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From the Test Tube to the Cell: Exploring the Folding and Aggregation of a β -Clam Protein

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Abstract

A crucial challenge in present biomedical research is the elucidation of how fundamental processes like protein folding and aggregation occur in the complex environment of the cell. Many new physico-chemical factors like crowding and confinement must be considered, and immense technical hurdles must be overcome in order to explore these processes in vivo. Understanding protein misfolding and aggregation diseases and developing therapeutic strategies to these diseases demand that we gain mechanistic insight into behaviors and misbehaviors of proteins as they fold in vivo. We have developed a fluorescence approach using FAsH labeling to study the thermodynamics of folding of a model β -rich protein, cellular retinoic acid binding protein (CRABP) in *Escherichia coli* cells. The labeling approach has also enabled us to follow aggregation of a modified version of CRABP and chimeras between CRABP and huntingtin exon 1 with its glutamine repeat tract. In this article, we review our recent results using FAsH labeling to study in-vivo folding and present new observations that hint at fundamental differences between the thermodynamics and kinetics of protein folding in vivo and in vitro.

Keywords

protein folding; protein aggregation; fluorescence; in-cell folding; molecular crowding

INTRODUCTION

Considerable progress has been made in our understanding of the mechanism of protein folding and aggregation in vitro, providing a foundation to elucidate protein misfolding pathologies such as the neurodegenerative diseases—Alzheimer's, Parkinson's, and other amyloid diseases.¹ However, the complexities of the cellular environment must be considered in order to face the challenges of protein folding and misfolding in vivo.^{2,3} Folding in vivo occurs in a crowded, spatially organized, heterogeneous cellular environment with a cast of folding assistants; this environment is drastically different from the highly dilute solutions and optimized buffers exploited for in vitro folding studies. Theoretical predictions as well as some exploratory experiments suggest that the high concentration of macromolecules (350–400 mg/ml) will markedly affect the conformational dynamics and energetics of folding polypeptides,

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favoring compact states.^{4,5} Moreover, proteins emerge vectorially from the ribosome with a rate comparable with the rates observed for fundamental steps in protein folding in vitro; the rate of translation in bacteria is ~ 10–50 amino acids per second and in eukaryotes, 4–5 amino acids per second. Thus, the N-terminal regions of a growing polypeptide in vivo will explore conformational space before the C-terminal regions have emerged from the ribosome. The sequence code for folding has arisen based on in vivo selection pressures and must necessarily reflect this vectorial conformational search process. Additionally, molecular chaperones interact with a large fraction of nascent and newly synthesized polypeptides and these interactions may influence the energy landscape for folding.³ Nonetheless, abundant evidence accumulated over the last half century argues persuasively that the intrinsic structural propensity of a protein's primary sequence contains the information for its eventual native fold. What is not yet well understood is how this sequence information is expressed in vivo.

Major technical challenges impede our ability to characterize protein folding in vivo using the wide array of sophisticated approaches that have been so informative in vitro. Observations of the protein of interest must be made at physiological concentrations in the background of all other cellular constituents. Kinetic experiments must monitor a population of synchronized molecules, in a system where the manifold of reactions is huge. Ideally, we seek to observe how structure formation occurs, what intermediate states are visited by a nascent or newly synthesized polypeptide, and what energetic differences exist among the accessible states. We must be able to 'see' the protein of interest first and foremost, leading to a need for signals that are observable above the cellular background. The signal must report on the conformational explorations of the labeled protein. And then we must be able to manipulate the system to pose questions about the formation of structure in the protein.

We have used a predominantly β -sheet protein, cellular retinoic acid-binding protein I (CRABP), whose in vitro folding we have explored in detail,^{6–9} to study folding and aggregation in vivo. We have incorporated a binding site (tetra-cysteine motif) for the fluorescent dye, 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FAsH),^{10,11} into a loop in both wild type CRABP and a slow-folding, aggregation prone mutant, P39A CRABP (Figure 1).¹² FAsH fluorescence has allowed us to assess the apparent in vivo stability of CRABP,¹² to elucidate the aggregation mechanism of P39A tetra-Cys CRABP in vitro,¹³ and to probe the behavior of chimeras of CRABP and exon 1 of huntingtin (Htt),¹⁴ the protein that aggregates in Huntington's disease. Our findings suggest that aggregation of P39A follows a nucleation–polymerization mechanism from a monomeric nucleus.¹³ Additionally, the osmolyte proline abrogates this aggregation both in vitro and in vivo.¹⁵ Lastly, expansion of the polyglutamine repeat in the Htt exon 1 destabilizes the flanking CRABP and leads to aggregation that is dominated early on by CRABP and later by the polyglutamine tract.¹⁴ These and other new results are enabling us to move from the test tube to the cell in our exploration of protein folding and aggregation.

In this article, we briefly review the results we have obtained to date and the questions these results raise. Then, we report intriguing new findings that suggest fundamental differences in the way folding occurs in vivo and how we are investigating the reasons for these differences.

MATERIALS AND METHODS

Protein Expression

Escherichia coli (*E. coli*) BL21(DE3) cells transformed with CRABP or tetra-Cys CRABP plasmids (in all cases the CRABP harbors a stabilizing mutation, Arg131Gln, and is thus a 'wild-type*' variant^{7,8}) were cultured at 37°C in LB medium with 100 μ g/ml ampicillin. Protein expression was induced at OD₆₀₀ = 1.0 by adding isopropyl- β -D-thiogalactoside (IPTG) to a final concentration of 0.4 mM for 4 h, and the tetra-Cys CRABP was purified from

the soluble fraction of the cell extract.⁸ Although it partitions nearly equally between the soluble and insoluble cell fractions, we isolated P39A tetra-Cys CRABP from the soluble fraction to insure the homogeneity of the samples used for in vitro studies. The concentration of the purified samples was determined spectrophotometrically using the ϵ_{280} value of 21750 $M^{-1}cm^{-1}$. Purified protein was stored in aliquots up to 200 μM at 4°C and used within 2 weeks.

In Vitro Urea Melts

Purified tetra-Cys CRABP and P39A tetra-Cys CRABP were labelled with FIAsh-EDT₂ (synthesized using published protocols^{10,11,16}) and ethane dithiol (EDT, ratio 1:5) at room temperature in 10 mM HEPES pH 7.5, containing 1 mM tris(carboxyethyl)phosphine as described.¹² Prelabeled protein (7 μM) was equilibrated in solutions at the desired urea concentration for a range of incubation times, and the unfolding transitions were monitored by Trp fluorescence (excitation at 280 nm; emission at 350 nm). The actual urea concentration was determined by measuring the index of refraction,¹⁷ and the curves were analyzed by a two-state model using the linear extrapolation method.¹⁸

In-Cell Labeling and In-Vivo Stability Measurements

E. coli cells transformed with either the tetra-Cys CRABP or P39A tetra-Cys plasmid were cultivated until OD₆₀₀ = 0.5 and after gentle lysozyme pre-treatment¹² were preloaded with FIAsh-EDT₂ along with EDT to suppress the labeling of endogenous cysteine pairs.¹¹ In the usual protocol, aliquots of 1.5 ml cells were treated with 3 μl FIAsh-EDT₂ (0.1 mM stock solution) and 3 μl EDT (0.5 mM stock solution). After one generation, at OD₆₀₀ = 1.0, tetra-Cys CRABP synthesis was induced with 0.4 mM IPTG and the protein expression continued for 2 h. Cells were aliquotted in 150 μl portions and sterile urea-LB solution (usually 9M urea) was added to different end concentrations. The volume of all aliquots was maintained equal by addition of fresh LB medium, and the incubation was continued at 37°C for different times. The actual urea concentration was corrected based on the measured index of refraction.¹⁷ In parallel, the viability of the cells at different denaturant concentrations and after various incubation times was monitored by counting colonies on an agar-LB plate after overnight incubation.¹² FIAsh fluorescence of the bulk suspension was monitored at 530 nm (excitation 500 nm) in a quartz cuvette maintained at 37°C with a water bath on a Photon Technology International QM-1 fluorometer. Fluorescence of cells treated identically but expressing CRABP without a tetra-Cys motif was used as a blank, and each point in the time-course was corrected by subtraction of the blank values. To derive the thermodynamic parameters, urea denaturation curves were fitted to a two-state model.¹⁸

Fluorescence Microscopy

For fluorescence microscopy studies, 2 μl of 10-fold concentrated cell suspension in 50 mM Hepes (pH 7.5) were immobilized in 1% agarose in LB and imaged by using a Nikon Eclipse E600 microscope, with excitation at 485 nm and a 510-nm emission barrier filter; the processing software used was OPENLABS (Improvision, Lexington, MA).

Kinetics of Unfolding In Vitro

To measure unfolding kinetics, protein in 10 mM Tris · HCl and 2 mM β -ME (pH 8.0) was added to buffered urea solutions (10 mM Tris · HCl, 2 mM β -ME at pH 8.0) to a final concentration of 5 μM . All solutions were preincubated at 37°C. The unfolding process was monitored using Trp fluorescence (excitation 280 nm; emission 350 nm) on a Photon Technology International QM-1 fluorometer with temperature control using a 1 ml quartz cuvette. Traces were collected until the signal reached saturation and were all buffer subtracted prior to analysis. A fluorescence scan from 300–380 nm was also performed to check whether the protein was completely denatured (λ_{max} = 350 nm). The rate constants of unfolding at a

particular urea concentration were determined by fitting to a single-exponential equation, consistent with a two-state model of unfolding. The quality of the fit was determined by looking at the residuals.

RESULTS AND DISCUSSION

Review of Our In Vivo Folding and Aggregation Studies

Our approach to study in vivo folding and aggregation has exploited the features of FAsH, a fluorescein analogue containing two arsenoxides that specifically ligates to a genetically encoded tetracysteine motif C-C-X-X-C-C,^{10,11,16} which is highly uncommon in the cellular proteome, here incorporated within the CRABP sequence (Figure 1A). An advantage of the system we designed is that by insertion of the FAsH-binding motif in the flexible Ω -loop of CRABP, the FAsH emission intensity reports on the conformational state of the protein; we find that the denatured ensemble is hyperfluorescent compared with the native state (Figure 1B). FAsH fluorescence can be used as additional read-out to follow the transition from native to unfolded CRABP during denaturant unfolding. Importantly, FAsH fluorescence can be also used to follow urea denaturation of the tetra-Cys CRABP in *E. coli* cells.¹⁵ Intriguingly, the transition in vivo, as we first observed and reported it,¹⁵ is apparently more strongly dependent on the urea concentration than the comparable in vitro curve, manifested by a steeper slope through the transition region than the test tube experiment. The apparent stability estimated from these first experiments was nearly the same in vivo as in vitro. At first consideration, this result was unexpected and seemed inconsistent with macromolecular crowding theory, which predicts that more compact states will be relatively favored in more crowded environments, and thus that the native state will be stabilized.^{4,5} However, multiple considerations led us to be cautious in pushing the interpretation of the in vivo data: First, our protocol was designed to err on the side of retaining viability of the cells exposed to urea by restricting the time of incubation as much as possible. Second, we recognized that a great number of urea-dependent phenomena could be taking place in the cells (stress responses, denaturation of other proteins, chaperone binding, etc.). Thus, a direct comparison of in vitro and in vivo data is confounded by the complex spectrum of denaturant dependent events in the cell. As described below, we have been pursuing followup studies to dissect in as much detail as possible these different complexities. Additionally, we were confident in concluding that FAsH labeling offers exciting prospects for following the cellular fate of the tetra-Cys proteins, exploiting the finding that the FAsH fluorescence signal reports on the conformational state of the protein in the cell.

A specific application of the sensitivity of FAsH fluorescence to the conformational state of CRABP presented itself when we observed different time courses upon induction of soluble and aggregation-prone variants of CRABP (Figure 2A).¹⁴ The normal fluorescence time course for soluble expression is a steady-state increase of the FAsH fluorescence, reporting on protein synthesis, which plateaus when de novo synthesis ceases and the cells enter stationary phase. In the case of aggregation-prone variants of tetra-Cys CRABP, the fluorescence is a cumulative signal of both synthesis and aggregate formation. Because the FAsH-bound protein has higher fluorescence intensity in an unfolded or partially folded state relative to the folded state, there is an abrupt rise in the FAsH fluorescence when expressed protein begins to accumulate misfolded and aggregated species (Figure 2B). Consistent with this interpretation are the results of fluorescence microscopy: The abrupt fluorescence increase coincides with the formation of hyperfluorescent aggregates. For highly aggregation-prone proteins, here exemplified by the tetra-Cys CRABP Htt53, a chimera of CRABP and exon 1 of Htt, a polyQ-containing protein whose aggregation is a hallmark of Huntington's disease, aggregation begins (signalled by the sharp rise in FAsH fluorescence) essentially as soon as expression of the protein commences.

The time course of in vivo fluorescence mirrors the solubility of the tetra-Cys proteins and enables tests of the impact of intrinsic (e.g., mutations) and extrinsic (e.g., chaperone over- or

under-expression, osmolytes) factors on aggregation. In a recent publication,¹⁵ we addressed the influence of natural osmoprotectants on the aggregation and were able to perform a fast screening of how the osmolality of the medium affected aggregation of the slow-folding mutant, P39A tetra-Cys CRABP. Increased osmolality of the nutrient medium triggers an osmotic shock response, and the cell starts to increase the cytoplasmic concentration of small protective organic molecules, termed osmolytes, which counteract the loss of water and protect cellular proteins from denaturation.^{19–21} Both active transport of exogenous osmolytes and synthesis of endogenous osmolytes are deployed to accomplish the increase in intracellular concentrations. The intra-cellular amount of the osmolyte (here proline¹⁵) depends on the salinity of the external medium and can reach concentrations $>0.4M$ by $0.3M$ NaCl.²¹ FIAsh fluorescence is sensitive to conformational changes and can be used as a read-out of solubility of the protein; a complete solubilization of the P39A tetra-Cys CRABP is observed at $0.3M$ NaCl, and consistent with this, the time course of FIAsh fluorescence for expression of P39A tetra-Cys CRABP under these high salt conditions resembles that of the soluble tetra-Cys CRABP. The effect of proline can be rationalized by its solvophobic destabilization of partially folded states and early aggregates (both of which expose excess hydrophobic surface) and its favorable effect on solubilization on the native state.^{15,22–25}

In addition to its utility for monitoring aggregation *in vivo*, FIAsh can be advantageously used as a read-out of aggregation *in vitro*. We found that the aggregation kinetics monitored by FIAsh fluorescence faithfully recapitulates the kinetics of monomer depletion in the time course of aggregation.^{13,14} This enabled us to address both the aggregation mechanism of P39A tetra-Cys CRABP, which forms amorphous aggregates, and of the tetra-Cys CRABP Htt53 chimera, which forms amyloid fibrils. The aggregation of both proteins is consistent with a nucleated-polymerization model,²⁶ and kinetic analysis revealed a monomeric nucleus in both cases, suggesting that a folding or unfolding reaction within a monomer was the thermodynamically unfavorable event prerequisite for aggregation. A monomeric nucleus was previously implicated in *in-vitro* aggregation of polyglutamine peptides,²⁷ making it difficult to distinguish which part of the tetra-Cys CRABP Htt53 chimera (either tetra-Cys CRABP or Htt domain) is mechanistically dominant in aggregation. Intriguingly, the diffraction pattern of isolated P39A tetra-Cys CRABP aggregates formed *in vivo* resembles the characteristic fingerprint pattern of ordered amyloid structures, suggesting a common microstructure for all types of aggregates.¹³

In addition to our work on the aggregation mechanism *in vivo* and *in vitro* of polyQ containing chimeras, we have been able to ask how an elongated polyQ stretch (in the Htt exon 1) leads to aggregation in the context of an adjacent stably folded domain (the FIAsh-labeled tetra-Cys CRABP).¹⁴ Attaching Htt exon 1 domains containing shorter polyglutamine repeats (Q20) (i.e., in the nonpathological range) to tetra-Cys CRABP, either at its N- or C-terminus, leads to constructs that remain soluble with no evidence of structural disruption of the tetra-CRABP protein. By contrast, chimeras of tetra-Cys CRABP and Htt exon 1 domains with expanded polyglutamine tracts (beyond the pathological threshold, i.e., >35) show perturbed structural integrity of the CRABP domain (again, whether the Htt exon 1 is N- or C-terminally attached). The tight correlation of these effects with the polyQ length argues that the repeat tract is solely responsible for the pathophysiology and that the indirect influence on adjacent domains (manifested as structural perturbation) are contributory to the disease pathologies.

In Vitro Versus In Vivo Protein Stability

Our initial experimental approach in measuring *in vivo* stability in *E. coli* cells by urea titration was designed to balance the competing considerations that (a) the viability of the cells decreases over time; and (b) the establishment of a true equilibrium between folded and unfolded populations requires a significant incubation time. In follow-up studies, we have examined the

change in the shape of the urea melt as a function of incubation time, both in vitro and in vivo, for both tetra-Cys CRABP and P39A tetra-Cys CRABP (Figure 3). Note that the time required for equilibrium, as indicated by the absence of further change in the urea melt as incubation time is increased, is strikingly different in vitro and in vivo. Equilibrium was reached at 37°C four to fivefold faster in vivo than in vitro. According to fundamental kinetics of reversible first-order reactions, we can calculate from the forward (folding) and reverse (unfolding) rate constants the time for equilibrium to be established in a two-state protein unfolding reaction starting with purely folded state. For example, using rate constants we determined for the P39A variant of CRABP,⁹ we calculate that at 20°C, it should take over 6 h for 95% equilibration at the C_m (5.9M urea) and up to 7M urea; this estimate is quite consistent with our observations, even at 37°C. For CRABP and its mutants in vitro, unfolding kinetics (in the unfolding branch of the urea-dependent so-called Chevron plot) are generally significantly slower than folding kinetics (in the folding branch).⁹ Thus, acceleration of the unfolding rate would be expected to be the most likely factor leading to faster equilibration in vivo. We have obtained preliminary data in support of this conclusion by monitoring the unfolding rate in vivo directly from the time course of FAsH fluorescence after suspending cells expressing tetra-Cys CRABP in solutions at varying urea concentrations. The apparent unfolding rate is approximately four times faster than the comparable rate of unfolding in vitro.²⁸ The fact that the C_m is shifted to lower urea concentration (consistent with the lower stability in vivo), and the possibility that the overall folding kinetics are also accelerated may lead to a more rapid establishment of equilibrium in vivo.

We are pursuing the origin of the altered equilibration time course, including making more quantitative measurements of the unfolding rate in vivo. Then we will seek an understanding of the attributes of the cellular environment that contribute to the changes. Factors that must be considered include interactions with other components in vivo (including chaperones) and macromolecular crowding, although the latter is predicted to accelerate the folding kinetics and not influence the unfolding kinetics.^{4,5} These predictions were based, however, on neutral or repulsive interactions between the folding polypeptide and the crowding species. The presence of attractive interactions, as might be expected for chaperones, could lead to exactly the opposite effects.

Now that the dependence on incubation time is established, we are in a position to compare much more confidently the difference in thermodynamic stability between the cellular environment and the highly dilute test-tube environment (Table I). As we reported previously, the apparent m value in vivo for tetra-Cys CRABP is significantly higher than that measured in vitro. The apparent stability is modestly lower in vivo than in vitro, consistent with our observation of a faster equilibration/faster unfolding in vivo. The in vivo experiment is very complicated to interpret, as the state of the cell is certainly changing in response to the urea treatment, and thus one cannot distinguish solution effects from interactions with other cellular components such as chaperones, and one must consider these parameters ‘apparent’.

To dissect the factors that contribute to differential behaviors in vitro and in vivo, we are exploring a number of conditions that may influence both kinetics and thermodynamics of CRABP folding. First, we have begun to assess the effect of crowding reagents.²⁹ Our preliminary results argue that the stability of CRABP is only modestly altered by the crowding and excluded volume factors characteristic of the cellular environment. We determined the stability of CRABP by urea titration in the presence of a crowding agent, Ficoll-70, at 100 and 186 g/l concentrations. A small destabilization effect is induced by the crowding agent, contrary to expectations from theory,⁴ but consistent with the trends observed in our in vitro and in vivo urea titrations.

Future Questions and Strategies

We are dissecting the various factors that may influence the behavior of a folding polypeptide *in vivo*. As illustrated above, we are exploring the impact of the high concentration of macromolecules through the use of ‘crowding agent’ and cell lysate samples. We will examine the impact of molecular chaperones through the use of strains in which chaperones are deleted, or reduced or elevated in concentration by inducible promoters. We will parallel these experiments *in vitro* by addition of chaperones to purified systems. Our most recent work is focusing on how the conformational ensemble is affected by the vectorial appearance of a polypeptide chain upon its biosynthesis on a ribosome. We are carrying out ensemble measurements of distance distributions in arrested nascent chains and single-molecule distance sampling measurements, both using fluorescence resonance energy transfer.

In all of this work, our hope is that we will be able to describe how the energy landscape for folding is shaped by the cellular environment, and how this landscape influences the propensity of polypeptides to aggregate *in vivo*, thereby shedding light on aggregation and misfolding diseases.

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FIGURE 1. Design of tetra-Cys CRABP. (A) Backbone structure of CRABP (PDB code 1CBI) showing the position of the tetra-Cys motif in the highly variable Ω -loop. (B) Fluorescence spectra of FIAsh-labeled tetra-Cys CRABP in its native state (folded) and denatured with 8M urea (unfolded). [Figure adapted from Ignatova, Z.; Gierasch, L. M. *Proc Natl Acad Sci USA* 2004, 101, 523–528.]

FIGURE 2.

Monitoring aggregation in vivo. (A) Time course of in-cell expression of FLAsH-labeled tetra-Cys CRABP I, P39A tetra-Cys CRABP and chimeras of tetra-Cys CRABP and exon 1 of Huntingtin with 40 and 53 glutamine residues followed at 530 nm.^{12,14} Cells were pre-loaded with FLAsH before induction of protein expression at time zero. Tetra-Cys CRABP (red) is soluble, P39A tetra-Cys CRABP (purple) partitions 50% into the insoluble fraction, tetra-Cys CRABP HD40 (orange) is 20% in the insoluble fraction, and tetra-Cys CRABP Htt53 (light blue) is isolated only in an insoluble state. For comparison, the fluorescence in all the samples is normalized against the highest fluorescence measured (tetra-Cys CRABP Htt53) and the cell number. (B) Fluorescence microscopy images of *E. coli* cells expressing P39A tetra-Cys CRABP showing the conversion of the uniformly distributed fluorescence (arising from the soluble state) into hyperfluorescent aggregates localized at the poles of the cells. [Parts of this

figure reproduced with permission from Ignatova, Z.; Gierasch, L. M. J Biol Chem 2006, 281, 12959–12967.]

FIGURE 3.

Urea denaturation curves as a function of incubation time in vitro and in vivo. (A) Urea melt of FIASH-labeled tetra-Cys CRABP in vitro after sample incubation for the indicated times, monitored by Trp fluorescence; (B) urea melt of FIASH-labeled P39A tetra-Cys CRABP in vitro after sample incubation for the indicated times, monitored by Trp fluorescence; (C) urea melt of FIASH-labeled tetra-Cys CRABP in vivo after sample incubation for the indicated times, monitored by FIASH fluorescence; and (D) urea melt of FIASH-labeled P39A tetra-Cys CRABP in vivo after sample incubation for the indicated times, monitored by FIASH fluorescence.

Table I

Thermodynamic Stability of Tetra-Cys CRABP In Vitro and In Vivo

Protein	ΔG (kcal $\cdot$ mol ⁻¹)	$-m$ (kcal $\cdot$ mol ⁻¹ $\cdot$ M ⁻¹)	C_m (M)
tetra-Cys CRABP			
In vivo (incubated 75 min)	5.2 $\pm$ 0.1	3.4 $\pm$ 0.1	1.52 $\pm$ 0.05
In vitro (incubated 360 min)	6.5 $\pm$ 0.3	1.8 $\pm$ 0.1	3.68 $\pm$ 0.06