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Protein folding on biosensor tips: folding of maltodextrin glucosidase monitored by its interactions with GroEL

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Protein folding has been extensively studied for the past six decades by employing solution-based methods such as solubility, enzymatic activity, secondary structure analysis, and analytical methods like FRET, NMR, and HD exchange. However, for rapid analysis of the folding process, solution-based approaches are often plagued with aggregation side reactions resulting in poor yields. In this work, we demonstrate that a bio-layer interferometry (BLI) chaperonin detection system can identify superior refolding conditions for denatured proteins. The degree of immobilized protein folding as a function of time can be detected by monitoring the binding of the high-affinity nucleotide-free form of the chaperonin GroEL. GroEL preferentially interacts with proteins that have hydrophobic surfaces exposed in their unfolded or partially folded form, so a decrease in GroEL binding can be correlated with burial of hydrophobic surfaces as folding progresses. The magnitude of GroEL binding to the protein immobilized on bio-layer interferometry biosensor inversely reflects the extent of protein folding and hydrophobic residue burial. We demonstrate conditions where accelerated folding can be observed for the aggregation-prone protein maltodextrin glucosidase (MalZ). Superior immobilized folding conditions identified on the bio-layer interferometry biosensor surface were reproduced on Ni-NTA sepharose bead surfaces and resulted in significant improvement in folding yields of released MalZ (measured by enzymatic activity) compared to bulk refolding conditions in solution.

Introduction

Numerous experimental techniques are available to study protein folding and the interactions between chaperonins and substrate proteins. *In vitro* protein folding studies have been done by monitoring the changes in secondary structure during the unfolding and refolding of substrates utilizing circular dichroism [1,2] and FTIR [3]. Where applicable, fluorescence spectroscopy can also monitor folding collapse near the microenvironment of the fluorophore and report global folding kinetics (intrinsic or extrinsic) provided more global orthogonal methods mirror the folding kinetics [4]. Many techniques like FRET [5], NMR [6], Cryo-EM [7], and HD exchange-based mass spectroscopy [8] have been previously employed for detailed analysis of protein folding process. Single molecule studies are now employed widely for studying protein folding [9]. *In vivo* experiments analyzing soluble protein content in cells and activity from whole

Abbreviations
BLI, bio-layer interferometry; GdnHCL, guanidine hydrochloride; MalZ, maltodextrin glucosidase; OD, optical density; Δnm, shift in wavelength interference.

cell lysates have also been used [10]. Some of these techniques usually require relatively high protein concentrations or need extensive preparation, expertise, and time to run the experiments. Thus, there is a need for designing a rapid, inexpensive, easy to use analytical tool to rationally identify superior conditions to aid in the protein folding process. In the present study, a bio-layer interferometry–GroEL chaperonin protein folding detection system has been utilized to monitor protein folding on the bio-layer interferometry biosensor surface. The use of the chaperonin as a detector for partially folded protein intermediates comes from the well-known observation that the nucleotide-free, high-affinity GroEL chaperonin tightly binds to protein folding intermediates to form complexes that then can be detected using simple gel filtration or immunoprecipitation assays [11]. In essence, the nucleotide-free, high-affinity GroEL can be thought of as a folding quencher provided that the folding reaction is sufficiently slow. The bio-layer interferometry technique is a label-free method that can monitor protein–protein interactions with similar outputs (i.e., kinetic readouts and signal amplitudes) to surface plasmon resonance (Fig. 1). A histidine-tagged version of maltodextrin glucosidase (MalZ), an aggregation-prone protein was selected as a model system for this study as this is a large protein and should easily interact with the large GroEL-binding site without steric interference from the Ni-NTA biosensor surface. In solution, unfolded MalZ does not refold very well due to deleterious self-aggregation. In the presence of the chaperonin GroEL alone, self-aggregation is prevented and refolding can be initiated by adding ATP and GroES [12]. As the chaperonin does not bind to properly folded proteins, the high-affinity, nucleotide-free chaperonin should only bind to partially folded conformers of MalZ [13] that are present on the bio-layer interferometry biosensor surface. The results presented herein show that as the immobilized protein refolds, the GroEL chaperonin binding signal progressively diminishes as a function of time.

Results

Construction of a MalZ biosensor and analysis of GroEL interactions

The construction of a bio-layer interferometry MalZ biosensor was accomplished by binding native folded functionally active His-tagged MalZ onto Ni-NTA biosensor tips. Binding of MalZ to the tip surface was observed by an increase in the $\Delta\lambda$ (recorded as Δnm) signal, and retention of this signal was observed upon washing with buffer containing ATP. Immobilized MalZ was challenged with ATP nucleotide because it is a potential chelator that has to be used in subsequent experiments to dissociate GroEL from immobilized MalZ. As shown in Fig. 2A, ATP addition did not affect the MalZ loading. The shaker speed of the equipment was kept at 2200 r.p.m. to avoid mass transfer errors during the MalZ loading and GroEL binding. The Ni-NTA biosensors are used because denaturation of immobilized protein with GdnHCl on the biosensor tip is possible without any significant dissociation of the His-tagged protein within the time frame of the experiments (Fig. 2C – step 11). MalZ bound to the biosensor with a magnitude of binding reaching up to 3 Δnm in a 300-s loading step with 0.5-μM concentration of MalZ. (Fig. 2A). The initial runs were used as proof of concept experiments and the loading concentration and magnitude were further optimized in subsequent experiments. GroEL did not tightly bind to the biosensor tip in the absence of MalZ. However, a low nonspecific upward deflection of 0.2 Δnm magnitude was recorded with a high concentration (1 μM) of GroEL in 300 s which rapidly reduced to 0.1 nm on washing with buffer. This small signal rise and rapid return is a hallmark of nonspecific binding interactions with the biosensor (Fig. 2B). Thus, any background nonspecific GroEL signal was subtracted from the main signal prior to analyzing the results. GroEL binding to native immobilized MalZ was analyzed and no increase in signal was observed upon addition of GroEL to native protein, indicating that GroEL does not bind to native MalZ. This suggests that no exposed hydrophobic surface is present on the native protein. However, an increase in GroEL binding signal was observed when GroEL was added after the immobilized protein was denatured. Denaturation was accomplished by dipping the MalZ biosensor into 4 M guanidine hydrochloride (GdnHCl) for 5 min. The loss of activity and loss of intrinsic fluorescence of MalZ within 5 min of 4 M GdnHCl treatment was confirmed through previous in solution experiments. The experimental results suggested that GroEL does not bind to the native MalZ, but associates with non-native conformations of MalZ (Fig. 2C). The time-dependent monitoring of the folding of denatured MalZ on biosensor tips is based on the observation that GroEL does not bind to native MalZ but readily binds to the non-native or unfolded forms. The GroEL binding and ATP-induced release steps were monitored in real time with the output recorded on the sensogram.

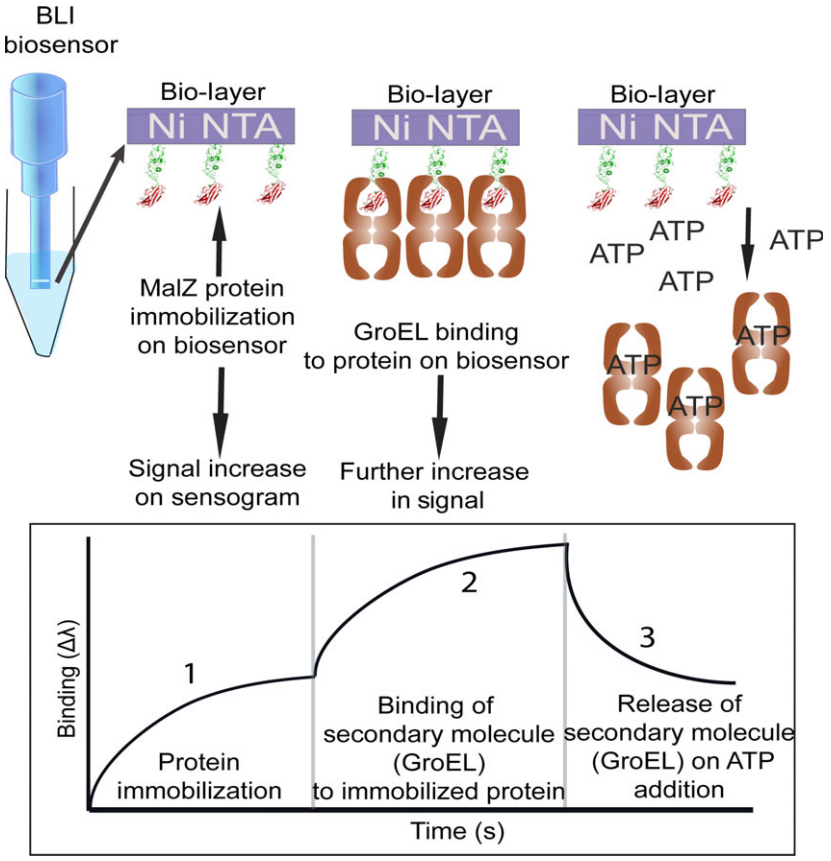

Fig. 1. The bio-layer interferometry method to analyze protein–GroEL interaction. The bio-layer interferometry method is utilized for analysis of protein folding based on protein–GroEL interactions. The biosensor is made of glass fiber having a biocompatible layer at the tip. White light is reflected through two interfaces, one between the glass fiber and biocompatible layer and the second at the bound protein–solution interface. The relative interference between the different wavelengths of light changes when the solution interface changes due to bound protein. These changes are monitored and processed to give a binding signal in terms of change in wavelength (Δλ). A plot of Δλ vs the time of interaction with proteins under investigation provides a sensogram which indicates the nature of binding. The present sensogram displays an increase in signal when the protein is immobilized (step 1). Further increase in the signal was observed due to GroEL binding (step 2). When GroEL dissociates upon ATP addition, the signal decreases to the initial MalZ binding signal (step 3).

MalZ folding on biosensor tip monitored through GroEL binding

In order to clearly understand the concept of monitoring protein folding in terms of its interaction with GroEL, a demonstrative proof of concept experiment was performed. A sequential BLI sensorgram was recorded to track the GroEL binding to native MalZ, denatured MalZ, refolded MalZ, and repeated denatured MalZ for demonstrating the utility and reproducibility of this method. Higher protein concentrations were used to enhance the signal to noise signals for the sensogram. Native MalZ was first bound to the biosensor to a magnitude of 5.5 Δnm in step 1 (Fig. 2C). No binding of GroEL to native MalZ was observed in step 2; there was no binding on addition of GroEL to native protein. In the next step (step 3), MalZ was denatured by dipping the MalZ biosensor into 4 M GdnHCl. Initial GroEL binding to the denatured MalZ (step 4) was reflected by an increase in the BLI signal of ~ 1.3 Δnm. After GroEL binding, the biosensor was then dipped into a 10 mM ATP solution. ATP binding to GroEL diminishes its binding affinity to denatured MalZ and results in the release of GroEL from the biosensor surface. Two separate ATP pulses (step 5 and 6) indicates a total release of GroEL binding since the signal returns to the original baseline prior to GroEL addition. The GroEL binding step was repeated (step 7) and it was observed that the GroEL-binding magnitude decreased from 1.3 to about 0.8 Δnm suggesting that a fraction of MalZ was

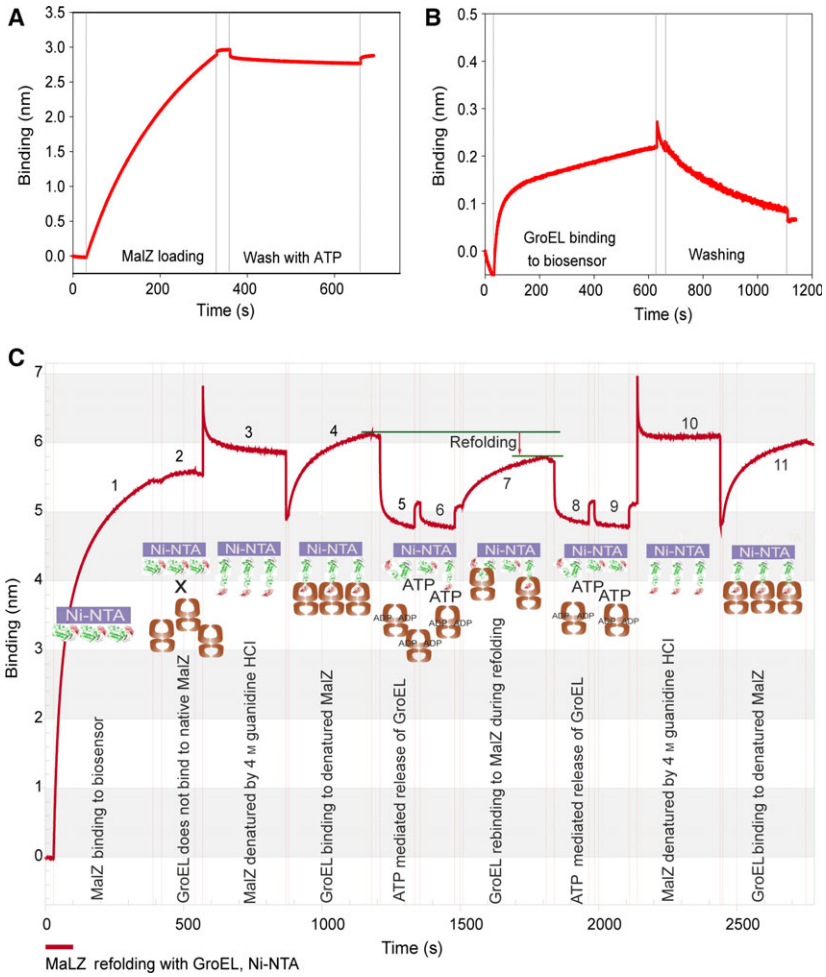

Fig. 2. Monitoring of protein folding process on the biosensor. (A) Binding of 0.5 μM MalZ on the bio-layer interferometry biosensor for 300 s and ATP (10 mM) wash for 300 s showing stable MalZ immobilization. (B) Biosensor immersed in 1 μM GroEL for 600 s as control. (C) The refolding experiment was done at 25 °C. MalZ and GroEL were used at 1 μM concentrations in 50 mM HEPES buffer with 20 mM magnesium acetate and 10 mM potassium acetate. The steps were (1) MalZ loading on biosensor, (2) GroEL binding to native MalZ, (3) GdnHCl-mediated denaturation, (4) GroEL binding to denatured MalZ, (5–6) ATP-mediated release of MalZ by GroEL, done twice with a baseline in between, (7) GroEL binding to partially refolded MalZ, (8–9) ATP-mediated release of MalZ by GroEL, done twice with a baseline in between, (10) repeated GdnHCl-mediated denaturation, (11) repeated GroEL binding to denatured MalZ.

folded during the 500 s between consecutive GroEL binding steps (steps 4 and 7). The percentage or fraction of folding was determined as the total decrease in terms of extent of GroEL binding. The amount of MalZ folding on the biosensor over this 500 s time span (step 5 and 6) results in decreased GroEL binding [only 62% of original binding (step 4)], corresponding to 38% MalZ folding. The GroEL was then removed once again by dipping the biosensor in 10 mM ATP solution (Step 8, 9). GroEL dissociated completely and the baseline returns back to the same magnitude as it was before GroEL association. When the refolded MalZ was denatured again (step 10) with the same concentration of GdnHCl used for the first denaturation. At this stage (step 11), the extent of GroEL binding was compared to the GroEL binding in the initial binding step (step 4). The binding magnitude in this step was about 1.25 Δnm, which was similar to the initial GroEL binding just after denaturation of MalZ (Fig. 2C). This suggests the decrease in GroEL binding to MalZ (in step 7) was due to refolding of a fraction of immobilized MalZ rather than a loss of MalZ from the biosensor surface. Thus, this experiment shows refolding of MalZ depicted by decrease in signal of GroEL binding in step 7. It also shows that GroEL binding and ATP-mediated release of GroEL from substrate proteins can be monitored in real time. This experiment demonstrates that one can

potentially monitor the time dependent folding of a protein on the basis of diminished chaperone interactions.

Optimization of MalZ loading concentrations and comparison with GroEL binding

In order to determine the concentration of MalZ that avoids saturating the biosensor tip and maintains linearity in binding of MalZ to GroEL for more quantitative experiments, the loading of MalZ was optimized. This protein folding screening tool is more quantitative when lower protein concentrations are used. Hence, lower concentrations should show a linear dependence of MalZ binding within a defined optimized range. It was found that MalZ loading signal on the biosensor follows a linear relationship from 12.5 to 0.25 μM concentration and then it starts to saturate at higher concentrations (Fig. 3A) after a 60-s loading step. The optimum concentration of MalZ protein was chosen at 50 nM as this avoids overloading of the biosensor surface. Optimal loading also prevents intrasurface aggregation from interfering with further analysis. The ratio of GroEL binding is linear to the MalZ load at lower concentrations (Fig. 3B). This experiment supports the concept that the GroEL binding to MalZ on the biosensor is proportional to the amount of unfolded or denatured protein loaded on the biosensor. This optimization was also important as normalization could be done in terms of MalZ loading if any minor differences in MalZ loading are observed in comparative experiments. Furthermore, GroEL binding to 50 nM MalZ load at different GroEL concentrations were plotted (Fig. 3C). Based on these results, GroEL concentration of 0.2 μM was used for further experiments with a MalZ loading concentration of 50 nM to minimize steric interference. The use of such low concentrations of protein and GroEL are definitely advantages for monitoring protein folding using this screening system.

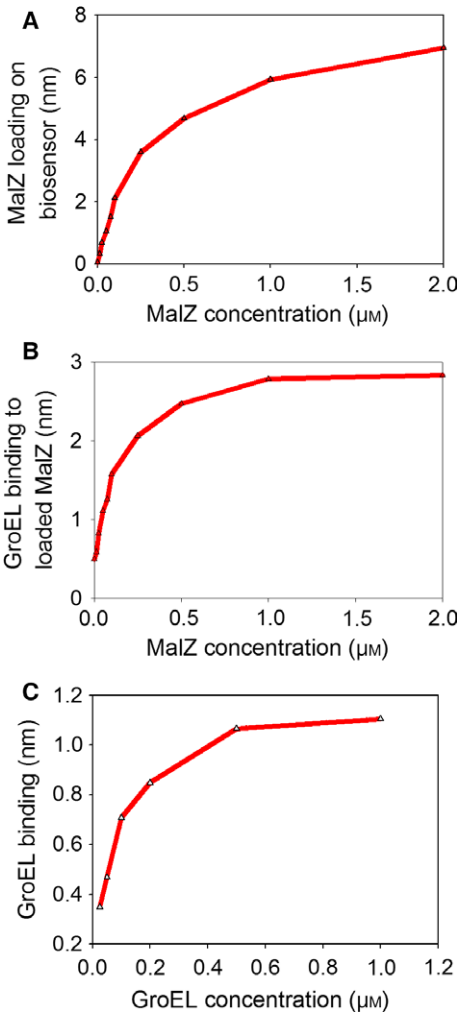

Fig. 3. Optimization of MalZ and GroEL concentrations for folding studies. (A) MalZ loading on the biosensor plotted against the concentration used for loading. The 50 mM HEPES buffer along with 20 mM magnesium acetate and 10 mM potassium acetate were used. The concentration of MalZ up to 250 nM was found to be in the linear range; (B) GroEL at 1 μM was added to MalZ at different loads, this strengthened the fact that GroEL binding is proportional to the amount of MalZ available in denatured state on the biosensor; (C) GroEL binding to MalZ at different GroEL concentrations, the concentration of MalZ was fixed at 50 nM (middle of linear loading/signal range). Optimized GroEL concentration of 0.2 μM was used for further experiments based on this result.

Identification of superior refolding condition using temperature in the presence of a physiologically relevant cation anion pair (potassium glutamate)

Folding of MalZ was measured using GroEL binding as a parameter. It was evident from the previous experiment, where a GroEL binding and release cycle is shown (Fig. 2C), that GroEL binding decreases as the immobilized protein is allowed to fold. The extent of MalZ refolding at different time points of 1, 2, 5, 10, 30, and 60 min was assessed using the GroEL quench protocol at three different temperatures of 12, 24, and 37 °C. The diminishing magnitude of GroEL binding (i.e., GroEL quench) to refolding MalZ was plotted as a function of time (Fig. 4A). The proposed folding profile at 12 °C showed slow MalZ refolding with a final recovery of 36% native signal after 1 h. The refolding profile did not reach a plateau value within this time frame suggesting that the refolding is

incomplete after 1 h. As the temperature is increased to 24 °C, the folding after 1 h was recorded to be 77% with little hint that the reaction is approaching a plateau value. At 37 °C, the folding magnitude detected by the GroEL quench protocol indicates that refolding saturates at around 86% as the values are similar at 30 and 1 h refolding. Based on reversibility experiments (ATP addition to release any bound GroEL and denaturation) on the biosensor tip (Fig. 2C), > 90% of the originally loaded MalZ remains attached to the biosensor surface at 37 °C throughout the immobilized refolding reaction even after 1 h.

To confirm on-surface folding conditions can be scaled-up, folding of MalZ under different temperatures was also analyzed using an equivalent Ni-NTA resin capture system where the readout for folding is assessed by measuring the activity of released MalZ at equivalent time points used in the chaperonin BLI refolding quench system. The efficiency of the immobilized Ni-NTA surface refolding was compared with the 'in-solution' refolding. In both cases, the protein was allowed to refold for 15 min at temperatures of 12, 24, and 37 °C. As previously observed [12], spontaneous refolding of MalZ in the absence of the GroEL chaperonin in solution is inefficient because under these concentrations the protein aggregates during refolding. In contrast, the immobilized refolding scheme resulted in a significant improvement in regain of activity at all temperature conditions (Fig. 4B). The refolding yields from resin-based immobilization and release were highest at 37 °C, in line with that observed for the BLI-GroEL quench protocol. Although the results cannot be exactly compared in a quantitative sense, the results were highly correlated. The conditions used here show that the rate of folding of MalZ is fastest at the physiological temperature of 37 °C in the presence of a physiologically relevant cation anion pair, potassium glutamate [14–16].

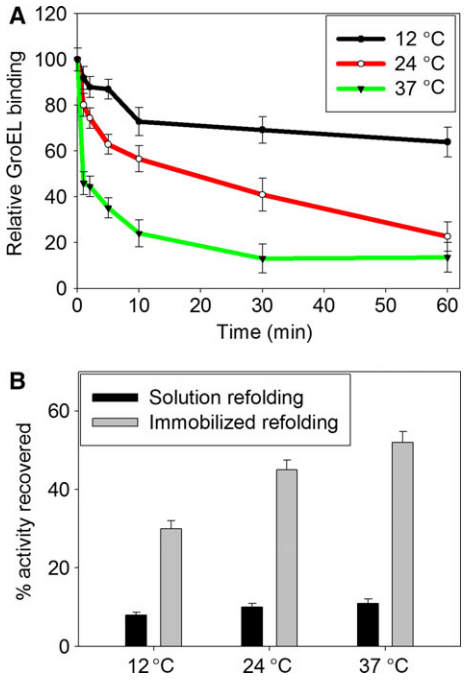

Fig. 4. Optimizing MalZ refolding conditions under immobilized conditions at different temperatures. (A) Relative GroEL binding after refolding at different temperatures for the plotted time durations. Separate MalZ biosensors were treated with 4 M GdnHCl and incubated at given temperatures for mentioned time points before analysis of GroEL binding. The assay was done in 20 mM sodium phosphate with 20 mM magnesium glutamate and 10 mM potassium glutamate; (B) comparison of relative enzymatic activity in solution and under immobilized conditions after 15 min refolding time. Normalization between solution- and resin-bound MalZ was done by comparing enzymatic activity and then using relative amounts. MalZ on beads was released in solution by 500 mM imidazole in the activity assay buffer. The plotted percentage is relative to the activity of native MalZ. An exact quantitative comparison was not possible due to the differences in experimental setups. However, the overall pattern of immobilized folding on Ni-NTA resin matches that of the screened conditions on bio-layer interferometry in panel a. The experiments were done in triplicate and error bars display the SD.

Conformational states of non-native MalZ required for GroEL binding on biosensor tip monitored through intrinsic fluorescence spectroscopy

It was evident from these experiments that GroEL binding to substrate protein is dependent on the amount of protein that is in a non-native hydrophobic state that is present at a particular time. To verify the relationship between the GroEL-binding magnitude to MalZ and the extent of conformational changes during unfolding of the MalZ protein, a comparison was made between the in-solution tryptophan fluorescence that monitors MalZ unfolding and the MalZ biosensor BLI-GroEL quench profile. To measure the MalZ denaturation progression on the MalZ BLI biosensor, a number of identically loaded MalZ biosensor were incubated in increasing concentrations of GdnHCl at 25 °C. The biosensor was then removed from the denaturant solution, washed for 5 s to remove any residual denaturant and then dipped into refolding buffer containing nucleotide-free GroEL to assess the amount of unfolded/partially folded protein that is present on the BLI biosensor surface. The increase in

magnitude of GroEL binding was compared to the change in fluorescence intensities of MalZ at a fixed emission wavelength (334 nm) as well as average emission wavelengths (Fig. 5A) at increasing concentrations of GdnHCl. The BLI sensogram signal (due to GroEL binding to denatured MalZ) saturates above 2 M GdnHCl concentration. Likewise, changes in fluorescence intensity also show a similar plateau signal above 2 M GdnHCl. The signals from the fluorescence changes and the GroEL binding magnitudes at intermediate denaturant concentrations were remarkably matched. The denaturation experiments were repeated in the presence of known protein stabilizers such as glycerol (Fig. 5B) and sucrose (Fig. 5C). The presence of the stabilizer osmolytes in the denaturant solution delays the protein denaturation yielding kinetically controlled denaturation profiles that are similar and are right shifted with respect to denaturant concentration for the GroEL-binding BLI biosensor signal and the decrease of fluorescence. This particular comparative scheme is useful in assessing potential stabilizing conditions for folding and refolded protein systems.

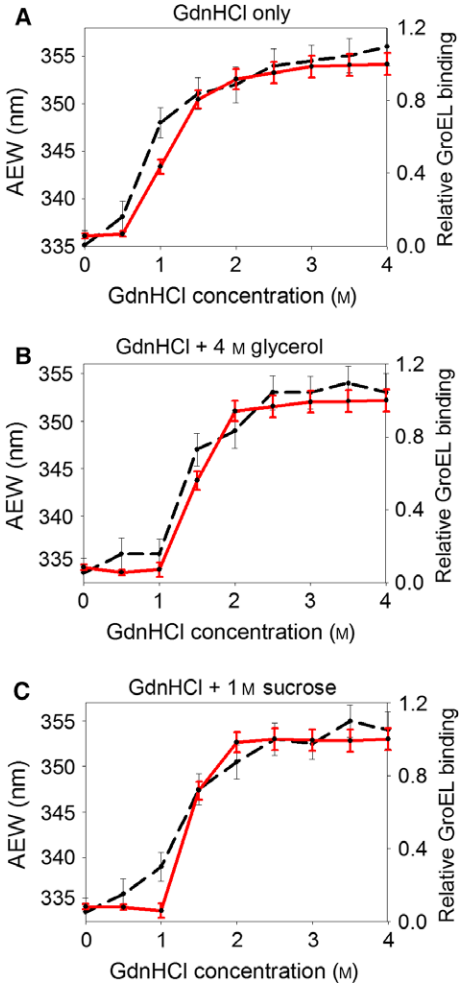

Fig. 5. GroEL binding related to MalZ unfolding. The unfolding of MalZ induced by GdnHCl is measured by tryptophan fluorescence and the unfolding is plotted against the GdnHCl concentration (black dotted lines). Both solution- and bio-layer interferometry-based unfolding was done at 25 °C for 5 min. The binding of GroEL to MalZ (red lines) under conditions (intervening 5-s wash to remove denaturant) clearly correlates with the changes in the tryptophan fluorescence measured as changes in average emission wavelength (AEW). (A) 0–4 M GdnHCl alone; (B) 0–4 M GdnHCl + 4 M glycerol; (C) 0–4 M GdnHCl + 1 M sucrose. The experiments were done in triplicates and the error bars represent SD. It can be seen through tryptophan fluorescence that osmolytes delay the denaturation of MalZ and same correlation can be seen with GroEL binding (compare signals at 1 M GdnHCl). Hence, GroEL binding to MalZ can be a good measure of the unfolded MalZ protein.

Discussion

In this work, we demonstrate that the BLI-GroEL detection system can be used to detect the time it takes for an immobilized protein on a BLI biosensor to acquire a collapsed conformation that does not or minimally interacts with the nucleotide-free, high-affinity GroEL chaperonin. The biosensor surface Ni-NTA chemistries can be used to immobilize His-tagged protein where unfolding and refolding reactions can occur without aggregation interference. The refolding on the microscale BLI surfaces correlates with the scaled-up Ni-NTA bead resin release assay and provides an orthogonal method to readily screen superior folding conditions. Chaperonin binding to a folding protein indicates that the folding reaction proceeds through folding intermediates that expose hydrophobic surfaces. Conditions that cause rapid burial of hydrophobic surfaces through simple folding will show decreased interactions with the chaperonin. This GroEL-BLI folding detection system can potentially identify solution conditions and/or ligands that can accelerate protein folding and diminish aggregation. The readout for extent of folding of a protein correlates with the reduction in signal intensity from GroEL binding to the folded protein as compared to the binding signal from fully denatured protein. Ready dissociation of GroEL from immobilized MalZ with ATP, but not with buffer alone, indicates that the binding of GroEL to partially folded MalZ is specific. As the promiscuous GroEL chaperonin generally binds to virtually any hydrophobic surface, this approach can be utilized for an extremely wide variety of proteins. The results from various experiments reported in this manuscript demonstrate how GroEL binding with denatured MalZ decreases

gradually, correlating with the refolding of the immobilized protein molecules and indicating that once refolded, the protein does not rapidly revert to its original unfolded form. The rate of folding of MalZ protein under immobilized condition on BLI tips clearly increases as the temperature increases from 12 to 37 °C. Understandably, the close correlation of tryptophan fluorescence changes for MalZ exposed for 5 min with increasing denaturing solutions compared with the increasing GroEL binding signal to MalZ on the BLI biosensor under the same denaturing conditions indicates that assessing the folding status of a protein on the biosensor surface is a valid approach. It has been demonstrated that the binding of MalZ to GroEL is maximum at a denaturant concentration where the change in intrinsic fluorescence signal is also maximum. The addition of polyol folding stabilizers such as glycerol and sucrose to the denaturant solution understandably delays the unfolding reaction and concomitantly results in comparable time-dependent delays in solution unfolding (Fig. 5).

The assessment of protein folding by employing a chaperone-based method on BLI can provide significant advantage in terms of time and protein concentrations required when optimization for better folding conditions is required prior to the experiments with more precise methods. This method is a fast and easy way to optimize/access the temperature and refolding buffer conditions for refolding while avoiding large-scale protein aggregation. One could conceivably use other chaperone proteins besides GroEL. However as discussed in Naik *et al*. [17,18], the nucleotide-free GroEL has the tightest binding affinity for partially folded or hydrophobic protein, is stable, binding is reversible, the binding site is large, and the binding is promiscuous. Any conformational alteration and destabilization in the structures of certain proteins in solution can also be identified by this same method [18]. This approach also works with a reverse-oriented (GroEL on the biosensor) BLI method [17]. Binding and detection of partially folded proteins can be done rapidly and with great ease. For example, with the single channel BLI unit (BLItz), minimum sample volumes of 4 μL at a concentration of 50 nM are sufficient for loading stock of MalZ. From a biotechnology standpoint, this technique could be used as an easy preliminary method to screen the initial state of folding for protein substrates and aid in screening refolding conditions at very low concentrations before proceeding to scale-up protocols that require larger protein concentrations.

In summary, the bio-layer interferometry coupled with a GroEL detection system provides an easy, convenient, and fast method for rapidly monitoring refolding of immobilized proteins. Refolding of aggregation-prone maltodextrin glucosidase has been demonstrated using this BLI-GroEL method, where a decrease in specific binding (i.e., reversible with ATP addition) correlates with the acquisition of a native active fold. This technique can prove to be a rapid, efficient, and convenient method to determine superior folding conditions. The results were validated by conventional experimental method like enzymatic activity assay and fluorescence spectroscopy, where the superior folding conditions can be determined using a microscale BLI chaperonin detection system. Once superior conditions are found, folding of the immobilized protein can be replicated on scaled-up bead-based platforms.

Materials and methods

Maltodextrin glucosidase preparation

Plasmid PCS19 containing the *malZ* gene was a generous gift from W. Boos, (University of Konstanz, Germany). The plasmid contains the *malZ* gene under control of a strong IPTG inducible T5 promoter and it contains ampicillin resistance for selection and a C-terminal 6× His tag on the protein for rapid purification. *Escherichia coli* BL-21 cells were transformed with the plasmid and grown at 37 °C in Luria–Bertani medium (HiMedia, Mumbai, India) containing 80 $\mu g \cdot mL^{-1}$ ampicillin (HiMedia). Induction for protein overexpression was done with 1 mM IPTG (SRL, Mumbai, India) when OD at 600 nm reached 0.6. The culture was kept for 8 h postinduction and protein expression was confirmed on a SDS/PAGE gel. MalZ purification was carried out by nickel affinity chromatography on a 5 mL His-Trap column using an Akta FPLC system (GE Healthcare, Little Chalfont, UK). About 20 mM sodium phosphate buffer (Merck, Darmstadt, Germany) containing 500 mM NaCl (Merck) was used for lysis and column equilibration. MalZ was eluted near 200 mM imidazole (Sigma Aldrich, St Louis, MO, USA) through a linear gradient of imidazole from 20 to 500 mM.

Chaperone preparation

GroEL containing plasmid pACYC-EL was gifted by H. Taguchi (Tokyo Institute of Technology, Yokohama, Japan). This plasmid contains the groEL gene regulated by *tac* promoter and it uses chloramphenicol resistance marker for selection. *Escherichia coli* BL-21 cells were transformed with this plasmid and were grown on Luria–Bertani medium containing 20 $\mu g \cdot mL^{-1}$ chloramphenicol for selection. Induction was done with IPTG to a final concentration of 1 mM when the OD at 600 nm of the culture was around 0.6. Purification of GroEL was done following the protocol

outlined in Wang *et al*. [19]. Q-Sepharose anion exchange column (GE Healthcare) was used for anion exchange chromatography. A Source 15ISO column (GE healthcare) was used for hydrophobic interaction chromatography to remove hydrophobic contaminants and the purified GroEL was further treated with affigel blue (BioRad, Hercules, CA, USA) for 18 h to obtain GroEL free of tryptophan containing contaminants [20]. The purity of GroEL was checked by fluorescence spectra and tryptophan fluorescence was negligible after affigel blue treatment as compared to considerable fluorescence before affigel blue treatment [21,22]. The second derivative wavelength scan in UV region did not show any indole absorbance in the 291–295 nm, thus confirming high purity GroEL preparation [23]. The purified GroEL was concentrated to 25–30 μM concentration and was stored with 10% glycerol at 4 °C.

Description of bio-layer interferometry

The principles of this technique are based on a light interference based signal. When molecules bind to the surface of the biosensor, the change in surface thickness is detected. This is based on developing an interference pattern from constructive and destructive waves of a reference layer compared with the reflection at the bio-layer. Molecules binding to the bio-layer portion results in both changes in thickness as well as local refractive index changes located at the end of the bio-layer interferometry biosensor tip. Changes in the constructive/destructive λ interference pattern of these two reflected beams results in a wavelength shift interference pattern. This shift in wavelength (Δλ, recorded as a Δnm) is proportional to the number and mass of molecules binding on the biosensor tip. This label-free technique is comparable to surface plasmon resonance and in all instances tested, yields similar quantitative and qualitative results. In the bio-layer interferometry-based method, an increase in Δnm signal is observed upon loading the subject proteins on to the biosensor. A further increase in signal is observed when interacting molecules bind to the immobilized subject protein (Fig. 1). Identification of structurally altered and aggregated states of proteins using GroEL as a probe on bio-layer interferometry has been previously described [17] and validated with SPR [18]. Chaperone binding to immobilized protein and ATP-mediated chaperone release can be monitored on bio-layer interferometry similar to previously done work on surface plasmon resonance [24,25].

Protein loading on the biosensor

Stock maltodextrin glucosidase was concentrated with an Amicon centrifugal concentrator to a final concentration of 80–100 μM in 20 mM sodium phosphate buffer containing 50 mM imidazole. The buffer used for this bio-layer interferometry control experiment consisted of 50 mM HEPES, 20 mM magnesium acetate, 10 mM potassium acetate at pH 7.4. MalZ was diluted in this buffer to a final concentration of 0.5 μM thus reducing the final imidazole concentration to 0.25 mM which does not affect loading of MalZ on the biosensor. Tube mode of the Blitz instrument (ForteBIO, Pall Life Sciences, Menlo Park, CA, USA) was used for all readings. The shaker speed of the instrument was kept at 2200 r.p.m. After 30-s initial baseline with the refolding buffer, MalZ was loaded on the tip for 300 s. A 30-s baseline was followed by ATP treatment for 300 s. As a control experiment, binding of 1 μM GroEL to the Ni-NTA biosensor was tested. The residual GroEL adherence to the free tip was measured during each experiment, and subtracted as a background from the actual readings during analysis. GroEL binding to MalZ in native state was analyzed and subsequently immobilized MalZ was denatured in 4 M GuHCl for 5 min, denaturant was washed off the tip and binding of GroEL to the denatured MalZ was observed.

Protein folding on bio-layer interferometry biosensor

MalZ protein at a concentration of 0.5 μM was loaded to the biosensor and GroEL binding was monitored after the immobilized MalZ has been denatured with 4 M GdnHCl. Buffer and conditions were the same as previous experiments and the complete cycle was done at 25 °C. GroEL was used at 1 μM concentration and magnitude of total GroEL binding was recorded. After a baseline step, the biosensor tip was immersed in 10 mM ATP solution so that GroEL releases the substrate protein due to conformational transition mediated by ATP hydrolysis. A baseline step was added after each cycle to remove the excess MalZ, GdnHCl, GroEL, or ATP.

The biosensogram construction begins with (1) loading His-tagged MalZ onto a Ni-NTA biosensor, (2) followed by baseline loading of GroEL to check for binding to native MalZ, (3) GdnHCl-mediated denaturation, (4) GroEL binding to denatured MalZ, (5–6) ATP-mediated release of MalZ by GroEL, done twice with a baseline in between, (7) GroEL binding to partially refolded MalZ, (8–9) ATP-mediated release of MalZ by GroEL, done twice with a baseline in between, (10) GdnHCl-mediated denaturation, (11) GroEL binding to denatured MalZ.

Optimization of MalZ concentrations for loading and GroEL binding

To avoid overloading, steric effects and intermolecular biosensor surface aggregation, loading signals were plotted and compared at different MalZ concentrations to identify the quantitative linear load region and to identify lower

protein requirements for screening method development. MalZ was diluted over a concentration range of 12.5 nM to 2 μM and the loading amplitude signal (at 60 s) onto the Ni-NTA biosensors were recorded for each concentration. The biosensor amplitude (Δnm) of MalZ loading was plotted against the respective concentration of MalZ. GroEL at 1 μM was added to these MalZ concentrations to identify linearity of GroEL binding to MalZ loading. The MalZ buffer contained 50 mM HEPES at pH 7.4, 20 mM magnesium acetate, and 10 mM potassium acetate. In the next experiment, MalZ is loaded at different concentration and denatured by 4 M GdnHCl, binding of 1 μM GroEL is done to the loaded MalZ. In a further step, GroEL association at different GroEL concentrations was observed to the MalZ loaded at 50 nM concentration. Any minimal nonspecific GroEL binding to the biosensor, when present, was subtracted in all cases to remove any nonspecific background signals.

Folding of immobilized MalZ at different temperatures monitored through GroEL binding

The experiments were performed in chloride-free buffer containing 20 mM sodium phosphate, 20 mM magnesium glutamate, and 10 mM potassium glutamate. Separate biosensors were used for each time point. MalZ was loaded on the biosensor at an optimized concentration of 50 nM. MalZ was loaded for 60 s on the biosensor tip and then after a brief wash, it was denatured by 4 M GdnHCl for 4 min. After denaturation, the biosensor was briefly washed with refolding buffer to remove denaturant, removed from the bio-layer interferometry instrument, and was incubated for a given time point at mentioned temperatures using a controlled temperature block. The biosensor was then again washed for 5 s to normalize to room temperature, and in all cases, GroEL binding step was carried out at room temperature of 25 °C only. The GroEL-binding magnitudes at these different points were plotted and compared to find out the dependence of the folding pattern of MalZ on these specific temperatures.

Folding of immobilized MalZ at different temperatures monitored through activity assay

An enzymatic activity assay was performed to analyze the refolding of MalZ protein in two sets. In one set, the solution-based refolding of MalZ was investigated and in the second case, the effect of immobilization was analyzed by immobilizing MalZ on Ni-NTA sepharose resin. Sodium phosphate buffer at a concentration of 20 mM was used with 20 mM magnesium glutamate and 10 mM potassium glutamate which is similar to the composition used for the bio-layer interferometry-based experiments. MalZ enzymatic activity was used to normalize protein concentrations in both cases so that equal amount of protein is present for comparison. MalZ was loaded on the Ni-NTA sepharose resin and washed thoroughly to remove any unbound protein. After washing, the resin was resuspended in imidazole-free buffer. At this stage, we took 250-μL resin (containing native protein) and added it to 1-mL substrate solution. MalZ enzymatic activity was measured by the substrate *p*-nitrophenyl α-D maltoside [26] (Gold Biotechnology, Missouri, USA), which on hydrolysis by MalZ releases *p*-nitro phenol that gives a characteristic yellow color and can be quantified by measuring absorbance at 405 nm. The substrate was prepared at 5 mM concentration in the mentioned refolding buffer, additionally containing 500 mM imidazole (MalZ elutes near 200-mM imidazole). This reading was compared with solution-based activity reading to the normalized amount of protein for the solution-based experiments. For the resin-based experiments, the resin was kept on mild shaking condition and equal volumes of resin suspension were taken for each set of experiment. For the refolding studies, the protein denaturation was done by 4 M GdnHCl. In the further steps, the protein in solution and on beads was diluted by refolding buffer and incubated at the temperatures 12, 24, and 37 °C. A 15-min incubation in refolding buffer was chosen so as to clearly identify the differences (15 min show significant differences in the BLI data). Enzymatic activity of each set was measured and analyzed to identify the refolding percentages by comparing the activity readings of native protein. There was no significant effect of imidazole concentration on the enzymatic activity of the protein in the given buffer conditions, as judged through measurement of MalZ activity at various imidazole concentrations.

Comparison of the MalZ unfolding process in solution vs under immobilized condition on biosensor tips

The same buffer was used as in previous experiments and MalZ at a final concentration of 0.5 μM was used for the fluorescence-based experiments and 50 nM for bio-layer interferometry-based experiments. GdnHCl was used at concentrations of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 M. GdnHCl concentrations above 4 M do not have further effect. For bio-layer interferometry experiments, native MalZ was immobilized on separate biosensors and each one was treated with one of the mentioned GdnHCl concentrations for 5 min. After a 10-s buffer wash to remove the denaturant from the biosensor tip containing immobilized MalZ, the tip was transferred into a GroEL solution to monitor binding amplitudes. These were plotted against the respective GdnHCl concentrations. For intrinsic tryptophan fluorescence, MalZ at 0.5 μM concentration was denatured in these GdnHCl concentrations. After a 5-min incubation with denaturant, emission spectra between 300 and 400 nm was recorded using excitation at 290 nm on

an Agilent cary eclipse fluorimeter (Agilent Technologies, Santa Clara, CA, USA). Both the GroEL binding experiment on bio-layer interferometry and the fluorescence measurement were repeated in the presence of 4 M glycerol and 1 M sucrose as stabilizers.

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Author contributions

AP and AKS performed all the experiments. TKC and MTF designed the experiments. AP, AKS, TKC, and MTF analyzed the data and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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