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In vivo detection and quantification of chemicals that enhance protein stability

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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

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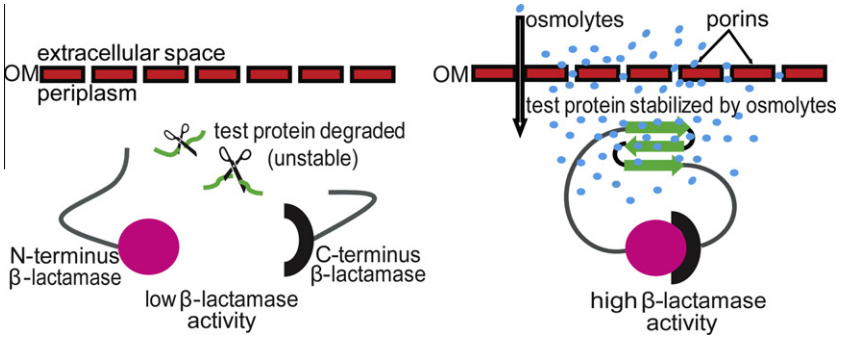

Fig.1. Schematic representation of a sandwich fusion system for assessing the influence of osmolytes on protein stability *in vivo*. An unstable test protein (green) is inserted into β–lactamase in the form of a sandwich fusion. In the absence of osmolytes, the inserted protein is subject to degradation 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.