) (13). In parallel, cell fractionation studies reveal the partitioning of the protein between the soluble and insoluble fractions.

amount of buffer. Measure fluorescence again. For any cellular protein, the measured fluorescence values of unwashed and washed cells should be within the measurement error, and the fluorescence of the supernatant should be comparable to the fluorescence of the supernatant of the control CRABP protein without a tetra-Cys binding motif. Hyperfluorescent supernatants are indicative of protein released into the medium or cell membrane debris that binds FlAsH nonspecifically.

5. To monitor the thermodynamic stability of the expressed protein in vivo, 2 h after induction the culture is split into equal aliquots (usually between 250 μL and 350 μL) and urea from a sterile stock (9 M urea in 10 mM Tris HCl buffer, pH 7.5) is added to each aliquot to various final concentrations (not higher than 3 M). Cells retain their viability in urea concentrations up to ~3 M urea (7,13), which should be the highest urea concentration used in in vivo urea titrations. The volumes of all samples in one urea titration set should be adjusted to equal amounts with sterile LB medium containing 100 μg/mL ampicillin. The samples are incubated at 37°C for at least 75 min to insure the equilibrium between folded and unfolded populations (Note 5) with constant shaking (longer incubation is also possible, but no longer than 120 min), and subjected to fluorescence measurements (emission at 530 nm; excitation 500 nm). Fluorescence of cells containing a plasmid bearing the wild-type CRABP without the tetra-Cys motif (labeled with FlAsH and under the same urea concentrations) is used as a blank, and the measured value at each urea concentration is subtracted from each point in the urea titration set. A representative result is shown in Fig. 7.1. To derive thermodynamic parameters, the urea denaturation curves were fitted to a two-state model (21) (Note 6) (Table 7.1). The actual urea concentration of each sample is determined using the measured index of refraction of the supernatant (after centrifugation of the cells at 1503 X *g* for 15 min) (22). In the urea titration experiment, the control experiments described above in step 4 (measuring the fluorescence of the supernatant and of washed and resuspended cells) are advisable to ensure that an observed increase in the fluorescence signal arises only from unfolding of the protein within the cell and is not a result of protein release into medium or hyperfluorescent cell debris.

3.2. Cell Viability

Cell viability and changes in the cell number during the incubation times in urea (Note 7) were qualitatively assessed by measuring the optical density at 600 nm (Fig. 7.2), using LB medium as a blank. This method alone, while rapid, is not sufficient to observe a potential deleterious effect of urea on cell viability, since dead cells can also contribute to the bulk absorbance at

[5]We observed that the incubation time required for establishment of equilibrium between folded and unfolded populations of CRABP in vivo is significantly shorter than in vitro (Figs. 7.1 and 7.4). Based on our preliminary measurements, we believe that acceleration of the unfolding rate is the most likely factor leading to faster equilibration in vivo (12), but studies are underway to dissect all possible factors. Our initial approach to measuring stability in vivo used a short incubation time of the cells in different urea concentrations (30 min) with a goal of minimizing the negative impact on viability of the cells at higher urea concentrations (13). However, follow-up studies on the incubation time dependence of quantitative in vivo stability experiments clearly indicate that the minimum time required for equilibration for either tetra-Cys CRABP or P39A tetra-Cys CRABP in vivo is 75 min, as indicated by the absence of further change in the urea melt as incubation time is increased (Fig. 7.1**A** and **B**) (12). The time dependence of establishing an equilibrium between folded and unfolded populations in vivo can vary significantly (i.e., fast-folding proteins might require shorter incubation times), and the optimal incubation time needs to be determined for each protein by monitoring in parallel the cell viability and the approach of the observed fluorescence to a constant value at any given urea concentration.

[6]To extract any thermodynamic data from the stability curves, the urea melts need to be reversible. In our case, the melts are reversible only for the completely soluble tetra-Cys CRABP protein (Fig. 7.5). To measure the stability of aggregation-prone proteins, which form detergent-resistant aggregates (i.e., amyloid aggregates), one might consider shorter induction times or lower expression levels in order to carry out the urea melt before insoluble structures are formed.

[7]Longer incubation times are accompanied by losses in cell viability (Fig. 7.2). To offset the impact of cell loss, prior to FlAsH-fluorescence measurements the optical density (OD_{600}) is recorded, and the OD_{600} of all the samples is normalized to the optical density of the 3-M urea sample by addition of fresh LB medium at the appropriate urea concentration.

600 nm. For more careful assessment of viability, the fraction of viable cells should be determined on solid nutrient medium by taking one μL samples of the cell suspension, diluting them 10,000-fold in fresh sterile LB medium, plating them on LB-agar (LB medium solidified by addition of 15 g/L agar), and incubating the plates at 37°C overnight. Colonies are then counted, and the number of the viable cells is calculated per mL (Fig. 7.2).

3.3. Cell Fractionation

It is essential for the determination of in vivo stability that the protein under study remain soluble throughout the measurement. Hence, we recommend a control to insure that the measured FlAsH signal arises from soluble protein; separation of soluble and insoluble protein by fractionation gives direct information on how the protein under study behaves in the expression system used. In comparing the behavior of different proteins and mutants of any given protein under study, it may be of interest to assess the extent to which a given protein partitions to the insoluble (pellet) fraction, and this same protocol can be used for this goal.

1. The 5 mL overnight culture prepared from freshly transformed *E. coli* BL21(DE3) cells with either tetra-Cys CRABP or P39A tetra-Cys CRABP plasmids is used to inoculate 100 mL of LB medium containing 100 μg/mL ampicillin and grown at 37° C with constant shaking (200 rpm). At OD_{600} = 1.0, IPTG is added to 0.4 mM to induce the T7 promoter-based expression of tetra-Cys CRABP or P39A tetra-Cys CRABP. At different time points, 10 mL aliquots are withdrawn with sterile handling, and bacteria are harvested by centrifugation at 2060 X *g* for 15 min at 4°C. All steps after the aliquot withdrawal may be carried out under nonsterile conditions.
2. Cells are resuspended in 1.5 mL of 50 mM phosphate buffer at pH 8.0 containing 300-mM NaCl. Cells are disrupted by lysozyme (at a final concentration of 500 μg/mL) for 30 min (Note 8) and DNase (at a final concentration of 50 μg/mL) treatment on ice for 15 min, followed by sonication on ice with 20 s bursts per minute for 3 min (30% duty cycle). The cell lysates are fractionated into soluble and insoluble fractions by centrifugation at 27,000 X *g* at 5°C in a tabletop centrifuge for 30 min. The insoluble pellet fraction is resuspended into 1.5 mL of 10 mM Tris HCl buffer, pH 8.0, containing 8 M urea. A volume of 20-μL aliquots of each fraction are mixed with 10 μL 3x SDS-loading buffer and preheated for 3 min at 95°C, then loaded onto an SDS-PAGE gel (12% acrylamide), and the tetra-Cys CRABP content in each fraction is quantified by optical densitometry. The density is related to a density of a standard sample containing purified tetra-Cys CRABP at 1 mg/mL.

3.4. Fluorescence Microscopy

During the time course of growth or urea incubation of the labeled cells expressing either tetra-Cys CRABP or P39A tetra-Cys CRABP (see **Section 3.1**), 20 μL of cell suspension is withdrawn and concentrated twice by centrifugation at 7,297 X *g* for 3 min and subsequent resuspension in 50 mM Hepes buffer, pH 7.5. A volume of 2 μL of this concentrated suspension is immobilized in 1% agarose in LB and imaged with a fluorescent microscope (Nikon Eclipse E600, Melville, NY) with excitation at 485 nm and a 510-nm emission cut-on filter. The images are processed with the Openlabs software (Improvision, Lexington, MA). Examples of micrographs are shown in Fig. 7.3.

[8]The lysozyme treatment converts the cell pellet into a very viscous suspension due to the release of DNA. The subsequent hydrolysis with DNase reduces the viscosity of the solution.

3.5. In vitro FlAsH-labeling and urea titration

1. Tetra-Cys CRABP contains an N-terminal His-tag, enabling it to be purified from the soluble fraction of the *E. coli* cell lysate using Ni^{2+}-NTA (Qiagen, Valencia, CA) affinity resin (14). The purification is monitored on 12% SDS-PAGE as above (3.3.2). The purest protein fraction appears between 175 mM and 260 mM imidazole. A typical purification of a completely soluble CRABP variant like tetra-Cys CRABP yields 7–10 mg of pure protein from 1L culture.
2. The fractions with pure protein are collected and dialyzed overnight at 4°C in 15 kDa MW cut-off dialysis tubingagainst 10 mM Tris HCl, pH 8.0, containing 2 mM β-mercaptoethanol (BME). BME is used to minimize oxidation of tetra-Cys-binding sites (15). The dialyzed protein is filtered through a Nalgene 0.45-μm syringe filter, and protein concentration is determined spectro-photometrically using die ε_{280} of 21,750 M^{-1} cm^{-1} for tetra-Cys CRABP (14). Purified proteins could be stored in aliquots up to 200 μM (preferably 100 μM) at 4°C for use within 2 weeks. When higher concentrations are desired, protein should be concentrated prior to the experiment using a Centricon system (molecular mass cut-off of 10 kDa) at 4°C.
3. Tetra-Cys CRABP from the purification stocks is diluted to the desired concentration in 10-mM HEPES pH 7.5, containing 1-mM tris(carboxyethyl)phosphine (TCEP) and labeled with FlAsH and ethanedithiol (FlAsH:EDT ratio 1:5) at room temperature for 2 h in the dark. The usual ratio of FlAsH:protein used is 2:1. The labeled tetra-Cys protein is stable for several days at 4°C. A typical labeling mixture for urea titration in vitro contains 7-μM labeled protein.
4. Labeled protein is aliquoted and mixed with various urea concentrations. After equilibration at 37°C for a minimum of 6 h, the aliquots are subjected to fluorescence measurements either using the intrinsic Trp signal (excitation 280 nm, 2-nm bandwidth; monitoring emission from 300 nm to 380 nm with a 2-nm bandwidth) or using the FlAsH signal (as described). The temperature of the cuvette holder is maintained at 37°C with a water bath. Representative results for tetra-Cys CRABP and P39A tetra-Cys CRABP (isolated using the same protocol) are shown in Fig. 7.4.

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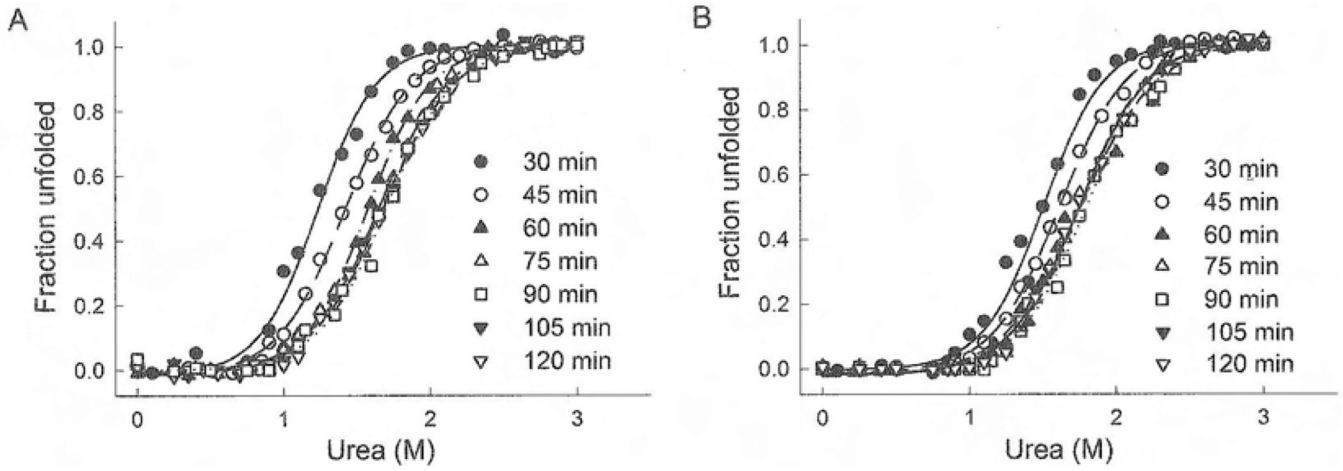

Fig. 7.1.

Urea titrations of FlAsH-labeled protein in vivo as a function of the incubation time: (**A**) tetra-Cys CRABP and (**B**) P39A tetra-Cys CRABP, after sample incubation for the indicated times in urea, monitored by FlAsH fluorescence. (Reproduced from **Ref.** (12) with permission from Willey InterScience.)

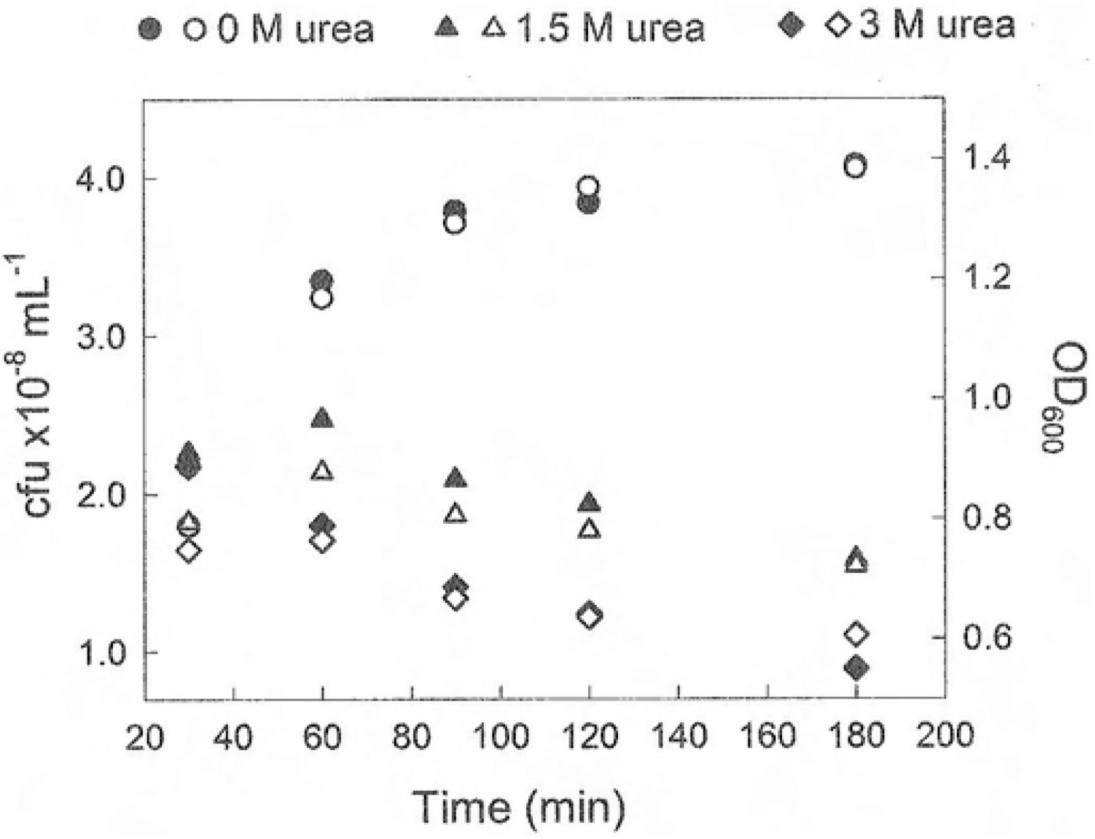

Fig. 7.2.

Cell viability. Change in the viability of the host cells as a function of the time of incubation in 0 M, 1.5 M, and 3 M urea. The viability was quantified by measuring the number of cells capable of forming colonies (*left axis, closed circles*) and optical density at 600 nm (*right axis, open symbols*).

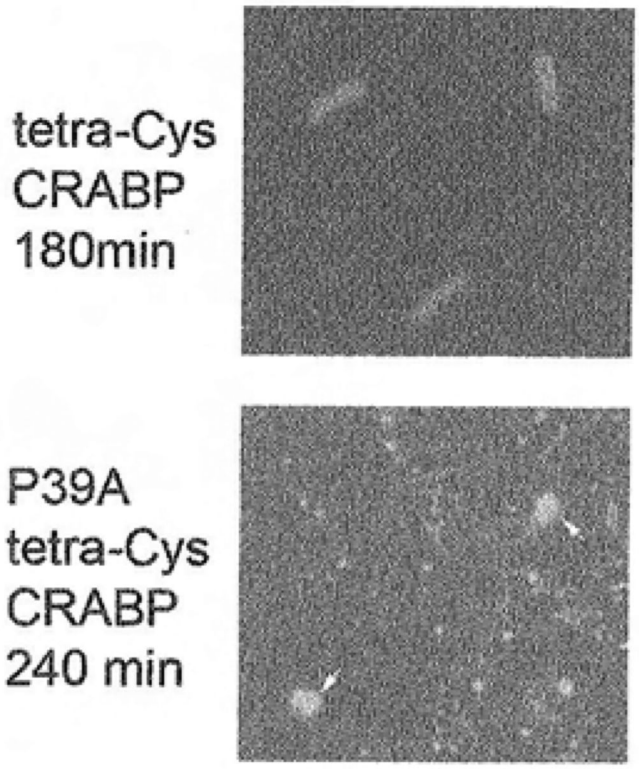

Fig. 7.3.
Fluorescence microscopy images showing uniformly distributed fluorescence of tetra-Cys CRABP (180 min after induction) and hyperfluorescent dense aggregates of P39A tetra-Cys CRABP at the poles of the cells (at 240 min after induction). Hyperfluorescent impurities in the extracellular medium are marked by an arrow.

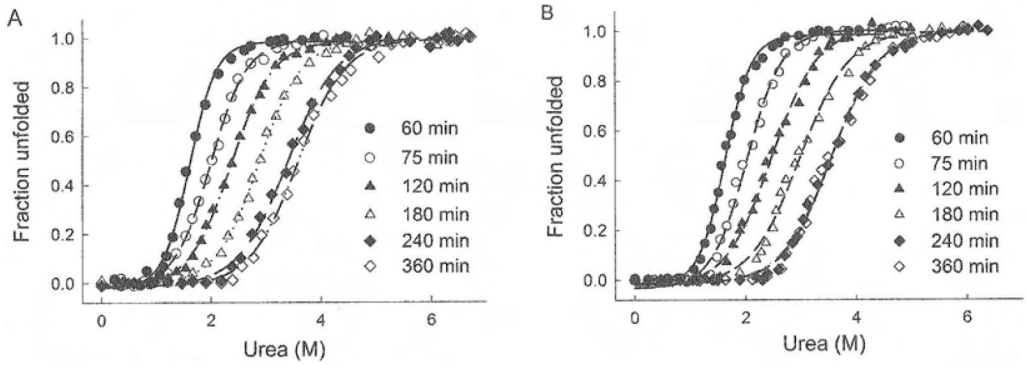

Fig. 7.4.

Urea titrations of FlAsH-labeled protein in vitro as a function of the incubation time (monitored by Trp fluorescence): (**A**) tetra-Cys CRABP and (**B**) P39A tetra-Cys CRABP. The actual urea concentration was determined by measuring the refractive index, and data are curve-fit to a two-state model. (Reproduced from **Ref.** (12) with permission from Willey InterScience.)

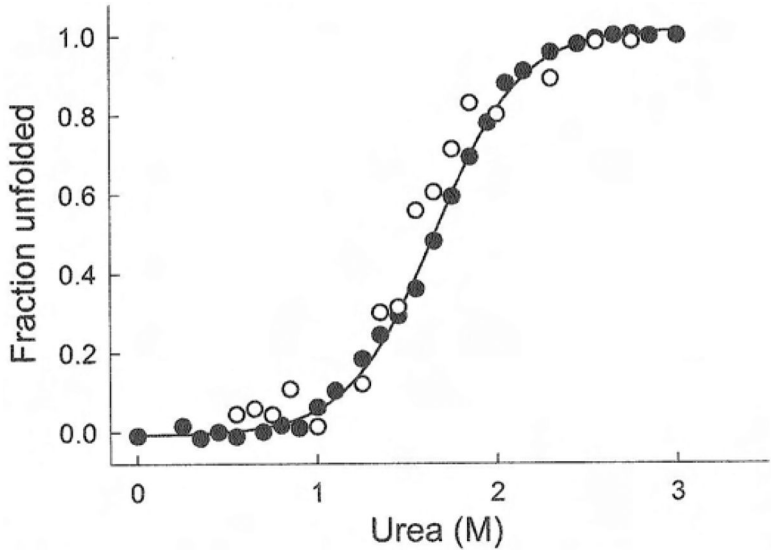

Fig. 7.5.

Reversibility of the in vivo urea titrations. FlAsH-labeled tetra-Cys CRABP-expressing cells were treated with 3 M urea for 75 min, and then refolding was initiated by dilution of aliquots into fresh LB medium containing 100 μg/mL ampicillin. After incubation of the cells for 60 min, the extent of return of the FlAsH signal, as a measure of refolding, was monitored by FlAsH fluorescence (*open symbols*). A urea melt of FlAsH-labeled tetra-Cys CRABP incubated for 75 min is given for comparison (*closed symbols*).

Table 7.1

Thermodynamic stability of tetra-Cys CRABP in vitro and in vivo. (Reproduced from Ref. (12) with permission from Wiley InterScience.)

	ΔG(kcal.mol^{-1})	m(kcal.mol^{-1}.M^{-1})	C_m(M)
In vivo (incubated 75 min)	5.2 ± 0.1	–3.4 ± 0.1	1.52 ± 0.05
In vitro (incubated 360 min)	6.5 ± 0.3	–1.8 ± 0.1	3.68 ± 0.06