Analytical Biochemistry 434 (2013) 181–186

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

In vivo detection and quantification of chemicals that enhance protein stability

Tsinatkeab T. Hailu [a,b], Linda Foit [a], James C.A. Bardwell [a,*]

[a] Howard Hughes Medical Institute, Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA
[b] Institute of Molecular Cell Biology, Faculty of Earth and Life Sciences, VU University Amsterdam, 1081 HV Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 9 June 2012
Received in revised form 27 November 2012
Accepted 27 November 2012
Available online 5 December 2012

Keywords:
In vivo
Folding sensor
β-lactamase
Osmolytes
Protein stability

ABSTRACT

We have devised protein-folding sensors that link protein stability to TEM-1 β–lactamase activity. The addition of osmolytes and other compounds with chemical chaperone activity to the growth medium of bacteria containing these sensors increases β–lactamase activity up to 207-fold in a dose-dependent manner. This enables the rapid detection and sensitive quantification of compounds that enhance *in vivo* protein stability.

Proteins are often frustratingly unstable, creating major problems in their biochemical and structural analyses [1]. Despite the presence of molecular chaperones that assist protein folding *in vivo*, as much as 30% of newly synthesized polypeptides form misfolded conformers in the cell [2–4]. Not surprisingly, a large number of disease states have been associated with protein misfolding. These include cystic fibrosis, Alzheimer's disease, and Huntington's disease [5]. A number of naturally occurring low-molecular-weight compounds, such as glycerol, sorbitol, and L–proline are known to stabilize proteins *in vivo* [6]. These compounds were first identified as natural osmolytes in living tissues. They act to maintain cell volume and stabilize macromolecules under conditions of environmental stress such as high osmotic and salt pressure [7–9]. Subsequent studies showed that these compounds stabilize proteins *in vitro* and assist in their refolding [10]. Intense efforts have also resulted in the discovery of compounds termed "pharmacological chaperones" that specifically stabilize disease-related mutant proteins [11,12]. The identification of chemical compounds or conditions that help to stabilize proteins typically involves an expensive and time-consuming trial-and-error process. For all of these reasons, a simple assay that allows for the rapid identification of stabilizing compounds would be very useful.

We present here a method that allows us to readily assess the influence of chemical compounds on the *in vivo* stability of proteins. Our approach is based on a sandwich fusion in which an unstable test protein is inserted into β–lactamase [1]. The *in vivo* stability of the test protein determines β–lactamase enzymatic activity, which can be measured by a simple colorimetric assay [13]. This results in a simple, sensitive, and quantitative assay that can be used to screen for chemicals that enhance protein stability *in vivo*.

In our sandwich fusion constructs that serve as protein stability indicators, a test protein is inserted between amino acids 196 and 197 of β–lactamase [1]. If the inserted protein is stable, the N- and C-terminal portions of β–lactamase will remain close enough together to form a functional entity that confers enzymatic activity. Conversely, unstable protein inserts will be targeted for proteasomal degradation. This results in the separation of the two halves of β–lactamase and reduced enzymatic activity (Fig. 1). This system provides a sensitive and convenient way of linking the *in vivo* stability of test proteins that may lack an easily assayable function to β–lactamase activity for which a simple colorimetric assay is available.

Due to the presence of outer membrane porins, the periplasm of gram-negative bacteria is permeable to molecules smaller than approximately 600 Da [14,15]. We reasoned that the periplasmic location of our protein folding biosensors allows for a simple enzymatic readout for the presence of osmolytes and chemical and pharmacological chaperones in the medium that positively affect the folding of unstable proteins.

Materials and methods

Chemicals and antibodies

Nitrocefin was purchased from Calbiochem (La Jolla, CA, USA). Stock solutions (10 mg/ml) of nitrocefin were prepared in dimethyl

* Corresponding author.
E-mail address: jbardwel@umich.edu (J.C.A. Bardwell).

0003-2697/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2012.11.022

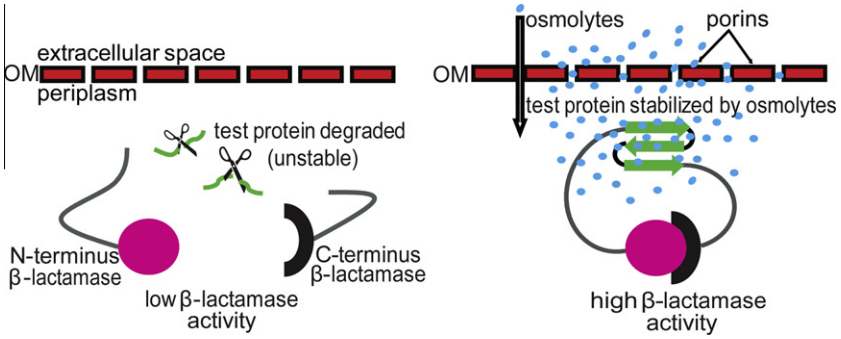

Fig.1. Schematic representation of a sandwich fusion system for assessing the influence of osmolytes on protein stability *in vivo*. An unstable test protein (green) is inserted into β–lactamase in the form of a sandwich fusion. In the absence of osmolytes, the inserted protein is subject to degradation by periplasmic proteases, symbolized by scissors. This results in a separation of the two β–lactamase fragments and, hence, decreased β–lactamase activity. Osmolytes (blue circles) diffuse into the periplasm through the holes generated in the outer membrane (OM) by endogenous porins. The presence of osmolytes favors a stable fold of the inserted protein. This allows an association of the N fragments (magenta circle) and C fragments (black semicircle) of β–lactamase, resulting in high enzymatic activity.

sulfoxide and stored at −20 °C. A 500-μg/ml working solution of nitrocefin was prepared in 100 mM sodium phosphate buffer (pH 7.0) prior to each experiment. Because nitrocefin is extremely light sensitive in solution, steps involving nitrocefin handling were performed in the dark and tubes were wrapped in aluminum foil. B-PER bacterial protein extraction reagent was purchased from Thermo Scientific (Rockford, IL, USA). Ethylenediaminetetraacetic acid (EDTA)[1]-free protease inhibitor cocktail was purchased from Roche (Indianapolis, IN, USA). EDTA, NaCl, urea, and glycerol were purchased from MP Biomedical (Solon, OH, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). All additives tested as osmolytes were of analytical grade and filter-sterilized after dissolving in Lysogeny Broth (LB) medium. Polyclonal anti-β–lactamase antibody was obtained from Millipore (Billerica, MA, USA). Polyclonal GroEL and DnaJ antibodies and monoclonal DnaK antibody were purchased from Stressgen (Ann Arbor, MI, USA).

Bacterial strains and expression vectors

NEB 10-beta cells were used for cloning. After cloning, plasmids were transformed into MG1655 *ΔampCΔhsdR* or NEB 10-beta strains for subsequent experiments. Wild-type (WT) β–lactamase was expressed from pBR322. Plasmids for the expression of β–lactamase–link (pBR322*link) or β–lactamase–Im7-β–lactamase (pBR322*–Im7, where Im7 is immunity protein 7) were constructed as described previously [1]. Human muscle acylphosphatase 2 (hAcP2) was expressed from the plasmid pMB1 (pMB1-AcyP2). pMB1 was constructed by cloning together the origin of replication and antibiotic resistance marker of pBR322 with the arabinose-inducible control elements of pBAD33. To achieve this, the β–lactamase gene with the linker-encoding region was amplified from pBR322*link and cloned into pBAD33 using *Eco*RI and *Xba*I sites. Whole plasmid polymerase chain reaction (PCR) was then performed to introduce additional restriction sites for cloning hAcp2 without altering the amino acid sequence of β–lactamase or the linker region.

A variant of hAcP2 where the cysteine at position 21 has been mutated to serine was used for assays. This variant is used to avoid folding kinetics complexities and has essentially the same properties as WT AcP2 [16].

Bacterial growth

Bacterial growth was performed in 5-ml tubes in LB medium. In all experiments, plasmids were maintained by growth in tetracycline (12 μg/ml). To prepare cultures for the β–lactamase assay, each strain containing the desired plasmids was inoculated from plates into 5-ml tubes and grown overnight in LB in a rotary drum incubator at 600 rpm at 37 °C. For strains expressing Im7 fusions, 10-μl aliquots of overnight cultures were used to inoculate 5-ml LB containing the appropriate additives and grown overnight at 37 °C. For hAcP2, 100 μl of overnight cultures was used to inoculate 5 ml of LB and grown for 2.5 h and then induced with 0.5% arabinose and grown for an additional 4 h.

Bacterial harvest and lysis

Either 2 or 4 ml of the bacterial cell cultures for the Im7- or hAcP2-expressing strains, respectively, was harvested by centrifuging at 13,000 rpm for 10 min at 4 °C, and the supernatant was discarded. The pellets were resuspended in 1 ml (or 0.5 ml for hAcP2) of ice-cold B-PER lysis buffer supplemented with one tablet per 10 ml of Complete mini protease inhibitor cocktail (EDTA-free) and 20 mM EDTA and incubated for 1 h with shaking at 1000 rpm at 4 °C to ensure efficient lysis. The lysate was then centrifuged at 13,000 rpm for 20 min at 4 °C to separate soluble proteins from the insoluble fraction. The lysate supernatant was transferred to new 96-well polypropylene blocks kept on ice and used for subsequent β-lactamase activity assays.

β-Lactamase colorimetric assay

To quantify β-lactamase activity, 10 μl of nitrocefin (100 μM) was added to each well of a clear-bottom 96-well microtiter plate (Costar, Corning, NY, USA). Then 90 μl of cell lysate, appropriately diluted in lysis buffer to ensure that the linear stage of hydrolysis reaction is monitored, was added to each well containing nitrocefin. Nitrocefin alone with buffer was used a blank. The increase in absorbance at 486 nm over time was recorded with a BioTek Synergy 2 plate reader (Winooski, VT, USA). Nitrocefin hydrolysis was monitored for 30 min, and rates of hydrolysis were calculated from plots of the linear range of increasing absorbance. The obtained hydrolysis rates were normalized to the cell optical density (OD) measured at 600 nm of the respective sample.

Western blot analysis

Whole cell extracts and Western blot analysis were performed as described previously [1]. Briefly, 1 ml of overnight culture was

[1] *Abbreviations used:* EDTA, ethylenediaminetetraacetic acid; LB, (EDTA)[1]-free protease inhibitor cocktail was purchased from Roche Lysogeny Broth; WT, wild-type; Im7, immunity protein 7; hAcP2, human muscle acylphosphatase 2; PCR, polymerase chain reaction; mRNA, messenger RNA; cDNA, complementary DNA; TMAO, trimethylamine *N*-oxide; CS, citrate synthase.

harvested and pelleted as described above. The cell pellet was resuspended with the appropriate amounts of 1× phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.0) to obtain an OD at 600 nm of 5. Then 100 μl of the resuspension was transferred into a new 1-ml tube and mixed with 25 μl of reducing loading dye, leading to a final cell OD at 600 nm of 4. The mixture was incubated at 100 °C for 10 min to lyse the cells and centrifuged at 13,000 rpm for 1 min. The resulting total cell extract was separated on a 7% Tris–acetate precast gel (Invitrogen, Carlsbad, CA, USA) and probed with antibodies.

Quantitative real-time PCR

Overnight cultures were diluted 1:100 in LB medium and grown until early log phase under tetracycline selection. Then 3 ml of these cultures was harvested and 6 ml of RNA Protect Reagent was added to the cultures to stabilize the RNA. RNA was extracted using Qiagen's RNeasy Mini Kit (Valencia, CA, USA). The total messenger RNA (mRNA) extracted was diluted to 0.75 μg/μl for complementary DNA (cDNA) synthesis. cDNA was synthesized using random hexamer primers to prime total mRNA by incubation at 65 °C for 5 min, followed by incubation on ice for 5 min. Reverse transcription was carried out by incubation of the total mRNA–random hexamer mix at 25 °C for 10 min and at 50 °C for 55 min, followed by incubation at 80 °C for 10 min to inactivate the reverse transcriptase. Following reverse transcription, the mRNA was digested with RNase H. Quantitative real-time PCR was performed with gene-specific primers using SYBR Green (Invitrogen) according to the manufacturer's instructions.

Presentation of data

The experiments presented in the figures were performed at least three times (unless stated otherwise). The data are shown as means ± standard deviations from representative experiments.

Results and discussion

We have previously developed fusion biosensors to assess protein stability *in vivo* [1]. Using these biosensors, we showed that there is a very good relationship between the thermodynamic stability of mutant variants of the Im7 test protein and the β-lactamase activity of strains containing tripartite sandwich fusions made between these test protein variants and β-lactamase [1]. This fusion approach enables one to link the *in vivo* stability of Im7, which is difficult to assay for, with the simple enzymatic assay for β-lactamase. We wondered whether our previously developed protein stability biosensors could be adapted to use β-lactamase activity to detect the presence of compounds that, when added to the medium, affected the stability, and therefore the steady-state level, of the biosensor. Examples of such compounds are osmolytes or chemical chaperones. β-lactamase is active only when expressed in the periplasmic space and is inactive when expressed in the cytosol [17]. Thus, protein stability biosensors based on monitoring β-lactamase activity are ideal for detecting changes in the periplasmic folding environment. We have extensively used Im7 as a test protein because of the ready availability of mutants with well-defined levels of stability and the well-studied nature of Im7's folding pathway [18]. We previously showed that very unstable mutants of the test protein Im7, when inserted into our stability biosensors, show up to a 4-fold enhancement of β-lactamase levels on overproduction of the periplasmic chaperone Spy [19]. More stable mutants of Im7 and WT Im7 show smaller or insignificant fold increases in β-lactamase levels on overproduction of this chaperone (unpublished data). Given that unstable insert proteins provide the possibility of substantial stabilization via the action of chemical chaperones and osmolytes [1,18], we reasoned that using unstable mutants of Im7 would allow us to very sensitively detect changes in the folding environment due to chemical chaperone or osmolyte additions to the medium. As our first insert protein, we tested Im7 I22V, which is known to be substantially unfolded [18], into β-lactamase via flexible glycine–serine linkers. This resulted in the protein stability indicators fusion β-lactamase–Im7 I22V–β-lactamase.

Insertion of Im7 I22V into β-lactamase led to a 500-fold decrease in enzymatic activity in cell extracts compared with cell extracts of strains expressing WT β-lactamase in the absence of any additive (Fig. 2A), reflecting the very poor stability of the inserted test protein. We then tested the effect of a number of additives that are known to act as protein-stabilizing agents or osmolytes *in vitro* and *in vivo* [6,7,10,20] on the β-lactamase activity of this protein stability indicator. Cultivation of cells expressing β-lactamase–Im7 I22V–β-lactamase in the presence of the osmolytes trimethylamine *N*-oxide (TMAO), betaine, arabitol, glycine, sucrose, L-proline, sorbitol, and glycerol substantially restored β-lactamase activity to 4% to 39% of that seen for WT β-lactamase in the absence of any insert in LB medium (Fig. 2A). These results correspond to 22- to 207-fold increases in the β-lactamase activities of the sandwich fusion β-lactamase–Im7 I22V–β-lactamase in the presence of additives compared with the cultivation of the strain in the absence of these additives in normal LB (Fig. 2B). The largest increases (157- and 207-fold) were observed for 1 M sorbitol and 2 M glycerol, which correspond to 29% and 39% restoration of WT β-lactamase activity in the absence of additives, respectively (Fig. 2A and B). We examined the four most potent osmolytes (glycerol, sorbitol, L-proline, and sucrose) further to determine whether the increase in β-lactamase activity was dependent on the concentration of the osmolyte added to the medium. We found activity to be very nicely dose-dependent for all four compounds, suggesting that a higher concentration of these osmolytes leads to increased stabilization of the test protein (Fig. 3).

The increase in the enzymatic activity of the reporter protein (β-lactamase) in the presence of osmolytes correlates well with an increase in steady-state protein levels of WT β-lactamase itself, as observed by Western blotting using anti-β-lactamase antibody (see Figs. S1, S2, and S3 in supplementary material). This suggests that the increase in β-lactamase activity for the β-lactamase–Im7 I22V–β-lactamase fusion is partly due to a stabilization of the reporter protein itself. However, because the fusion protein shows a much larger increase in β-lactamase activity and steady-state protein level in the presence of chemical chaperones and osmolytes compared with the increase observed for WT β-lactamase or a fusion containing the short 30-amino-acid linker containing the multiple cloning sites for guest protein insertions (e.g., Im7) (Figs. S1A and S3), the osmolytes seem to preferentially stabilize the insert protein rather than the reporter itself (e.g., sucrose showed a 16.5-fold increase in steady-state protein level for strains expressing β-lactamase–Im7 I22V–β-lactamase vs. only a 1.9-fold increase in steady-state level for strains expressing WT β-lactamase). Such preferential stabilization of Im7 I22V over β-lactamase by chemical chaperones is consistent with the relative stabilities of the two proteins that have free energies of unfolding of −16 and −41.86 kJ/mol for Im7 I22V and WT β-lactamase, respectively. These results indicate that osmolytes work at least in part by increasing the levels of the sandwich fusion in the bacterial periplasm. These increased levels could be the result of the osmolytes either enhancing the *in vivo* folding yield of the fusion protein or enhancing their *in vivo* proteolytic resistance or a synergistic combination of these effects. Although it is possible that osmolytes can also change the specific activity of enzymes by directly interacting with them [21], it is unlikely that these potential changes would be

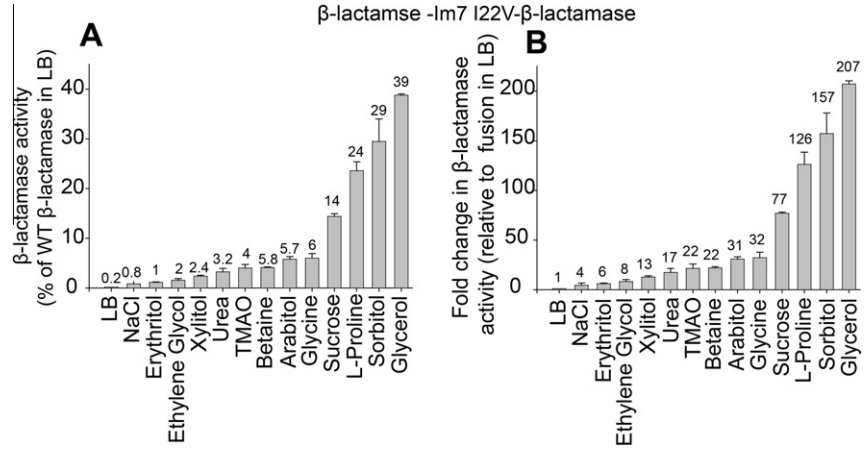

Fig.2. Influence of selected osmolytes on the β–lactamase activity of tripartite fusions containing the destabilized Im7 variant Im7 I22V. (A) β–Lactamase activity (determined by measuring absorbance at 486 nm over time and adjusted to the cell OD of the cell extract) in whole cell extracts of *Escherichia coli* MG1655 *ΔampCΔhsdR* expressing the tripartite fusion β–lactamase–Im7 I22V–β–lactamase in the presence or absence of protein-stabilizing agents in the cultivation medium. Enzymatic activity is expressed as percentage of the activity of cells expressing WT β–lactamase in LB medium (100%). (B) Fold change in β–lactamase activity in the presence of osmolytes in the cultivation medium compared with activity in LB of the same strain. NaCl (0.25 M) was used as a negative control. All of the additives were present at 0.5 M except glycerol, which was used at 2 M; sorbitol, which was used at 1 M; urea, which was used at 200 mM; and glycine, which was used at 0.1 M. Mean values ± standard deviations are shown for independent triplicate experiments.

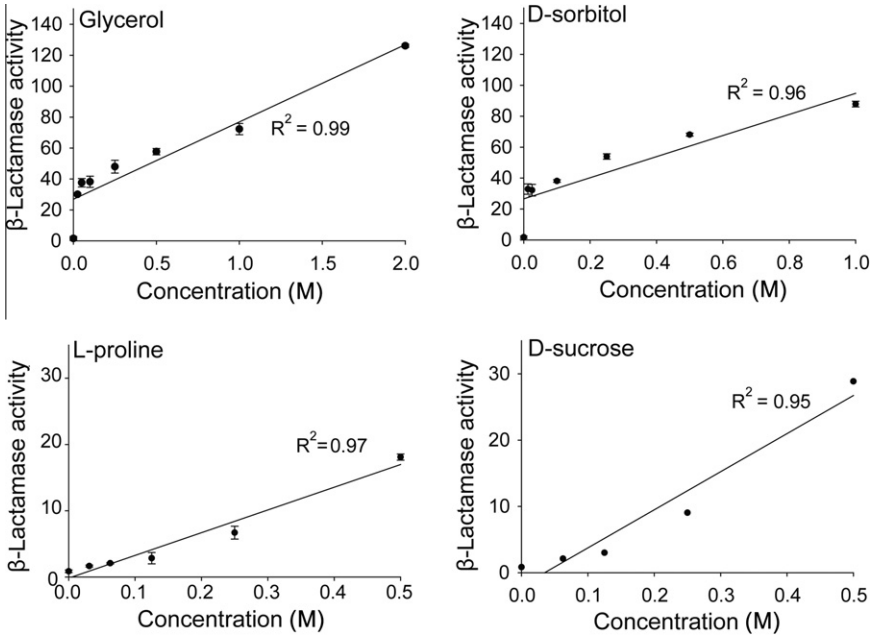

Fig.3. Addition of the osmolytes glycerol, D-sorbitol, L-proline, and D-sucrose to the cultivation medium restores β–lactamase activity in a dose-dependent manner in extracts of *E. coli* MG1655 *ΔampCΔhsdR* expressing β–lactamase–Im7 I22V–β–lactamase. Absolute enzymatic activity is expressed as the rate of hydrolysis of nitrocefin (determined by measuring absorbance at 486 nm over time) adjusted to the cell OD of the cell extract. Mean values ± standard deviations are shown for independent triplicate experiments except sucrose (for which data are from one experiment).

detected in our system given that the β-lactamase assays were done after removing the osmolytes.

Even though molar quantities of osmolyte are generally used *in vitro*, our in vivo assay is sensitive enough to detect the effects of even millimolar quantities. For example, the addition of 25 mM glycerol or sorbitol led to 30- and 33-fold enhancements in β-lactamase activity, respectively, for the Im7 I22V fusion (Fig. 3). That these compounds are effective at such low concentrations *in vivo* is surprising. Although the precise mechanism of protein stabilization by osmolyte and chemical chaperones is still unclear [3,6,20,22,23], many models propose that they act indirectly by inducing molecular crowding or by changing the structure and dynamics of water. These models suggest that the osmolytes must be present in high concentrations to be active. However, a direct interaction with the polypeptide backbone has also been proposed; this model might allow these compounds to be effective at lower concentrations, as we observed [22]. For instance, TMAO, a protecting osmolyte, appears to act by promoting H-bonding, an interaction that exists even within unfolded polypeptide chains such as in glycine–serine linkers [24–26]. Our

finding that these additives are active at lower concentrations *in vivo* is interesting and potentially important because it may make their clinical use more feasible.

One result that was initially surprising is that we saw a slight increase of 3.2-fold in β-lactamase activity in the presence of 0.2 M urea. On searching the literature, we found that a previous study showed that the two most common denaturants, urea and guanidinium hydrochloride, can act as protein stabilizers when used in subdenaturing concentrations [27]. The authors of that study suggested that these denaturants can act as chemical cross-linkers that help to establish hydrogen bonds and van der Waal's interactions between main chain and side chain atoms of proteins leading to limited freedom of motion. This effect has been termed "protein stiffening" [27].

As described above, Im7 I22V was selected as a test protein because it is a very unstable Im7 variant destabilized by 8.9 kJ/mol relative to WT Im7. We thought that this high degree of instability would provide the potential for a high degree of stabilization through the action of osmolytes and chemical chaperones and reasoned that tripartite fusions between β-lactamase and Im7 I22V might make for a sensitive chemical chaperone biosensor. The large enhancement of β-lactamase enzymatic activity on the addition of osmolytes and chemical chaperones shows that this choice was appropriate and enabled our development of a very sensitive sensor. Most of our experiments were performed using the β-lactamase–Im7 I22V–β-lactamase protein stability indicator. To determine whether other similarly destabilized mutants of Im7 were also useful as osmolyte/chemical chaperone biosensors, we performed a number of experiments with an alternate protein stability indicator sandwich fusion, β-lactamase–Im7 L34A–β-lactamase. This biosensor uses a different destabilized Im7 mutant (Im7 L34A), which is destabilized by 7.7 kJ/mol relative WT Im7 and similar to the degree of destabilization shown by Im7 I22V. The chemical chaperone biosensor constructed using the Im7L34A variant gave very similar results to the one made with Im7 I22V in terms of both the fold increase of β-lactamase activity and the β-lactamase steady-state protein level (see Figs. S1C and S4 in supplementary material). To see whether other destabilized test proteins could also be used to construct chemical chaperone biosensors, we studied the response of tripartite fusions between β-lactamase and two destabilized mutants of hAcP2: C21S/M61A and C21S/L65V. These biosensors still responded to the chemical chaperones glycerol, sorbitol, and trehalose, albeit to a much lesser degree than β-lactamase–Im7 I22V–β-lactamase or β-lactamase–Im7 L34A–β-lactamase (Fig. S5).

We also tested the effects of osmolytes on a β-lactamase variant that did not contain an Im7 insert but instead contained a 59-residue glycine–serine linker (β-lactamase–link long–β-lactamase). Cells expressing this construct that were cultivated in LB medium exhibited very low β-lactamase activities compared with WT β-lactamase. However, the activities were comparable to those measured in extracts from cells expressing tripartite fusions containing the destabilized Im7 mutant β-lactamase–Im7 I22V–β-lactamase or β-lactamase–Im7L34A–β-lactamase (Fig. S4a). This suggested that the insertion of this 59-residue linker into β-lactamase substantially destabilized the protein. Adding osmolytes to the medium increased the β-lactamase activity of β-lactamase–link long (59 aa)–β-lactamase 19- to 77-fold (Fig. S4b), suggesting that these osmolytes not only help to stabilize proteins but also can stabilize proteins that contain flexible unfolded structures such as the long glycine–serine linker construct. Previously, we showed that β-lactamase activity *in vivo* could be used to quantitatively monitor the thermodynamic stability of various proteins inserted into β-lactamase [1]. Our results here raise the possibility that many kinds of polypeptides inserted into β-lactamase, including unstable mutants of Im7, hAcP2, and even a simple 59-residue glycine–serine linker, can be used as osmolyte and chemical chaperone detectors.

We also considered the possibility that the osmolytes were exerting their effect on β-lactamase activity indirectly, either by inducing expression of the fusions at the transcriptional level or by causing the induction of host-encoded molecular chaperones [6,28,29], which then acted to stabilize the β-lactamase fusions. Therefore, we performed quantitative real-time PCRs on total mRNA extracted from cells expressing WT β-lactamase, β-lactamase–link–β-lactamase, or β-lactamase–Im7 I22V–β-lactamase cultured in the presence or absence of 2 M glycerol, 1 M sorbitol, or 0.5 M sucrose. We found no increase in mRNA encoding the fusion proteins; rather, a small osmolyte-dependent decrease was observed (see Fig. S6 in supplementary material). Similarly, quantitative real-time PCRs on mRNA levels of periplasmic chaperones or proteins that influence chaperone expression, including DegP, Spy, RpoH, SurA, Skp, and CpxP, showed no significant differences in the presence or absence of the osmolyte (Fig. S7). Furthermore, Western blots with antisera against the molecular chaperones DnaJ, DnaK, and GroEL showed only small osmolyte-dependent increases and decreases that were of neither sufficient magnitude nor direction to explain the increased levels of the tripartite fusions observed (Fig. S8).

Because of the relatively insensitive and laborious nature of previously available assays for osmolytes and chemical chaperones, most studies have tested the activity of only one or two of these compounds [28,30], making it difficult to evaluate the relative efficiency of different osmolytes. Mishra and coworkers' in vitro study [10], however, used the same conditions and compared the ability of five different chemical additives (glycerol, sorbitol, xylitol, ethylene glycol, and erythritol) to enhance citrate synthase (CS) refolding yield at 25 °C. Consistent with our *in vivo* results, they found that glycerol and sorbitol were the most effective as osmolytes, with 2 and 7 M glycerol enhancing CS refolding yield 1.5- and 2.25-fold, respectively. Sorbitol at 1 M was also effective, enhancing CS refolding yield 1.5-fold [10]. Like us, they also observed that ethylene glycol and erythritol were less effective as osmolytes than glycerol and sorbitol. Erythritol, which we found to be a very weak osmolyte, actually decreased refolding yield in their studies. Similar to our results, they found 0.5 M xylitol (the concentration we used) to be relatively ineffective. Thus, the rank order of effectiveness of the osmolytes *in vitro* was very similar to what we observed *in vivo*. However, our assay appears to be much more sensitive than the CS refolding assay, has a much greater dynamic range, and scales much better to osmolyte concentration (see Figs. 2 and 3).

In particular, we note that the increases that Mishra and coworkers observed in CS refolding yield (up to 2.3-fold) are much smaller than the up to 207-fold increases that we observed in stability biosensor activities. Furthermore, Mishra and coworkers observed a very poor correlation between osmolyte concentration and refolding yield [10]. Higher concentrations of osmolytes in general were found to actually decrease CS refolding yields, and (as noted) some osmolytes such as erythritol even decreased folding yields at all concentrations tested. Consistent with our observations, Mishra and coworkers observed that the addition of 3 M glycerol increased the midpoint of guanidine hydrochloride denaturation of CS from approximately 1.3 to 2 M. However, measurement of midpoints of guanidine denaturation is laborious and required large amounts of purified protein. In contrast, our *in vivo* folding biosensor-based assay for osmolytes not only shows much larger fold increases in the observed signal but also shows an excellent and positive correlation between osmolyte concentrations and β-lactamase activity (Fig. 3). It is clear that the relatively small effects that osmolytes have on *in vitro* refolding make these types of assays much less suitable to screen for chemical

chaperone activity of compounds compared with our *in vivo* biosensor screen.

Conclusion

Taken together, these results suggest that the increased β-lactamase activity and the increased steady-state protein levels observed in the presence of osmolytes are most likely attributable to the direct stabilizing effect that osmolytes have on the unstable protein constructs. Our method relies on a simple colorimetric assay and, therefore, should be useful in screening for compounds that either generally enhance protein stability or enhance the stability of specific proteins. Because there is a thermodynamic link between ligand binding and protein stability [31], this assay may also be useful in screening for compounds that bind tightly to and, therefore, either stabilize or inhibit specific proteins, including those involved in human disease [32]. This capability may enable the rapid discovery of pharmacological chaperones or protein inhibitors of pharmacological interest and may facilitate the identification of compounds that stabilize proteins for crystallography.

Acknowledgment

We thank Isabell Bludau for help with cloning of hAcP2 and Timothy Tapley for helpful suggestions during assay design.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2012.11.022.

References

[1] L. Foit, G.J. Morgan, M.J. Kern, L.R. Steimer, A.A. von Hacht, J. Titchmarsh, S.L. Warriner, S.E. Radford, J.C. Bardwell, Optimizing protein stability in vivo, Mol. Cell 36 (2009) 861–871.
[2] F.U. Hartl, M. Hayer-Hartl, Converging concepts of protein folding in vitro and in vivo, Nat. Struct. Mol. Biol. 16 (2009) 574–581.
[3] F.F. Liu, L. Ji, L. Zhang, X.Y. Dong, Y. Sun, Molecular basis for polyol-induced protein stability revealed by molecular dynamics simulations, J. Chem. Phys. 132 (2010) 225103.
[4] U. Schubert, L.C. Anton, J. Gibbs, C.C. Norbury, J.W. Yewdell, J.R. Bennink, Rapid degradation of a large fraction of newly synthesized proteins by proteasomes, Nature 404 (2000) 770–774.
[5] C. Voisine, J.S. Pedersen, R.I. Morimoto, Chaperone networks: tipping the balance in protein folding diseases, Neurobiol. Dis. 40 (2010) 12–20.
[6] T. Arakawa, D. Ejima, Y. Kita, K. Tsumoto, Small molecule pharmacological chaperones: from thermodynamic stabilization to pharmaceutical drugs, Biochim. Biophys. Acta 1764 (2006) 1677–1687.
[7] E. Papp, P. Csermely, Chemical chaperones: mechanisms of action and potential use, Handb. Exp. Pharmacol. 172 (2006) 405–416.
[8] P.H. Yancey, Organic osmolytes as compatible, metabolic, and counteracting cytoprotectants in high osmolarity and other stresses, J. Exp. Biol. 208 (2005) 2819–2830.
[9] P.H. Yancey, W.R. Blake, J. Conley, Unusual organic osmolytes in deep-sea animals: adaptations to hydrostatic pressure and other perturbants, Comp. Biochem. Physiol. A 133 (2002) 667–676.
[10] R. Mishra, R. Seckler, R. Bhat, Efficient refolding of aggregation-prone citrate synthase by polyol osmolytes: how well are protein folding and stability aspects coupled?, J Biol. Chem. 280 (2005) 15553–15560.
[11] J.H. Zhao, H.L. Liu, H.Y. Lin, C.H. Huang, H.W. Fang, S.S. Chen, Y. Ho, W.B. Tsai, W.Y. Chen, Chemical chaperone and inhibitor discovery: potential treatments for protein conformational diseases, Perspect. Med. Chem. 1 (2008) 39–48.
[12] W. Zheng, J. Padia, D.J. Urban, A. Jadhav, O. Goker-Alpan, A. Simeonov, E. Goldin, D. Auld, M.E. LaMarca, J. Inglese, C.P. Austin, E. Sidransky, Three classes of glucocerebrosidase inhibitors identified by quantitative high-throughput screening are chaperone leads for Gaucher disease, Proc. Natl. Acad. Sci. USA 104 (2007) 13192–13197.
[13] A. Galarneau, M. Primeau, L.E. Trudeau, S.W. Michnick, β-Lactamase protein fragment complementation assays as in vivo and in vitro sensors of protein protein interactions, Nat. Biotechnol. 20 (2002) 619–622.
[14] L.N. Csonka, A.D. Hanson, Prokaryotic osmoregulation: genetics and physiology, Annu. Rev. Microbiol. 45 (1991) 569–606.
[15] H. Nikaido, Molecular basis of bacterial outer membrane permeability revisited, Microbiol. Mol. Biol. Rev. 67 (2003) 593–656.
[16] F. Chiti, N. Taddei, P. Webster, D. Hamada, T. Fiaschi, G. Ramponi, C.M. Dobson, Acceleration of the folding of acylphosphatase by stabilization of local secondary structure, Nat. Struct. Biol. 6 (1999) 380–387.
[17] R. Tan, X. Jiang, A. Jackson, P. Jin, J. Yang, E. Lee, B. Duggan, L.L. Stuve, G.K. Fu, *E. coli* selection of human genes encoding secreted and membrane proteins based on cDNA fusions to a leaderless β-lactamase reporter, Genome Res. 13 (2003) 1938–1943.
[18] A.P. Capaldi, C. Kleanthous, S.E. Radford, Im7 folding mechanism: misfolding on a path to the native state, Nat. Struct. Biol. 9 (2002) 209–216.
[19] S. Quan, P. Koldewey, T. Tapley, N. Kirsch, K.M. Ruane, J. Pfizenmaier, R. Shi, S. Hofmann, L. Foit, G. Ren, U. Jakob, Z. Xu, M. Cygler, J.C. Bardwell, Genetic selection designed to stabilize proteins uncovers a chaperone called Spy, Nat. Struct. Mol. Biol. 18 (2011) 262–269.
[20] V. Vagenende, M.G. Yap, B.L. Trout, Mechanisms of protein stabilization and prevention of protein aggregation by glycerol, Biochemistry 48 (2009) 11084–11096.
[21] P. Leandro, M.C. Lechner, I. Tavares de Almeida, D. Konecki, Glycerol increases the yield and activity of human phenylalanine hydroxylase mutant enzymes produced in a prokaryotic expression system, Mol. Genet. Metab. 73 (2001) 173–178.
[22] T.O. Street, D.W. Bolen, G.D. Rose, A molecular mechanism for osmolyte-induced protein stability, Proc. Natl. Acad. Sci. USA 103 (2006) 13997–14002.
[23] Y. Zhang, P.S. Cremer, Chemistry of Hofmeister anions and osmolytes, Annu. Rev. Phys. Chem. 61 (2010) 63–83.
[24] L.M. Holthauzen, J. Rosgen, D.W. Bolen, Hydrogen bonding progressively strengthens upon transfer of the protein urea-denatured state to water and protecting osmolytes, Biochemistry 49 (2010) 1310–1318.
[25] A. Moglich, K. Joder, T. Kiefhaber, End-to-end distance distributions and intrachain diffusion constants in unfolded polypeptide chains indicate intramolecular hydrogen bond formation, Proc. Natl. Acad. Sci. USA 103 (2006) 12394–12399.
[26] C. Nick Pace, B.M. Huyghues-Despointes, H. Fu, K. Takano, J.M. Scholtz, G.R. Grimsley, Urea denatured state ensembles contain extensive secondary structure that is increased in hydrophobic proteins, Protein Sci. 19 (2010) 929–943.
[27] R. Kumar, N.P. Prabhu, M. Yadaiah, A.K. Bhuyan, Protein stiffening and entropic stabilization in the subdenaturing limit of guanidine hydrochloride, Biophys. J. 87 (2004) 2656–2662.
[28] T. Majtan, L. Liu, J.F. Carpenter, J.P. Kraus, Rescue of cystathionine β-synthase (CBS) mutants with chemical chaperones: purification and characterization of eight CBS mutant enzymes, J. Biol. Chem. 285 (2010) 15866–15873.
[29] Z. Ignatova, B. Krishnan, J.P. Bombardier, A.M. Marcelino, J. Hong, L.M. Gierasch, From the test tube to the cell: exploring the folding and aggregation of a β-clam protein, Biopolymers 88 (2007) 157–163.
[30] S. Paul, S. Punam, T.K. Chaudhuri, Chaperone-assisted refolding of *Escherichia coli* maltodextrin glucosidase, FEBS J. 274 (2007) 6000–6010.
[31] J.W. Shriver, S.P. Edmondson, Ligand-binding interactions and stability, Methods Mol. Biol. 490 (2009) 135–164.
[32] F.E. Cohen, J.W. Kelly, Therapeutic approaches to protein-misfolding diseases, Nature 426 (2003) 905–909.