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Chaperonin-Based Biolayer Interferometry To Assess the Kinetic Stability of Metastable, Aggregation-Prone Proteins

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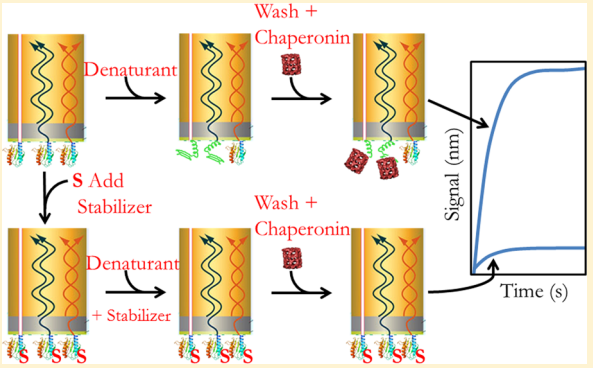
Supporting Information

ABSTRACT: Stabilizing the folded state of metastable and/or aggregation-prone proteins through exogenous ligand binding is an appealing strategy for decreasing disease pathologies caused by protein folding defects or deleterious kinetic transitions. Current methods of examining binding of a ligand to these marginally stable native states are limited because protein aggregation typically interferes with analysis. Here, we describe a rapid method for assessing the kinetic stability of folded proteins and monitoring the effects of ligand stabilization for both intrinsically stable proteins (monomers, oligomers, and multidomain proteins) and metastable proteins (e.g., low T_m) that uses a new GroEL chaperonin-based biolayer interferometry (BLI) denaturant pulse platform. A kinetically controlled denaturation isotherm is generated by exposing a target protein, immobilized on a BLI biosensor, to increasing denaturant concentrations (urea or GuHCl) in a pulsatile manner to induce partial or complete unfolding of the attached protein population. Following the rapid removal of the denaturant, the extent of hydrophobic unfolded/partially folded species that remains is detected by an increased level of GroEL binding. Because this kinetic denaturant pulse is brief, the amplitude of binding of GroEL to the immobilized protein depends on the duration of the exposure to the denaturant, the concentration of the denaturant, wash times, and the underlying protein unfolding−refolding kinetics; fixing all other parameters and plotting the GroEL binding amplitude versus denaturant pulse concentration result in a kinetically controlled denaturation isotherm. When folding osmolytes or stabilizing ligands are added to the immobilized target proteins before and during the denaturant pulse, the diminished population of unfolded/partially folded protein manifests as a decreased level of GroEL binding and/or a marked shift in these kinetically controlled denaturation profiles to higher denaturant concentrations. This particular platform approach can be used to identify small molecules and/or solution conditions that can stabilize or destabilize thermally stable proteins, multidomain proteins, oligomeric proteins, and, most importantly, aggregation-prone metastable proteins.



Rapidly assessing protein stability and identifying potential ligands and stabilizers that bind to metastable proteins are challenging areas in protein science. To identify potential small molecule stabilizers or stabilizing solutions, one must document the effects these compounds or solutions have on protein structure, dynamics, and more commonly thermal or chemical stability. There are numerous general approaches available for assessing protein stability. Solution phase protein stability measurements usually rely on monitoring thermally or chemically induced midpoint transition shifts of the protein in the presence or absence of stabilizing (or destabilizing) ligands or solutions. Methods such as differential scanning calorimetry,[1] changes in light scattering,[2] global spectroscopic signatures (CD spectroscopy), or other direct physical measurements are commonly used to identify stabilizers of protein structure. Current high-throughput strategies that typically identify protein stabilization conditions rely on

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kinetically perturbing these states *in solution* using thermal or chemical denaturation. Potential stabilizing ligands and/or solutions are tested by determining how they delay the measured perturbation. Specifically, these methods often rely on detecting shifts in solution isotherms caused by ligand or solution stabilization.

A majority of the most popular protein stability measurements in solution use spectroscopic measurements that depend on a combination of thermal perturbation coupled with fluorescence or optical methods (e.g., thermofluor, aggregation assays). Currently, the most frequently used high-throughput screening approach to identifying potential protein stabilizers is differential scanning fluorimetry (DSF). This protein stability method entails a gradual heating of the protein solution and monitoring the amount of fluorescent dye that binds to the exposed hydrophobic regions as the protein undergoes a partial unfolding transition to a molten globular state.[3] If a stabilizing ligand is present, the shift toward the denatured state is delayed, resulting in a shifted protein unfolding profile. The stability of a test protein in solution is usually measured in a gradual manner, using a thermal scan rate of 1 °C/min or incubating the protein with various denaturants until equilibration is reached. This method can be conveniently executed with high-density microplates using thermal cyclers. Direct binding evidence between well-behaved target proteins and small molecules is easily discerned through protein kinetically controlled denaturation transition (T_m) shifts.[4] Compounds promoting protein stability have been extensively characterized or confirmed using DSF as screens for a number of drug targets.[5−8] This solution-based method examines protein kinetic T_m upon incubation with small molecule ligands. Unfortunately, the hydrophobic binding dyes used in these types of assays, such as SYPRO Orange, can contribute to the protein unfolding reactions, subsequently shifting the folded protein population to denaturation or aggregation. Therefore, caution is needed to minimize the preincubation time between the dye and target protein.[9,10] Also, many test compounds have an intrinsic absorbance spectrum that interferes with spectroscopic measurements. In addition, ligand rank order and binding affinities derived from the T_m shift cannot be easily translated to those at physiological temperatures because of a lack of knowledge of other thermodynamic parameters, such as enthalpy and heat capacity changes.

In contrast to thermal denaturation, equilibrium chemical denaturation is an alternative for evaluating protein stability and binding constants at low temperatures (lower than T_m or physiological temperature).[11,12] Equilibrium chemical denaturation performed on automated platforms, such as AVIA 2304, relies on changes in intrinsic or extrinsic fluorescence of target proteins during the unfolding process as a measurable parameter.[13] However, as observed with the thermal denaturation protocols, the equilibrium chemical denaturation processes *in solution* can also result in aggregate formation, particularly as proteins approach intermediate denaturant conditions where aggregation-prone partially folded species become highly populated.

There are some other protein specific caveats that prohibit widespread use of these methods for a broader range of proteins. The gradual thermal denaturation approach works best for well-behaved proteins that produce smooth reproducible kinetically controlled denaturation profiles. Unfortunately, oligomeric proteins or multidomain proteins sometimes fail to produce recognizable denaturation profiles, often reporting multitransitions when subunits or domains fail to melt cooperatively due to aggregation, making the interpretation of data difficult. Thermally based assays also fail for thermally stable oligomers such as transthyretin (TTR) because no signal is generated during thermal ramps under physiological solution conditions. On the other end of the stability spectrum, the solution-based DSF or chemical denaturation assays are also problematic in situations when the protein is marginally stable because aggregation is even more problematic at physiological temperatures (i.e., low equilibrium T_m). This latter class of proteins includes many missense mutants that are responsible for misfolding diseases.[9] In addition, the method often requires a large quantity of protein to obtain a visible melt transition for large screening sets. It is also very challenging to use thermal denaturation approaches to monitor the stability of membrane proteins because the presence of the lipid and/or solubilization medium itself leads to extensive hydrophobic interactions generating uninterpretable signals.

For the aggregation-based approaches, potential test stabilizers may also inhibit the aggregation reactions directly rather than physically stabilizing the protein of interest.[14] For example, generation of smaller aggregates reduces the magnitude of the light scattering signal, yielding false positive results. Furthermore, protein aggregation-based assays are notorious for their slow kinetic readouts, particularly during oligomer dissociation processes.[15,16] Aggregation assays based on the binding ability of dyes such as thioflavin T also suffer from a lack of complete specificity in dye binding, are subject to false negatives, and include instances in which the compounds stabilize folding intermediates (misfolded) without stabilizing the native protein. Problems can also arise from the physical properties of the protein stabilizer (e.g., time-dependent self-aggregation and optical interference) where spectroscopic interference may compromise specific enzyme assays.

To assess the stability of slowly dissociating oligomers or metastable proteins, there is a need for a general rapid perturbation system that prevents aggregation while permitting assessment of ligand-induced stabilization of these types of proteins. Here we present a simple protein kinetic stability evaluation system using a GroEL chaperonin-based, label-free BLI approach that avoids general aggregation, allowing detection of partially folded transient populations immobilized on biosensor surfaces. Normally, this chaperonin can allow proteins to acquire their functionally folded conformations by binding these transient forms, unfolding, and releasing them to fold either inside a hydrophobic cavity or, in the case of a large protein, releasing them back into solution. Both the encapsulated folding (*cis*) and release (*trans*) folding processes involve elaborate allosteric ATP-dependent cycling mechanisms.[17] The protein stability assessment system described here uses the high-affinity nucleotide-free form of the GroEL chaperonin state that has the ability to tightly bind partially folded proteins (molten globules) or unfolded states as hydrophobic regions become exposed to solvent. The substrate binding interactions with the high-affinity GroEL chaperonin often have binding affinities that approach nanomolar to subnanomolar K_ds.[18] Most importantly, dynamic unfolding ↔ refolding equilibria can populate protein states that can partition onto the nucleotide-free chaperonin, but this partitioning reaction is suppressed when either small molecule stabilizers (e.g., natural ligands) or stabilizing conditions (e.g., folding osmolytes and/or nonoxidizing conditions) shift the natural dynamic equilibria toward folded states.[19−22] In most

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cases, perturbing the initial unfolding ↔ refolding equilibria with minimal temperature elevation or low denaturant concentrations is enough to generate larger partially unfolded populations that kinetically partition onto the high-affinity chaperonin.[9,21] For the system illustrated herein, transitions toward partially folded conformers capable of binding GroEL are dictated by the intrinsic stability and the solution conditions that kinetically stabilize or destabilize the immobilized target protein. This work describes the development of a broadly based chaperonin BLI detection system capable of assessing the kinetic stability of almost any folded protein species.

MATERIALS AND METHODS

Ultrapure (MB grade) guanidine hydrochloride (GuHCl) was purchased from USB Corp. (Cleveland, OH). Urea (ACS grade) and Tween 20 were obtained from Fisher Scientific (Pittsburgh, PA). Resveratrol was obtained from Tocris (Bristol, U.K.). Glutathione (GSH), ethacrynic acid (EA), and diethyldithiocarbamate (DDC) were purchased from Sigma-Aldrich (St. Louis, MO). GT1b (bovine brain) was purchased from Enzo LifeSciences as a lyophilized powder and resuspended in chloroform at a concentration of 20 mg/mL (9.1 mM). Working dilutions were made by two 1/10 serial dilutions in Millipore water. Amine reaction second-generation (AR2G) biosensors, Ni-NTA biosensors, and anti-GST biosensors were procured from *forté*BIO. The 53BP2 (490−498), p53 binding loop (CDB3) p53 binding peptide (NH_2-REDEDEIEW-COOH, MW of 1220.2 Da) was purchased from Peptide 2.0 (Chantilly, VA) and stored at −20 °C prior to use.

A monoclonal IgG was obtained as a gift from *forté*BIO, and His-tagged MalZ was purified according to a previously described procedure.[23] Repeated automated runs of IgG denaturation profiles were performed with *forté*BIO anti-GST antibody biosensors. The von Willebrand factor (vWF) A1-A2-A3 protein with a His tag on the A3 domain was expressed and purified following the methods outlined by Tischer et al.[24] *Schistosoma japonicum* glutathione *S*-transferase (*Sj*GST) was obtained from Genscript (Piscataway, NJ), while His-tagged GST was obtained from EMD Millipore (Temecula, CA). His-tagged recombinant human transthyretin and superoxide dismutase were obtained from Sino Biological Inc. (Beijing, China). A His-SUMO-tagged nucleotide binding domain of cystic fibrosis transmembrane regulator (CFTR-NBD1) was obtained as a gift from the Cystic Fibrosis Foundation. Maltodextrin glucosidase (MalZ) plasmid PCS19 containing the *malZ* gene was a generous gift from W. Boos (University of Konstanz, Konstanz, Germany).

Chaperonin Purification and Storage. Highly purified GroEL was obtained using the purification scheme outlined in a previous work.[25] Because GroEL does not contain tryptophan, the purity was determined by following the diminishing contributions from tryptophan (proteins or peptides) as assessed by contributions from contaminant tryptophan fluorescence (excitation at 297 nm) or by noting the indole contributions using second-derivative UV analysis.[26] It is crucial to obtain highly pure GroEL because small amounts of contaminating proteins and peptides diminish the effectiveness of protein capture and binding. In addition, highly purified GroEL has a tendency to slowly dissociate into heptamers and monomers, where monomers can bind to remaining oligomeric GroEL, further compromising the GroEL preparation. To avoid dissociation of the GroEL tetradecamer, the purified GroEL lots are stored in 50% glycerol at 4 °C. This storage solution is removed prior to immediate GroEL use and replaced with a GroEL buffer (preferred in protein refolding assays) containing 50 mM Tris, 50 mM KCl, 10 mM $MgCl_2$, and 0.5 mM EDTA (pH 7.5) at 25 °C. For elevated temperatures (37 °C), the Tris-HCl buffer can be replaced with 50 mM HEPES, but the pH decline is only to 7.2 for the Tris buffer.

Maltodextrin Glucosidase Purification. MalZ was produced and purified according to the protocol outlined by Paul et al.[23] The PCS19 plasmid contained the *malZ* gene under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T5 promoter and ampicillin resistance selection marker. *Escherichia coli* strain BL-21 was used for protein overexpression. The purification of the N-terminally His-tagged MalZ was conducted by nickel affinity chromatography. A 20 mM sodium phosphate buffer containing 500 mM NaCl was used for lysis and nickel column equilibration. The bound MalZ protein was eluted with a linear imidazole gradient, with MalZ eluting near 200 mM. Appropriate fractions of MalZ were evaluated using sodium dodecyl sulfate−polyacrylamide gel electrophoresis gels stained with Coomassie blue. Fractions corresponding to pure single-band fractions were pooled.[27]

TeNT Subcloning, Expression, and Purification. DNA encoding tetanus neurotoxin (TeNT) was cloned into a modified pET28a expression vector (Merck KGaA, Darmstadt, Germany) such that the resulting fusion protein contained an N-terminal His tag and a C-terminal Strep tag. To generate a catalytically inactive form of TeNT for use in downstream experiments, two point mutations within the light chain (R372A and Y375F) were generated using the QuikChange II Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). Recombinant TeNT was expressed in *E. coli* BL-21 AI cells and purified by sequential chromatography on Ni-NTA agarose, Sephacryl S-200, and Strep-Tactin sepharose. Peak fractions from the Strep-Tactin sepharose column were concentrated to ∼1.5 mg/mL using an Amicon filtration device (YM-100 type filter), dialyzed into TeNT buffer [30 mM HEPES-NaOH and 500 mM NaCl (pH 7.6)], and stored at −80 °C until use. A typical preparation yielded 1−3 mg of purified toxin/L of batch culture.

p53 Subcloning, Expression, and Purification. A plasmid with the gene for TP53 aka p53 (AY891243) was obtained from the Harvard PlasmID Database (HsCD00001269). The DNA binding domain (DBD) of p53 (residues 94−312) was subcloned into pTBMalE provided by F. P. Gao. The resulting construct had an N-terminal His tag followed by maltose binding protein (MBP), a TEV recognition sequence, and the p53 DBD. The plasmid was transformed into BL21(DE3) competent *E. coli* cells obtained from New England BioLabs (Ipswich, MA). For expression, a 10 mL preculture was grown overnight in LB broth with 100 μg/mL ampicillin from Fisher Scientific (Fair Lawn, NJ). One liter of LB with ampicillin was inoculated with the preculture and induced with 0.24 g of IPTG from EMD Millipore (Billerica, MA) when the OD_{600} reached 0.6. Cells were grown overnight at 21 °C, then harvested by centrifugation at 5000 rpm, and resuspended in 50 mM Tris (Fisher Scientific, Fair Lawn, NJ) (pH 7.2), 1 mM DTT (Applichem GmbH, Darmstadt, Germany), and 50 mM imidazole (Acros). Protein was purified by affinity chromatography with Ni-NTA agarose (Thermo Scientific, Rockford, IL) and concentrated with an Amicon Centrifugal filter unit from Merck Millipore (County Cork, Ireland). Protein was buffer exchanged into 50 mM Tris (pH

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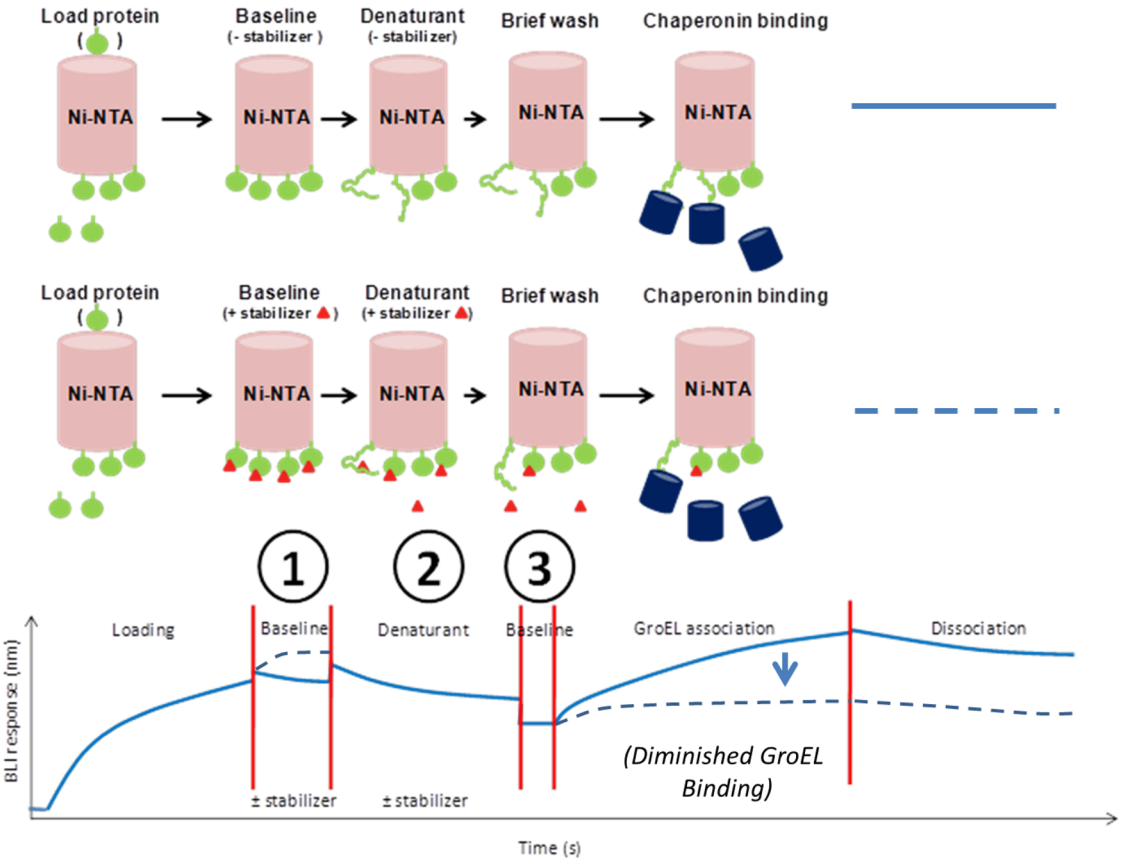

Figure 1. Schematic illustration of a generic denaturation pulse platform for identification of kinetic protein stabilizers. Key steps include loading of target protein onto biosensors followed by dipping into a buffer with or without a potential stabilizer (baseline), dipping into a denaturant solution with or without a potential stabilizer, a wash step to remove denaturant (and compound if any), and subsequent association of GroEL with protein species that expose hydrophobic residues. The following steps highlight the processes that can be varied, including (1) pretreatment time with a potential stabilizer to ensure adequate binding, (2) denaturation time to increase the unfolded population, and (3) wash time to remove denaturant and compound. In the presence of a stabilizer (red triangle) prior to and during the denaturant pulse phase, the GroEL binding amplitude and kinetics are diminished (dotted line). As a necessary control, profiles with any evidence of stabilizer dissociation in the absence of GroEL need to be subtracted from the GroEL association phase.

7.2) with 10 mM DTT using a PD10 desalting column (GE Healthcare, Buckinghamshire, U.K.) and concentrated again to ~1−5 mg/mL, followed by subsequent storage at −20 °C in 50% glycerol (Fisher Scientific, Fair Lawn, NJ).

Biolayer Interferometry. In most instances, preliminary biolayer interferometry experiments were performed on a single-channel BLItz instrument (*forté*BIO) to establish conditions (loading, GroEL concentrations, buffers, etc.), and kinetically controlled denaturation isotherm generation was performed on the automated eight-channel Octet RED96 instrument (*forté*BIO) unless otherwise stated. Biolayer interferometry is an all-optical technique measuring biomolecular interactions. The biosensor tip is a glass fiber optic system that measures the changes in the reflected white light interference patterns between a reference layer and a test biolayer. Any shift in the interference pattern of the reflected white light obtained at the detector indicates changes in the number of bound molecules. This change is monitored in real time, resulting in a phase shift reflecting both the kinetics of the binding process and increases in amplitude caused by binding.[28] The temperature was set at 25 °C unless otherwise specified. The loading of the protein substrate was optimized to avoid crowding on the biosensor surface and was within a linear range of BLI signal to protein concentrations (Figure S1). Shaking platforms were set at 2200 and 1000 rpm for the single-channel BLItz and automated Octet RED96 system, respectively. At excess GroEL concentrations, the binding signals are stabilized at these two shaker settings, diminishing mass transport effects. Sample volumes in all steps were provided in 250 μL aliquots in 500 μL black microcentrifuge tubes for the BLItz single-channel unit and 200 μL aliquots for the 96-well formats with the automated Octet RED96 system. Ni-NTA tips were regenerated following a previously established protocol.[29] All the protein targets described in this work, except IgG, were immobilized onto Ni-NTA tips via the strong and highly specific interaction between the His tag and nickel ions. In contrast, immobilization of the IgG molecules was accomplished using a standard 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS) (Thermo Fisher Scientific, Waltham, MA) protocol and amine reactive second-generation (AR2G) biosensors.[30,31] The anti-GST IgG biosensors were manufactured and supplied by *forté*BIO.

Optimizing Loading of Protein onto BLI Biosensors. Loading of protein [e.g., GST as an example (Figure S1)] onto the biosensor surface was within the lower end of the linear response range (signal vs protein concentration). Using lower protein concentrations diminishes tip surface overcrowding and intrasurface protein interactions. Overcrowding could result in self-association between loaded protein molecules, leading to a weakened GroEL binding signal. Because the loading of the

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protein and the concomitant GroEL binding was kept within a linear response range, one could correct for slight GroEL binding variations per protein load by performing a load correction based on the valid assumption that the GroEL binding signal was directly proportional to the amount of protein loaded and/or partially unfolded (Figures S1 and S2). At high GroEL concentrations, the sensorgram traces would rise rapidly and approach a saturated signal amplitude due to favorable collisional rates and optimally attached (within the linear loading range) target proteins.

BLI Denaturant Pulse Method for Generating Kinetically Controlled Denaturation Isotherms. The kinetic denaturant pulse method (Figure 1) was performed using the following general steps. Each protein target was individually loaded onto specific affinity biosensor tips followed by a buffer wash step (with or without a ligand) to establish a buffer baseline. Biosensor tips were subsequently exposed to a series of increasing denaturant solutions (with or without a ligand) for a defined incubation (pulse) time (1, 3, 5, or 10 min) followed by a dip of the probe into a brief wash solution (usually original loading buffer) to remove any adhering denaturant for anywhere between 5−60 s. Following the brief wash, the biosensor tips were immersed in solutions containing GroEL where chaperonin binding to denatured protein populations that were present on the tip resulted in a time-dependent kinetic rise in the BLI sensorgram. For experiments performed on the automated Octet RED96 (as was the case for GST, TTR, SOD, and vWF), Ni-NTA tips were regenerated at the end of each denaturant pulse and return cycle following an established regeneration procedure.[29] The TeNT protein was not regenerated, and fresh Ni-NTA tips were used for each replicate of constructing the TeNT kinetically controlled denaturation isotherm. For the regeneration procedure, Ni-NTA tips with the protein−GroEL complex were first cycled through 10 mM glycine at pH 1.7 followed by neutralization buffer (1× kinetics buffer, *forté*BIO) three times, with each pulse lasting for 5 s. The NTA regenerated tips were then recharged with Ni by immersing the tips in 10 mM $NiCl_2$ for 1 min. If regeneration protocols cannot be implemented, then new tips should be used, provided that loading amplitudes continue to remain constant (e.g., no compromise in protein integrity). The regenerated surface was reused to bind His-tagged protein for the next round of experiments. The reloading was highly reproducible for proteins that remained stable throughout the regeneration procedures, varying by a standard deviation of only ∼3−5%, as assessed by noting the variation of the final loading signal amplitudes (Figures S2 and S4). As a necessary control, any evidence of stabilizer dissociation sensorgram traces (nonspecific or specific declines if observed) in the absence of GroEL was always subtracted from the GroEL association phase to create the final corrected sensorgrams (corrected kinetics and amplitude).

Generating Kinetically Controlled Denaturation Profiles of MalZ in the Presence of Urea and Osmolyte Stabilizers. His-tagged MalZ at a concentration of 80−100 μM was prepared with a reduced imidazole concentration by buffer exchange and concentration on an Amicon 30 kDa centrifugal concentrator. The buffer used for BLI experiments consisted of 20 mM sodium phosphate, 20 mM $MgCl_2$, and 10 mM KCl (pH 7.4). Initial MalZ experiments were performed using the single-channel BLItz system, and under these conditions, MalZ did not show dissociation from Ni-NTA biosensors. To construct the automated kinetically controlled denaturation profiles of the MalZ protein, a His-tagged natively folded MalZ construct concentration of 50 nM (within linear range) was loaded onto Ni-NTA tips over 2 min. Once MalZ is loaded onto the Ni-NTA biosensors, these tips were automatically removed from the loading solution, briefly washed in loading buffer (30 s), and subjected to separate but increasing urea concentrations in a 5 min pulse followed by a 10 s wash in refolding buffer to remove denaturant. The tips were then immersed in GroEL binding buffer containing 0.5 μM GroEL for 5 min. Solid urea was added to refolding buffer to make an 8 M stock solution. The same solution procedures were used with the denaturation series where urea solutions also contained stabilizing osmolyte 1 M sucrose or 4 M glycerol. The resulting GroEL binding amplitudes were plotted against the respective urea concentrations to generate kinetically controlled denaturation isotherms, and the isotherms were compared with isotherms generated with the urea denaturant alone. Each data point represents the mean of three separate automated experiments with error bars present for the standard deviation per denaturant concentration using the automated Octet RED96 system.

Effect of Denaturation Time on His-Tagged GST. As a model protein, His-tagged GST was used to explore the effect of denaturation time on its kinetic transition. The loading of GST showed a nanometer linear response throughout a GST concentration range of 0.05−1 mg/mL. At a response signal of 1 nm loading (∼0.25 mg/mL GST), the GroEL concentration was varied to determine the binding saturation. The GroEL concentration that was chosen for the GST kinetic denaturant pulse experiments was 0.5 μM. The denaturant pulse times were varied from 3 min to 5 min to 10 min for GST alone and GST that was covalently modified with EA/GSH adduct. Loading of GST was relatively constant throughout the repeated runs (Figure S2), and the GroEL binding amplitudes were load corrected for slight variation. The raw data traces and load-corrected traces revealed the same trend in GroEL binding responses (Figure S2).

His-tagged GST attached to the Ni-NTA biosensor was tested for functional integrity through a titration with the known inhibitor, tannic acid, using the Octet RED96 BLI instrument (sensitive enough to observe binding of small molecules of >150 Da to immobilized proteins). The results from this assessment indicate that the estimated binding affinity of the immobilized GST for tannic acid is 17 ± 2.3 nM, well within the same order of magnitude as in the solution binding constant range (49 nM) (Figure S1C).

Ligand-Induced Stability Assessment of Oligomeric Protein Dimers (TTR and SOD1). The thermal stability of TTR and SOD dimers is often difficult to assess because of the inherent stability of these oligomers. (Note that TTR is usually a tetramer, which is substantially more stable than the dimeric form used in these studies.) A known TTR stabilizer, resveratrol, and a known SOD1 inhibitor, DDC (chelates Cu^{2+} metal ligand), both applied at 200 μM, were added in both baseline steps before and during the denaturant pulse step, respectively. Following a 5 s buffer wash to remove denaturant and the compound ligand solution, binding of GroEL to the immobilized target oligomers was monitored after the protein-loaded biosensors were stabilized or destabilized due to ligand binding or metal ligand removal, respectively.

Evaluation of Metastable Proteins. *CFTR-NBD1*. The wild type nucleotide binding domain 1 (NBD1) fragment of the cystic fibrosis transmembrane regulator (CFTR) engi-

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neered with an N-terminal His-SUMO tag was loaded onto Ni-NTA tips at a concentration of 0.01 mg/mL (optimized loading amplitudes to avoid intrasurface aggregation). Because CFTR-NBD1 is metastable in the absence of a stabilizing ligand, ATP was added to maintain CFTR-NBD1 stability during the loading phase and loading amplitudes remained essentially constant throughout the experimentation on both BLItz and the Octet RED96 system. Specifically, for the Octet RED96 system, a 0.01 mg/mL CFTR-NBD1 solution [diluted in 50 mM Tris-HCl, 150 mM NaCl, and 3 mM $MgCl_2$ (pH 8.0)] was prepared and dispensed into one column in a 96-well solid bottom plate at a volume of 200 μL/well. The Octet RED96 chamber temperature was maintained at 25 °C, and the plate containing CFTR-NBD1 protein solutions was kept in the chamber for the entire duration of the experiment. Ni-NTA tips were hydrated in the aforementioned CFTR-NBD1 buffer to establish a baseline before protein loading. The loading magnitude was observed for a 5 min period of time before the tips were dipped into CFTR-NBD1 buffer again to re-establish the baseline. The same set of Ni-NTA tips were regenerated and reused to load CFTR-NBD1 repeatedly within a 40 min time window. Separately, the same experiment was performed using CFTR-NBD1 solutions that contained 2 mM ATP, a natural stabilizing ligand for the protein. Weakly binding ATP that was bound to CFTR-NBD1 was essentially washed away during the buffer wash step and the denaturant pulse step. Comparisons of CFTR-NBD1 kinetic stabilities in the presence and absence of GTP in the postload step and denaturant pulse step were performed using the single-channel BLItz system. GTP, a known nucleotide stabilizer, does not bind to GroEL,[18] nor does this nucleotide facilitate release and folding of GroEL-bound protein substrates. Both the initial GroEL binding amplitude differences and a limited kinetic isothermal denaturant curve were obtained for CFTR-NBD1 in absence and presence of GTP.

Testing Osmolyte and CDB3 Peptide Stabilization of the p53 Core DNA Binding Domain. The His-tagged maltose binding protein (MBP) p53 core DNA binding domain was loaded at a concentration of 0.1 μM, which was well within the linear concentration range for this protein (results in a 1 nm amplitude deflection during loading). The GroEL concentration used for binding immobilized p53 was 0.15 μM (yields an ~1 nm deflection when binding completely denatured p53, which was achieved upon pulse exposure to 6 M GuHCl). The osmolyte stabilization assessment was performed on the automated Octet RED96 BLI system using the denaturant pulse protocols (25 °C). The potential stabilization effect due to binding of the CDB3 peptide to the p53 core was assessed by monitoring the potential decline in the level of binding of GroEL to native and pulse-denatured p53. The effects of CDB3 peptide binding and/or stabilization of p53 on the GroEL interactions after the denaturant pulse step were determined on the single-channel BLItz system.

Developing a Kinetic Stability Isotherm for Multidomain Proteins. *IgG Kinetically Controlled Denaturation and Stabilization.* To generate kinetically controlled denaturation profiles of a known multidomain protein, immobilized IgG was tested. For these experiments, two sets of IgG biosensors were used. In one instance, the IgG is randomly attached via reactive amine coupling to AR2G biosensors. In the second instance, commercial anti-GST IgG biosensors were used in the multiple-denaturant pulse replicate experiments. Each of these particular IgG biosensors was briefly exposed to increasing denaturant concentrations for 5 min using either urea or GuHCl at pH 7.5 in refolding buffer. After the denaturation pulse, tips were washed in refolding buffer for either 10 or 60 s followed by exposure to 1 μM GroEL using the same buffer. Wash times were varied to examine potential folding of correctly disulfide-bonded IgG. The amount of bound GroEL represents the amount of destabilization of the IgG, and the amplitudes were measured at each denaturant concentration. GroEL binding amplitudes were plotted as a function of denaturant concentration to generate kinetically controlled denaturation isotherms. In a separate set of experiments, immobilized IgG was exposed to mixtures of 4 M urea and a number of folding osmolyte/protein stabilizer solutions (2−4 M) to mimic an excipient screen. Following the urea denaturant pulse with or without stabilizers and a 10 or 60 s wash, the GroEL binding sensorgrams were recorded and the amplitude at 5 min was recorded. In this instance, the wash times were varied to examine the extent of on-tip refolding. Each data point in the kinetically controlled denaturation isotherms was the mean value of three separate runs with error bars of the standard deviation recorded.

von Willebrand Factor (triple A domain): Constructing Kinetically Controlled Denaturation Isotherms Using Varying Denaturation Times. To determine if the kinetic denaturant pulse method could be used to detect the stability of the von Willebrand factor (vWF), a multidomain protein containing three individual integrin-like domains (triple A domains) connected by flexible linkers, vWF biosensors were constructed to generate and compare a kinetically controlled denaturation isotherm with the multiphase denaturation transitions that are observed during the solution equilibrium denaturation of vWF.[32] After the linear range for loading His-tagged wild type vWF (attached through the A3 domain) had been determined, 0.6 μM wild type vWF triple A domain was loaded onto Ni-NTA biosensor tips over a 5 min time period. After a brief 30 s wash, biosensor tips were exposed to a urea denaturant pulse for 1, 5, or 10 min. After a second 10 s wash to remove any residual denaturant, tips were dipped into refolding buffer containing 0.5 μM GroEL. The GroEL binding amplitude, recorded after each pulse time, was plotted as a function of denaturant pulse concentration, and the kinetically controlled denaturation isotherms for each denaturant pulse time were constructed.

Generating a TeNT Kinetically Controlled Denaturation Profile with and without a Natural Ligand. TeNT was supplied with an N-terminal His tag at a concentration of 1.5 mg/mL in TeNT buffer [30 mM HEPES and 500 mM NaCl (pH 7.6)]. This buffer was used during the biolayer interferometry experiments for baselines, protein loading, and urea denaturation. TeNT denaturant pulse isotherms were generated on the Octet RED96 system using nonbinding black well plates, containing 240 μL/well. After the linear response range for loading TeNT protein (linear from 2.5 μg/mL to 0.75 mg/mL) had been determined, a concentration of 7.5 μg/mL (50 nM) was chosen to generate the kinetically controlled denaturation isotherm. *forté*BIO recommends using the lower end of the protein range to prevent tip surface crowding. Ni-NTA biosensors were used to immobilize TeNT for the assay. Once bound to the biosensors, the protein was denatured for 300 s in urea at a concentration of 0, 1, 2, 3, 4, 5, 6, or 7 M in refolding buffer. After denaturation, the tip was briefly washed for 10 s in TeNT buffer. GroEL, at 0.5 μM in refolding buffer, was allowed to bind to the denatured TeNT for 5 min.

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Biochemistry XXXX, XXX, XXX−XXX

Following GroEL association, the GroEL−TeNT complex was allowed to dissociate for 5 min in refolding buffer. The GroEL signal was load-corrected by dividing the amplitude of GroEL binding by the amplitude of TeNT loading. To determine the association of GroEL with partially unfolded TeNT, the load-corrected GroEL signal was plotted against urea concentration. To examine the effect of GT1b on TeNT stability, the protocol described above was slightly modified. (1) An additional step consisting of 1 μM GT1b in TeNT buffer was included before the urea denaturation pulse step to allow for ligand association. (2) GT1b was present in the urea solutions at 1 μM. (3) The 10 s wash after the denaturant pulse was split into two 5 s washes, the first with 1 μM GT1b and the second with just TeNT buffer. To assess the effects of folding osmolytes on delaying potential toxin transitions and/or unfolding, a separate set of experiments in which 1 M sucrose was included in the denaturant pulse (as was previously described for most of the proteins examined herein) were performed, and a kinetically controlled denaturation isotherm was generated using the automated Octet RED96 system using the experimental protocols mentioned above.

RESULTS

Using the GroEL Chaperonin To Detect Partially Folded States Immobilized on Surfaces. The use of the high-affinity nucleotide-free GroEL chaperonin system as a detector of partially folded or misfolded proteins on immobilized surfaces stems from the frequent observation that GroEL chaperonins (naturally occurring or overexpressed) bind to misfolded proteins. Interestingly, the GroEL chaperonin is a common contaminant protein binding to the affinity-tagged protein isolated on affinity column matrices[33] due to the fact that GroEL binds to affinity-tagged proteins that remain partially folded. To avoid *in vivo* aggregation, methods were developed to construct chimeric proteins in which difficult folding domains could be stabilized by attaching an engineered easy-to-fold chimeric affinity tag (e.g., SUMO, glutathione *S*-transferase, maltose binding protein, etc.). Even when the affinity tag readily folds and binds to the biospecific tag, the protein of interest may still fail to completely fold and bury its hydrophobic surface, allowing the high-affinity nucleotide-free chaperonin to readily bind to the protein while immobilized on the biospecific affinity surface.[34] Previous work indicates that the rates of folding of immobilized protein can be facilitated by adding specific folding osmolytes, increasing the temperature, or including small molecule stabilizers to enhance proper folding and weaken GroEL protein binding.[34]

In the examples that follow, dynamic populations of folding intermediates are generated by immersing target proteins attached to BLI biosensors in a denaturant solution for a defined period of time (defined as a denaturant pulse). In all of these instances, the amount of GroEL binding to the partially folded immobilized substrate when loaded within a defined linear concentration range is predicted to directly correlate with the extent and number of partially folded/unfolded protein molecules that remain on the BLI biosensor once the denaturant is removed and the biosensor tip is immersed in a GroEL solution (GroEL binding step) (Figure 1).

Extending Stability and Folding Analysis for a His-Tagged Recombinant Protein MalZ. The first example of examining binding of GroEL to a large immobilized protein uses the *E. coli* protein MalZ, which has been shown to be efficiently folded by the GroE system. MalZ is a 69 kDa monomeric enzyme functioning to degrade maltodextrins to shorter oligosaccharides[35] and has also been used to examine GroEL folding of larger proteins that cannot be sequestered inside a GroEL−GroES cavity. Chaudhuri and colleagues demonstrated that MalZ appears to fold from GroEL by a *trans* mechanism with the assistance of GroEL/GroES.[23] To generate a kinetically controlled denaturation profile, His-tagged MalZ was immobilized onto a series of Ni-NTA biosensor tips, and each was briefly immersed in varying denaturant concentrations (Figure 2). The urea denaturant was subsequently washed away with a 10 s wash step, and the population of unfolded/partially folded species on the tip at the time of measurement was assessed by monitoring the chaperonin binding amplitude recorded for 5 min. The amplitude of chaperonin binding is directly correlated with the amount of folded versus unfolded/partially folded protein populations that are present on the biosensor surface at the time of measurement, resulting in an increasing amplitude change as the denaturant concentration increases. The presence of any stabilizing conditions (ligands or folding stabilizers) is predicted to slow the kinetics of unfolding of the immobilized protein populations. The observed decrease in the chaperonin binding amplitude with an increase in denaturant concentration when stabilizers are included in the denaturant pulse solution agrees with this prediction (Figure 2). The presence of folding osmolytes, such as 4 M glycerol or 1 M sucrose, within each denaturant pulse solution resulted in weakened chaperonin binding at the time of measurement, indicating that a lower percentage of the attached protein had unfolded during the duration of the pulse. As the denaturant concentrations increased further, the GroEL binding ampli-

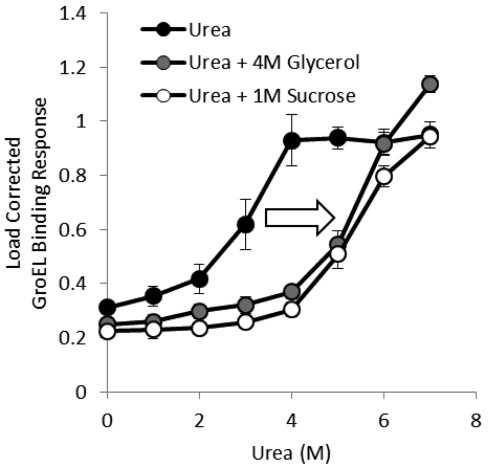

Figure 2. Kinetic stabilization of MalZ in the presence of different osmolytes. The MalZ protein was loaded onto Ni-NTA biosensor tips at 1 μM, and loading was consistent throughout the titration experiment. Each MalZ tip was exposed to various concentrations of urea, and the GroEL binding amplitude was measured for 5 min. A separate set of MalZ-attached tips were exposed to urea supplemented with sucrose or glycerol. This figure denotes the GroEL binding response as a function of urea concentration with or without osmolytes. Diminished GroEL binding amplitudes were observed at each urea titration point upon the addition of the stabilizing osmolytes (1 M sucrose or 4 M glycerol), indicating a delay in the kinetically controlled denaturation isotherms, which results in an apparent stabilization of MalZ. Each isotherm generated represents three separate runs. The error bars denote one standard deviation, and the Z' factor at 4 M urea is 0.41 (Table S1). A clear rightward shift in the GroEL binding amplitude response (denoted by an arrow) is observed.

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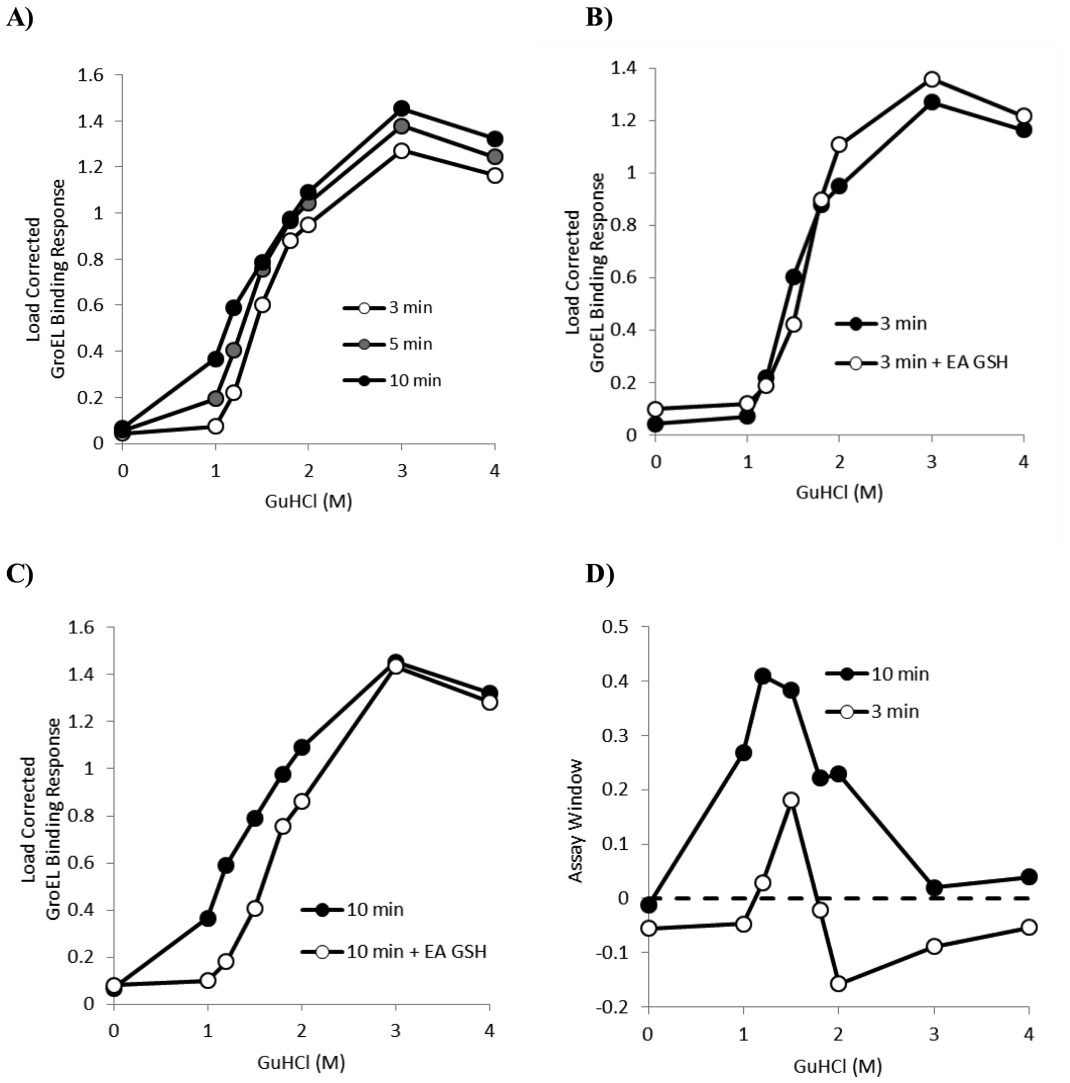

Figure 3. Kinetically controlled denaturation profiles exhibiting the effect of denaturation time on the GroEL binding amplitude. (A) Kinetically controlled denaturation of His-GST at various denaturation times (3, 5, and 10 min). (B) Kinetically controlled denaturation of His-GST in the absence (●) and presence (○) of 200 μM EA and 2 mM GSH with a 3 min denaturation time. (C) Kinetically controlled denaturation of His-GST in the absence (●) and presence (○) of 200 μM ethacrynic acid and 2 mM GSH with a 10 min denaturation time. (D) Differences in GroEL binding amplitudes derived from stabilizer-bound and stabilizer-free kinetically controlled denaturation profiles with 3 and 10 min denaturation times. The 10 min denaturation time data indicate that stabilizer-induced differences were optimal within the lower denaturant concentration range.

tudes in the presence of the osmolytes did approach the same maximal GroEL binding amplitude as was observed for MalZ biosensors that were not stabilized by osmolytes (Figure 2). It was important to note that each tip was removed from the osmolyte/denaturant pulse solution and was washed briefly (10 s) to remove the denaturant/osmolyte mixture before immersion in buffer containing the chaperonin. This procedure ensures that bulk osmolyte removal is complete and will not interfere with the binding of chaperonin to the partially folded substrate. Separate control experiments indicated the immobilized MalZ, which was completely denatured at high urea concentrations at 25 °C, refolds very slowly ($t_{1/2}$ at 25 °C of ~10 min) relative to the wash times that were used to remove an adhering denaturant or a ligand solution.[36] It was also shown that the refolded MalZ immobilized on and released from Ni-NTA surfaces was functionally active with yields that were substantially improved compared to those of solution refolding.[36] These control measurements indicated that no substantial refolding was detected (i.e., chaperonin binding amplitudes remain high) if two separate MalZ attached tips were compared before and after a 10 s wash period.

Initial denaturant pulse experiments were performed on a single-channel BLItz system and showed a predicted denaturation isotherm profile and subsequent delayed GroEL binding response due to osmolyte stabilization. These experiments were repeated with the automated Octet RED96 system, in which eight biosensor tips were simultaneously loaded and subjected to varying denaturant concentrations during the 5 min denaturant pulse. A total of three sets of separate biosensor tips were used for each point, and the GroEL binding amplitudes in all instances were highly reproducible (Figure 2). The predicted rightward shift and delay of GroEL binding for the kinetically controlled denaturation isotherms with stabilizers present in the denaturant solutions indicate that MalZ denaturation rates are slowed in the presence of a common stabilizing osmolyte such as 4 M glycerol or 1 M sucrose. For these particular experiments, the maximal difference between the 5 min denaturation profiles without

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Biochemistry XXXX, XXX, XXX−XXX

and with osmolytes was at 4 M urea with a resulting Z' factor of 0.42 (Table S1). The maxima for the denaturation profile differences between unstabilized and stabilized biosensor-loaded protein may be dependent on the programmed denaturant pulse times. The predicted shift in maximal GroEL binding differences is examined in the next section.

Potential for Small Molecule Stabilizer Development and Lead Compound Validation. To illustrate the general utility of this platform in identifying or validating small molecule stabilizers and eventually accelerating pharmacological chaperone drug discovery for aggregation-prone protein folding diseases, we tested a set of model or disease relevant protein targets that had different well-established inhibitors or stabilizers. As stated in the introductory section, proteins involved in folding diseases are difficult to study because they tend to aggregate under physiological conditions. To facilitate the search for stabilizers while preventing aggregation, we immobilized the proteins presented in the following sections on BLI biosensors to evaluate their propensity to bind the chaperonin GroEL as they increasingly underwent transitions to partially folded or fully unfolded populations by varying concentrations of denaturant pulses.

Glutathione S-Transferase (GST). Glutathione *S*-transferase (GST) was initially chosen to demonstrate the presence of general protein stabilizers through the detection of shifts in the kinetically controlled denaturation isotherms. GST is one of the most commonly applied purification tags as well as an enzyme involved in phase II biotransformation of xenobiotics in different organisms.[4] GSTs have been extensively associated with cancer, and their inhibitors exhibit potentiation in cancer therapy. GST protein structures are typically represented by either a homo- or heterodimer with an active site in each two-domain-containing subunit (N- and C-terminal domains). A highly conserved GSH binding site (G site) is located at the N-terminal domain, while a larger hydrophobic substrate binding site (H site) resides in the C-terminal domain. At least two main classes of GST inhibitors exist, including glutathione (GSH, GST native ligand) mimetics (i.e., GSH competitive inhibitors) or compounds forming adducts with GSH (non-competitive with respect to both G and H sites). The compound ethacrynic acid (EA) is a covalent inhibitor of GST with a reported IC_{50} in the submicromolar range against *Sj*GST.[37] Initially, a non-tag-containing version of GST was immobilized onto amine reactive tips through EDC/NHS chemistry. In spite of sufficient concentration load optimization, this particular nonspecific attachment scheme failed to generate highly reproducible kinetically controlled denaturation curves (data not shown). This may not be surprising as prior reports have shown that specific and homogeneous orientation of immobilized protein molecules relative to the sensor surface could be crucial in maintaining the integrity of protein function and activity, particularly for small proteins.[38] When a His-tagged version of GST was immobilized on Ni-NTA biosensors using an Octet RED96 system, the controlled loading and multiple-regeneration protocol resulted in highly reproducible kinetically controlled denaturation isotherms. In addition, this particular His-tagged attachment scheme appears to provide a homogeneously oriented binding surface for GroEL. To ensure GST was properly oriented on the tip to retain functionality, the His-tagged GST protein was titrated with the glutathione inhibitor, tannic acid, using the Octet RED96 BLI system. The K_d values that were obtained agreed with previously published values (see Figure S1C and a reference in its legend). This same His-tagged version of GST was loaded onto Ni-NTA tips with both protein loading and GroEL concentration optimized [this early optimization work was conducted on BLItz (Figure S1A,B)]. Because this is a kinetically controlled denaturation process, the extent of GroEL binding is expected to vary with the duration of the denaturant pulse. A comparison of the kinetically controlled denaturation curves at varying denaturant pulse times through the denaturant concentration range (GuHCl, 0−4 M) shows an increase in the GroEL binding magnitude in the ligand-free GST sample compared with the GST sample that is modified with the covalent inhibitor and/or stabilizer EA.

Curiously, the initial 3 min denaturation pulse failed to yield any substantial separation in the kinetic denaturation isotherms between EA covalently modified GST and unliganded GST. To determine if any differences in the GST kinetic stability could be observed, the time of the denaturant pulse was progressively increased. The His-GST kinetically controlled denaturation isotherms were examined as a function of denaturant pulse exposure time [3, 5, or 10 min (Figure 3A)]. As the denaturant time increases, the kinetic denaturant curves for the ligand-free GST showed a distinct left shift toward earlier destabilization. Understandably, more immobilized protein should unfold as the time period of the denaturation pulse is extended. EA forms a covalent conjugate with GSH (either enzymatically or nonenzymatically), and the resulting adduct not only is a more potent inhibitor of GST but also covalently modifies the enzyme's active site, which has been previously shown to result in thermal stabilization.[39] For these experiments, EA was present at a concentration of 200 μM with 2 mM GSH in both the baseline step preceding the denaturant step and during the denaturant pulse step. Unlike the wild type ligand-free GST, the GroEL binding amplitudes for the EA-GST biosensors showed very little change in the kinetically controlled denaturation isotherms as the denaturant pulse time increased from 3 to 10 min (compare the 3 min EA-GSH GST profile in Figure 3B with the 10 min EA-GSH GST profile in Figure 3C). Both showed significant delays in GroEL binding, particularly at the low denaturant concentration (compare amplitudes in the 1−1.5 M GuHCl range).

The ligand-induced shift in the kinetically controlled denaturation isotherm obtained at different denaturant exposure times shows the covalent EA adduct led to a decrease in the level of GroEL binding, suggesting that the EA modification leads to an observable kinetic stabilization of GST. Specifically, when 200 μM EA along with 2 mM GSH was preincubated with immobilized GST prior to the GuHCl denaturation, there was little difference (no reduction) in GroEL binding throughout the GuHCl titration range after the 3 min denaturation pulse (Figure 3B), but this difference between wild type and EA-modified GSH was more apparent after 10 min [see the kinetically controlled denaturation isotherm (Figure 3C)]. Furthermore, the differences between the load-corrected GroEL binding responses were plotted as a function of denaturant concentration (Figure 3D). The difference seemed to reach its maximum near 1.5−2 M GuHCl. Curiously, the apparent kinetically controlled transition slope appears to be sharper for the EA-GSH modified protein than for the wild type GST after a 10 min denaturant pulse.

Transthyretin (TTR). To demonstrate the potential of using this platform to screen for pharmacological chaperones of oligomers, the kinetically controlled denaturation profiles of

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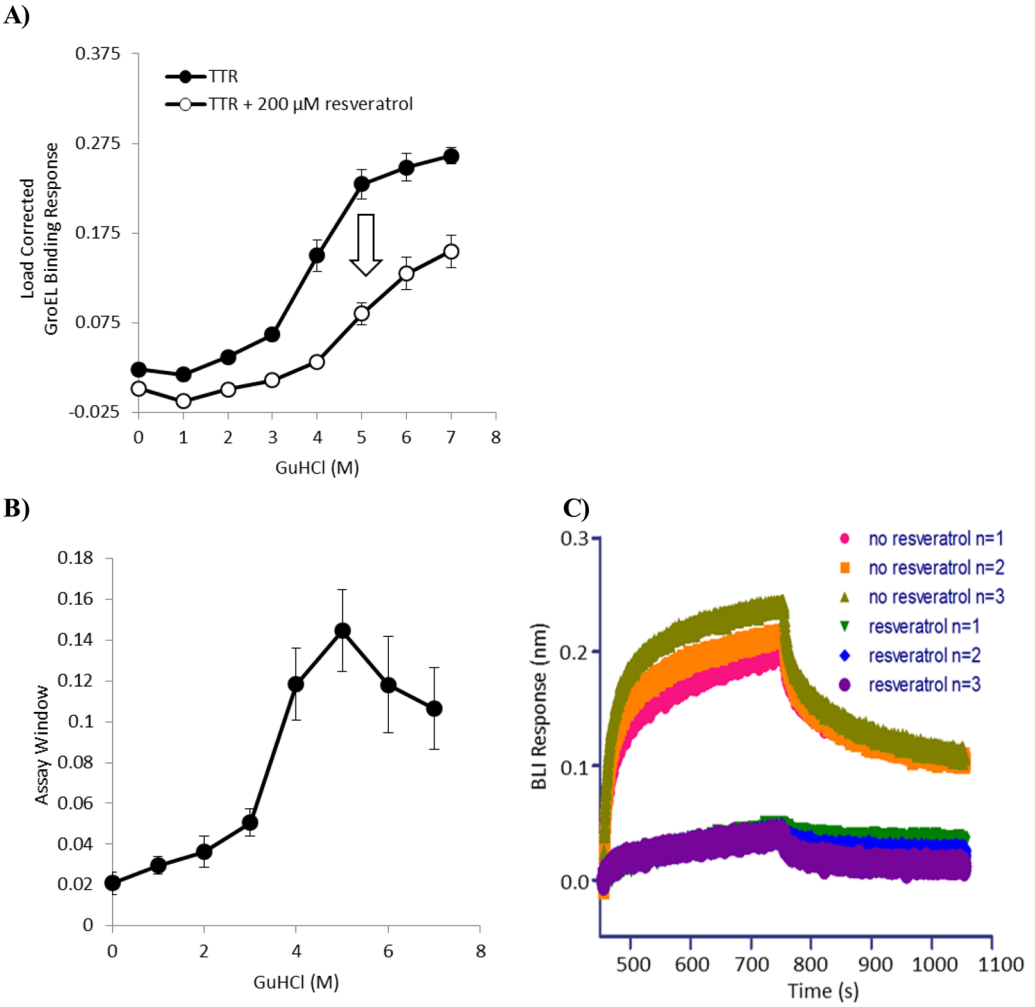

Figure 4. Detection of the resveratrol stabilization effect on TTR. Kinetically controlled denaturation of His-tagged TTR in the absence (●) and presence (○) of a known TTR stabilizer, resveratrol. Experiments were performed on the Octet RED96, with each data point comprising three separate measurements and error bars representing the standard deviation. A reduced GroEL binding signal amplitude was observed at each GuHCl titration point when TTR was pretreated with resveratrol, indicating the compound's stabilizing effect on the protein delays the unfolding of TTR. (B) Differences between the wild type and resveratrol-stabilized kinetic isotherms were generated to determine an optimal assay window. The optimal GuHCl concentration to readily observe stabilizer effects was in the 4−5 M GuHCl pulse range [Z' factor of 0.51 at 4 M (Table S1)]. (C) Aligned sensorgram traces for three sample automated runs showing association (binding of GroEL to TTR that had been pretreated with or without resveratrol) and dissociation steps after a 4 M GuHCl pulse.

important disease relevant oligomeric proteins were subsequently evaluated using this BLI denaturant pulse procedure. One such target considered is TTR, which is prone to fibrillogenesis for a number of missense mutants as the oligomer dissociates into monomers, or when the native oligomer is destabilized under low-pH conditions and high salt concentrations *in vitro*.[40] Wild type TTR and more than 80 mutants have all been found to be capable of amyloid formation and are linked to a group of amyloid diseases, including senile systemic amyloidosis (SSA), familial amyloid polyneuropathy (FAP), and familiar amyloid cardiomyopathy (FAC).[40] TTR, a 55 kDa homotetramer, carries thyroid hormone thyroxine (T4) and retinol binding protein in blood and cerebrospinal fluid. TTR possesses two distinct dimer−dimer interfaces, a robust interface stabilized by hydrogen bonding and a weaker dimer−dimer interface where its two T4 binding sites are located.[16,41] Currently, kinetic stabilization of the TTR native state conformation by binding of a ligand to the T4 binding site(s) represents an effective strategy for preventing TTR dissociation of the tetramers into dimers or amyloidogenic monomers,[41] with the goal of ameliorating amyloidogenesis. In the past, several different compounds, identified through either rational design[42,43] (including substructure combination strategy)[44] or high-throughput screening,[45] have been reported to stabilize the TTR native state,[15,46−49] with their stabilization effect correlating to decreasing subunit dissociation constants. In this study, a less stable dimeric form of the protein was captured on Ni-NTA tips through the engineered N-terminal His affinity tag because the tetrameric form may not undergo sufficient denaturant pulse destabilization in GuHCl.[50] After loading densities had been optimized [following the general protocol used for His-tagged GST (Figure S1)], kinetically controlled denaturation isotherms were generated for the protein in the absence and presence of the stabilizer resveratrol (Figure 4A). As predicted, the kinetically controlled denaturation isotherm somewhat mirrors the equilibrium denaturant isotherm of the TTR dimer (used in this study) rather than the tetrameric form that does not show denaturation until ∼6 M GuHCl.[50] The ligand stabilizer resveratrol is a natural polyphenol and an antioxidant found in red wine and several

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Biochemistry XXXX, XXX, XXX−XXX

plants. The *trans* isomer of resveratrol preferentially binds to the T4 binding sites, reducing the extent of TTR fibril formation.[42] The BLI denaturation pulse experiments showed that there was a decrease in the GroEL binding amplitudes in the kinetically controlled transition of the wild type TTR when 200 μM resveratrol was included both prior to and during the denaturant pulse (Figure 4A). This observation is in line with the prediction that resveratrol binding should stabilize or kinetically slow the dissociation and/or unfolding of TTR dimers against GuHCl unfolding. In results similar to those found with GST, the assay window that was generated from differences in the load-corrected GroEL binding response (Figure 4B) shows that the largest difference in the kinetic response in the presence of the stabilizer resveratrol was achieved in the 4−5 M GuHCl pulse range. The raw sensorgram data show a dramatic decline in the level of GroEL binding (Figure 4C) when the biosensor-immobilized TTR with resveratrol present is denaturant pulsed at 4 M GuHCl for 5 min. At this particular GuHCl concentration, the calculated Z' factor between the unstabilized and resveratrol-stabilized TTR dimer was 0.50 (Table S1).

Superoxide Dismutase 1 (SOD1). Another oligomeric disease relevant target tested is dimeric human copper−zinc SOD1. Biochemically, superoxide dismutase catalyzes the conversion of superoxide into oxygen and hydrogen peroxide,[51] thus serving as an antioxidant defense for the cellular components against reactive oxygen species. Structurally, eukaryotic SOD1 is a homodimer with high thermal stability (melting temperature reported to be >80 °C, when fully metalated).[52] Each protein subunit contains two metal binding sites, and each subunit is further stabilized by the presence of an intramolecular disulfide bond.[52] Superoxide dismutase can be stabilized by binding different metal ions, including copper/zinc, manganese, or nickel. In humans, there are three types of superoxide dismutases, with SOD1 and SOD3 both containing copper and zinc and SOD2 containing manganese in its reactive center. The linkage between more than 100 mutations in human SOD1 (hSOD1) and familiar amyotrophic lateral sclerosis (FALS; ALS is also commonly known as Lou Gehrig's disease)[53] was first reported ~20 years ago,[54] with A4V and G93A mutations being the two most common mutations. Evidence provided by crystal structures of mutant SOD1 suggests a structurally defective form of the SOD dimer with limited catalytic activity.[54] The ability of SOD1 mutants to form higher-order structures, perhaps due to dimer dissociation, that in turn could eventually lead to motor neuron death makes hSOD1 an intensely interesting target to study to improve our understanding of the mechanism and pathology of FALS. Specifically, the steps involved in the formation of deleterious SOD1 aggregates may include demetalation and reduction in disulfide bond formation,[55] both side reactions that destabilize the SOD dimer.

As the metal cofactors contributed significantly to the kinetic stability of SOD1 (likely through protein restructuring as structural differences exist between the apoprotein and holoprotein),[6,56,57] we constructed and compared the kinetically controlled denaturation profiles of His-tagged dimeric SOD1 in the absence and presence of a known SOD1 inhibitor, diethyl dithiocarbamate (DDC), that chelates metal away from SOD1.[58] The addition of 200 μM DDC significantly destabilized SOD1 as seen from a leftward shift and increased GroEL binding amplitudes compared to those of the native protein (Figure 5A). Predictably, the raw chaperonin binding

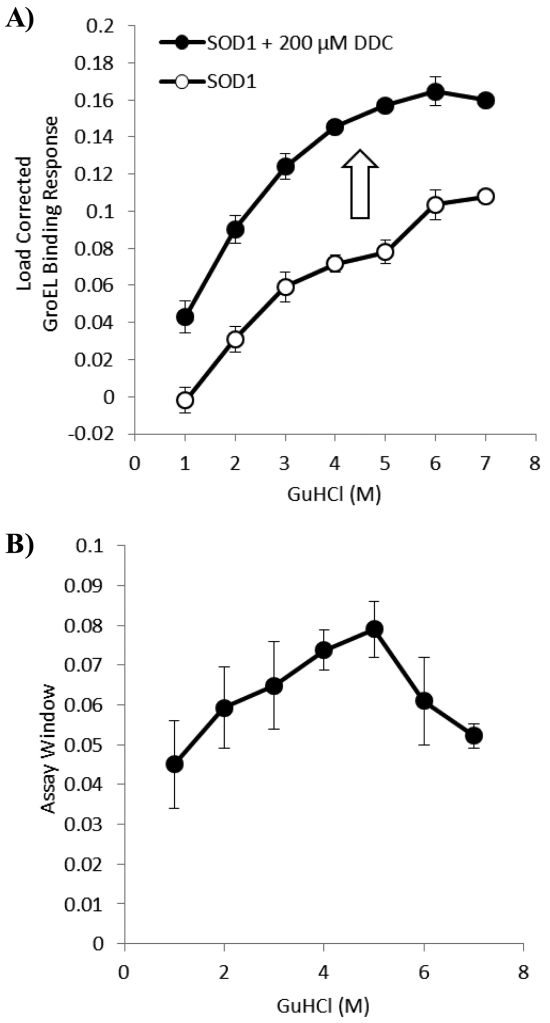

Figure 5. Assessment of the stability of SOD1 and effect of a known metal chelator DDC on SOD1 stability. (A) Kinetically controlled denaturation profiles of SOD1 in the absence (○) and presence (●) of 200 μM DDC. Experiments were performed on the Octet RED96 with three replicates for each data point. Error bars represent the standard deviation. A larger GroEL binding response was obtained at each GuHCl denaturant concentration when SOD1 was exposed to 200 μM DDC, a known inhibitor of SOD1 to destabilize the protein via the metal chelation mechanism (denoted by the arrow). (B) Differences between the wild type SOD1 and the destabilized Cu^{2+}-chelated apo-SOD1 kinetic isotherms were generated to determine an optimal assay window. In this instance, the differences showed a slight maximum in the 4−5 M GuHCl pulse range [Z' factor 0.73 at 4 M (Table S1)].

amplitude for the demetalated form was significantly higher than that of the untreated SOD1 protein, showing that one could observe destabilization due to a loss of ligand/cofactor. The difference between the chaperonin binding the metal-free versus metal-bound SOD shows a fairly even difference between the apo-SOD and the Cu^{2+}-bound form throughout the range of GuHCl pulse concentrations used (Figure 5B). This difference has a maximum at 4 M GuHCl under these experimental denaturant pulse conditions with a calculated Z' factor of 0.70 (Table S1). Because these denaturant pulse profiles were kinetically controlled, our observations agreed with previous kinetic data that showed an acceleration in unfolding for demetalated SOD compared to that of metalated protein in an acid-induced denaturation experiment.[6] Cupric

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Biochemistry XXXX, XXX, XXX−XXX

ion replenishment of wild type SOD1 pretreated with DDC showed restored stability, similar to that of wild type SOD1 (Figure S3).

Evaluating the Kinetic Stability of Marginally Stable Proteins. *Cystic Fibrosis Transmembrane Regulator Nucleotide Binding Domain (CFTR-NBD1).* Misfolding and subsequent mistrafficking of CFTR are sources of another prominent folding disease. A lack of functional CFTR caused by more than 1500 mutations identified to date is the cause of cystic fibrosis, a lethal genetic disease, most commonly found in Caucasians. CFTR contains two nucleotide binding domains (NBDs) and two transmembrane domains (TMD). One common CFTR deletion mutation ΔF508 occurs on NBD1 and adversely affects post-translational folding and transport of the protein to the cell surface.[59] In solution, the CFTR-NBD1[60] fragment is stabilized by binding of a nucleotide by ATP or GTP.[61] NBD1 has been considered as a surrogate target in the search of small molecule stabilizers for CFTR because the most prominent single-site mutation causing cystic fibrosis is located within this domain. This fragment has a low T_m, readily unfolds, and aggregates at room temperature, which explains why designing assays to search or validate stabilizers of this fragment has been problematic. In contrast to the stable automated loading of protein substrates GST, TTR, and SOD1 (see Materials and Methods and Figure S2), CFTR-NBD1 protein showed a lack of stable loading with time, most likely because of its thermally liable nature at room temperature. To stabilize this thermally liable protein fragment prior to immobilization on BLI biosensors, the intrinsic ligand ATP (applied at 1 mM, approximately 20-fold higher than its K_d[62]) was added to the loading solution. As predicted, CFTR-NBD1 exhibited more consistent load amplitudes over time when ATP was present (Figure S4A). Once the CFTR biosensor was dipped into buffer without ATP, the stabilizing effect of ATP on NBD1 was removed because ATP binding was relatively weak and ATP dissociation readily occurred in the buffer wash pulse steps. Because the nucleotide-free form is aggregation-prone, the removal of ATP from the His-SUMO-tagged CFTR-NBD1 fragment was predicted to be able to bind GroEL at room temperature. As shown in Figure 6, GroEL exhibited significant binding to the immobilized CFTR-NBD1 protein fragment on the BLI biosensor at room temperature alone, without the need for a denaturant pulse. Because the nucleotide binding to CFTR has an affinity in the micromolar range, there was little effect on the binding magnitude when CFTR-NBD1 was preincubated with GTP because this ligand was washed away from CFTR during the 1 min wash step prior to the GroEL binding step. The stability of this fragment is particularly liable because the ligand-induced stability of the CFTR fragment is also lost once GTP rapidly dissociates from the NBD1 fragment.[63] Because previous experiments have shown that GTP does not interfere with GroEL capture and release,[64] adding GTP to the GroEL buffer should show a decrease in the level of binding of GroEL to the CFTR biosensor. As predicted, a clear decrease in the GroEL binding magnitude was observed (Figure 6). This set of data agreed well with our prior observation in which a chaperonin bead-based setup was utilized, and the presence of GTP prevented the partitioning of CFTR-NBD1 to GroEL that was immobilized on sepharose beads.[64] The ligand response also supports the contention that the Ni-NTA-immobilized His-SUMO-tagged CFTR-NBD1 protein is functional in nucleotide binding. Without a denaturant, the raw biosensor traces with the automated BLI system show nearly the same GroEL binding amplitudes in the absence and presence of GTP (Figure 6B). The calculated Z' factor for these replicate runs is 0.85.

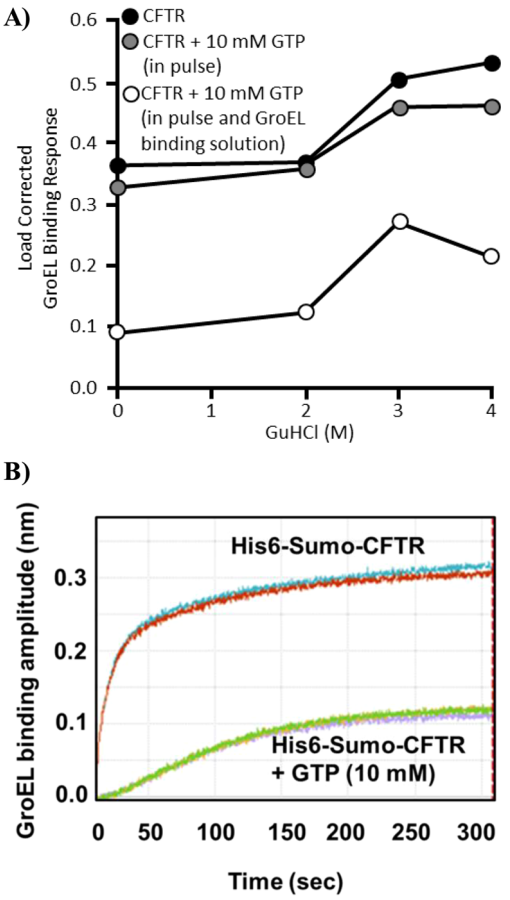

Figure 6. Kinetically controlled denaturation of His-SUMO-tagged CFTR-NBD1 with and without pretreatment with its native ligand GTP. (A) The trace highlighted by closed black circles represents a kinetically controlled denaturation isotherm of CFTR. In the line containing the filled gray circles, immobilized CFTR was exposed to 10 mM GTP followed by various concentrations of GuHCl with 10 mM GTP. Both denaturant and GTP were subsequently washed off before the sample was dipped into a GroEL solution. In contrast, in the bottom trace highlighted by the empty circles, all other steps were performed as described above except the GroEL solution also contained 10 mM GTP. (B) Raw sensorgrams at 0 M GuHCl in the absence and presence of the stabilizer GTP (10 mM) show considerable overlap of GroEL binding sensorgrams from three automated runs. The calculated Z' factor is 0.85.

Thus, it appears that a variation to the denaturant pulse method can be applied for protein fragments, because protein fragments are dynamically unstable and/or metastable (i.e., rapidly fluctuating between folded and partially folded forms). In this instance, one can simultaneously test ligands provided that they do not interfere with GroEL capture and release.[9,64] One can simply determine if GroEL binding is diminished when the immobilized protein is incubated with GroEL and the test ligand in question. In this instance, one needs to run a secondary assay with another protein system to rule out ligand interference with GroEL binding. There are two options that one could implement. First, one could use any other immobilized protein that completely unfolds during a high-denaturant pulse as a comparative test run with or without a test ligand. Second, and as in this paper, the GroEL-dependent

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Biochemistry XXXX, XXX, XXX−XXX

refolding of the unfolded green fluorescent protein was examined in the absence or presence of the test ligand stabilizer GTP. No differences in folding yields were observed, nor does GTP interfere with the intrinsic fluorescence of GFP.[14]

DNA Binding Core of Tumor Suppressor p53. Another protein domain fragment that exhibits a low T_m (i.e., significant denaturation at body temperature) and in which missense mutations result in disease is found in the DNA binding core domain of general tumor suppressor protein p53.[65] As noted in the method development section, the first step in constructing valid and reproducible kinetically controlled denaturation isotherms involves demonstrating that loading of the protein onto BLI Ni-NTA biosensor surfaces remains constant. Unlike the case with the metastable CFTR fragment, there are no easily dissociable ligands that can prevent aggregation of p53 long enough to ensure constant loading onto the biosensor tip. However, one possible method for limiting p53 aggregation to improve reproducibility of p53 loading onto the biosensor is to stabilize the p53 DNA binding domain with an osmolyte stabilizer solution in the loading well. Of the osmolytes tested, glycerol seemed to yield the most uniform loading amplitudes (Figure S3B). Once constant loading of this normally metastable protein was achieved using loading buffer containing 4 M glycerol, the multiple kinetically controlled denaturation profiles generated by the denaturant pulse showed that this protein begins to kinetically denature at lower denaturant concentrations. When glycerol was added to the denaturant pulse solution (and removed during the wash phase), the overlaid GroEL binding amplitudes were dramatically diminished, verifying that glycerol was a good kinetic stabilizer of the p53 DNA binding core domain (Figure 7A). At 1.5 M GuHCl, the calculated Z' factor was 0.27 (Table S1). The lower Z' factor value is due to the larger standard deviations for this particular protein upon generation of the denaturation isotherm in the absence of stabilizers. The implications, importance, and suggested approaches to improve the error in measurement for some metastable proteins are presented in the Discussion.

The tumor suppressor p53 DNA binding core domain has been the focus of a large number of stability studies because many cancer outcomes are linked to missense mutants of this protein that misfold.[65] Thus, the search for small molecules and peptides that stabilize this protein through specific binding mechanisms is an attractive approach to reverse misfolding and to restore normal p53 function.[66] One such molecule that binds and stabilizes the core domain (through the DNA binding surface) is a peptide that has been rationally designed from the p53 core domain−53BP2 complex interface resolved from the X-ray crystallographic structures by Fresht and colleagues.[67] They found that the binding of the CDB3 peptide stabilizes the p53 core domain, induces refolding of equilibrium-denatured p53, and restores sequence specific DNA binding to I95T, a highly destabilized p53 mutant. In addition, binding of the CDB3 peptide to wild type p53 and a mutant of p53 resulted in thermal stabilization as assessed by differential scanning calorimetry measurements.

To examine the effect of this stabilization on the denaturant pulse p53 kinetic unfolding transition, the CDB3 peptide was added at a concentration of 200 μM prior to and during the denaturant pulse phase. Although the binding affinity decreases in the presence of an increasing denaturant concentration,[67] it is predicted that the binding of the peptide to p53 on the BLI biosensor should slow the kinetics of unfolding of p53 and

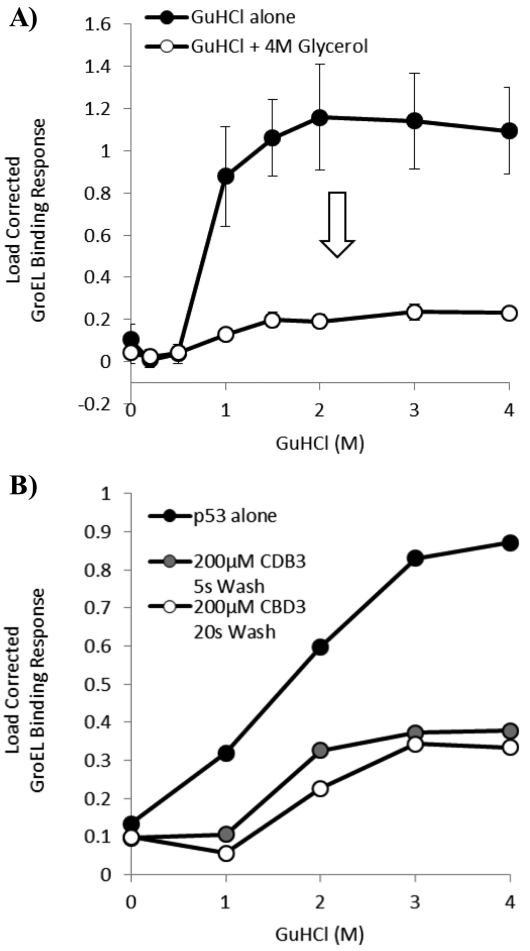

Figure 7. Effect of kinetic stabilization on p53 by an osmolyte or CDB3 on the kinetically controlled denaturation. (A) Wild type p53 was attached to BLI biosensors and subjected to a 5 min denaturation pulse. When 4 M glycerol was included in the denaturant pulse phase, the GroEL binding signal amplitude to the p53-loaded tip was significantly reduced. Glycerol was washed away prior to the performance of the GroEL binding phase. The data were load-corrected to account for slight variations in p53 loading (>4% standard deviation). In this instance, larger error bars in the sample loading that was not stabilized by osmolyte diminished the Z' factor between these two kinetic denaturation isotherms that show a maximum at 1.5 M GuHCl of 0.27 (Table S1). (B) CDB3 peptide at a final concentration of 200 μM was added both at an initial wash step after p53 was loaded onto the BLI biosensor and in the following denaturant pulse step. After the biosensor was removed from the denaturant solution and dipped into the loading buffer (to wash away denaturant and excess CDB3 peptide) for a 5 or 20 s wash step, the biosensor was then immersed in the GroEL buffer containing 0.15 μM GroEL.

significantly diminish the level of GroEL binding, consistent with the stabilization of p53 by this peptide. Here, the peptide binding experiments were conducted on BLItz, a system that is insensitive to small molecule binding interactions (10 kDa sensitivity). The weak GroEL binding response that is observed when the stabilizing peptide is present in the denaturant pulse step remains nearly the same regardless of the time of the wash pulse (no peptide present during the wash phase) (Figure 7B). The very slight differences in GroEL binding amplitudes that decrease with longer wash times may be due to some slow refolding. The notion of refolding during the wash step following the denaturant pulse is an important parameter to

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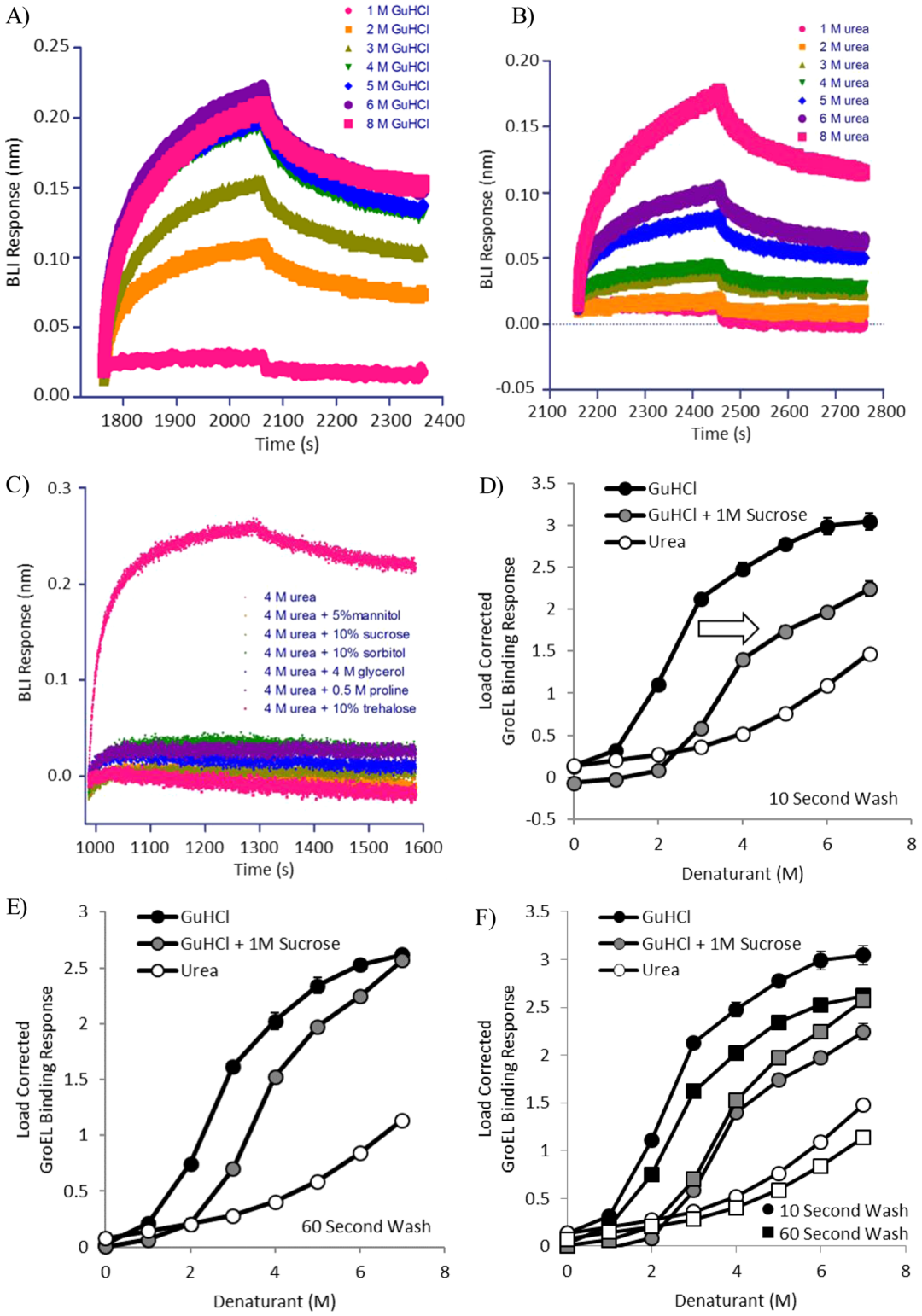

Figure 8. Generation of IgG kinetic isothermal denaturation curves and assessment of the osmolyte stabilization effect for attached IgG monoclonal (A–C) and *forté*BIO anti-GST antibody sensor tips (D–F). (A) Aligned sensorgram traces (raw data) showing GroEL association and dissociation steps after the GuHCl pulse. GroEL binding showed a GuHCl concentration-dependent response. The higher the GuHCl concentration used in a previous denaturation step, the stronger the GroEL binding response. The GroEL binding signal appeared to approach saturation at 5 M GuHCl (GuHCl was titrated from 1 to 8 M). (B) Aligned sensorgram traces (raw data) showing GroEL association and dissocation steps after the urea pulse. GroEL binding also exhibited a urea concentration-dependent response. A higher urea concentration led to higher GroEL binding signal amplitude. The GroEL binding signal continued to increase within the urea concentration range that was titrated (1–8 M). (C) Aligned sensorgram traces (raw data) showing kinetic stabilization of IgG by different osmolytes and/or excipients. (D) Comparative GroEL binding responses on progressively denatured anti-GST *forté*BIO biosensor tips where each amplitude represents three separate automated runs. The error bars of one standard deviation are so small that they are barely visible. The kinetically controlled denaturation isotherms for which an automated 10 s wash was implemented are recorded as a function of increasing GuHCl concentration (black circles), GuHCl concentration with 1 M sucrose (gray circles),

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Biochemistry XXXX, XXX, XXX–XXX

Figure 8. continued

and urea denaturant concentration (white circles). (E) The same experimental setup was followed (see panel D), the only difference being was the implementation of a 60 s wash step. (F) Comparative kinetically controlled denaturant isotherms comparing the 10 s (circles) and 60 s (squares) wash protocols. Slight differences in GroEL amplitudes indicate that some folding may occur within the lengthened wash step time span. The calculated Z' factors at 3 M GuHCl with or without sucrose were 0.88 and 0.87 for 10 and 60 s wash times, respectively (Table S1).

measure. As will be observed in the next section (IgG unfolding), any refolding effects do not appear to affect the calculated Z' factor provided that the loading of the sample protein is conducted with high precision.

More importantly, these results illustrate that CDB3 peptide binding stabilizes the p53 core domain, slowing the unfolding and resulting in a diminished chaperonin binding signal amplitude. In contrast to the weak binding of the GTP nucleotide to the CFTR-NBD1 binding domain, the CDB3 peptide binds tightly to the DNA binding p53 core domain, which slows the p53 unfolding rate, weakening chaperonin binding. Fersht and colleagues demonstrated that, at 3 M urea, the p53−CDB3 complex (K_d of 0.53 μM) has a dissociation $t_{1/2}$ of 6×10^3 s.[69] Again, these experiments illustrate the utility for using this method to identify ligand stabilizers of dynamically unstable proteins that slowly dissociate from the protein once the denaturant pulse is completed (in this instance 5 min).

Assessing the Kinetic Stability of Multiple Domain Proteins. *Generating a Kinetically Controlled Denaturation Isotherm Using Immobilized IgG.* It has been repeatedly demonstrated that partially folded proteins as large as antibodies easily bind to GroEL.[68,69] It is known that IgG shows more complex unfolding by equilibrium-monitored methods.[70] To determine a kinetically controlled denaturation isotherm for the multidomain IgG molecule, two different sets of coupled human IgG biosensor tips, one of which was demonstrated to be fully functional (*forté*BIO anti-GST antibody tips), were simultaneously exposed to increasing concentrations of either urea or GuHCl during a 5 min denaturant pulse. The tips were then removed from these denaturant conditions and dipped into a buffer solution (1 min) to remove residual denaturant. The washed tips were then dipped into a 1 μM GroEL solution, and binding of GroEL to the partially folded IgG population produced a signal correlating with the amount of partially folded protein present on the tip surface at the time of assay. The raw data for generating the kinetically controlled denaturation isotherms for IgG show a progressive rise in the GroEL binding signal amplitude with an increasing denaturant concentration. Predictably, the individual GroEL binding signals following the urea-induced unfolding lagged behind the stronger GuHCl kinetic denaturant pulse profile (Figure 8A,B).

To demonstrate that one can rapidly identify potential solution stabilizers that are predicted to slow IgG unfolding, a series of known antibody excipient stabilizers such as polyols (mannitol, sucrose, glycerol, and trehalose) or amino acid osmolytes (proline) were then mixed with 4 M urea (final concentration) using the multiwell Octet RED96 system, and the denaturant pulse was again implemented. In all instances, the level of binding of GroEL to the tip following a brief buffer wash was significantly reduced (Figure 8C) for the 5 min denaturant pulse, indicating that, as predicted, the excipient/stabilizer conditions slowed the unfolding reaction of the attached IgG. Because the stabilizing excipients were also washed and diluted away with the buffer wash and the GroEL incubation steps [wash and binding volumes of 200 μL (see steps 2 and 3 of the scheme in Figure 1)], interference of the osmolyte with GroEL binding would not be possible.

Using commercially supplied immobilized anti-GST antibody BLI biosensors, complete kinetically controlled denaturation isotherms were generated with three separate biosensor tips per data point in the presence of increasing GuHCl and urea concentrations. The GroEL binding amplitude differences between the GuHCl and urea denaturation isotherms are due to the denaturant strength differences of GuHCl and urea. The addition of a protein-stabilizing osmolyte such as 1 M sucrose to the GuHCl denaturant pulse solutions diminishes the GroEL binding amplitudes (Figure 8D,E).

Because the disulfide bonds of the immobilized antibodies were not reduced, the effect of varying the wash times on potential refolding (reflected as weaker GroEL binding) was examined with the commercial *forté*BIO anti-GST antibody biosensors. Initial experiments showed that the immobilized antibody does not completely refold to its original native conformation even after the 1 min wash at low denaturant concentrations (Figure 8A,B). To indirectly determine the extent of refolding that may proceed during the wash step phase, the kinetically controlled denaturation isotherms generated from GroEL binding were compared between assays programmed with 10 or 60 s wash steps on the automated Octet RED96 BLI system (Figure 8D−F). These experiments show that there is most likely some refolding that occurs for the immobilized IgG systems because the GroEL binding amplitudes that define the denaturant isotherms with a 60 s wash steps are slightly smaller than those generated when a 10 s wash step was implemented (Figure 8F). The maximal Z' factor between the IgG biosensor alone and one that was partially stabilized by 1 M sucrose was calculated to be around 0.88 or 0.87 at 3 M GuHCl with a 10 or 60 s wash time, respectively (Table S1). Interestingly, the Z' factor between GuHCl alone and GuHCl with 1 M sucrose for the lower concentrations (1 M GuHCl) was 0.73 for the 10 s wash and dropped to −0.19 when the 60 s wash was programmed in the run (Table S1). This indicates that the wash times influence the Z' factors upon examination of the effects of stabilizers on the immobilized target proteins. The error bars for the triplicate runs per GroEL binding data point (at 10 and 60 s wash steps included after the denaturant pulse) are smaller than the symbols used to plot the isotherms (Figure 8D−F).

Comparison of vWF Kinetically Controlled Denaturation Profile to Equilibrium Denaturation. von Willebrand disease is a bleeding disorder affecting roughly 1% of the world population. This hereditary disease is caused by a deficiency or mutation in the von Willebrand factor (vWF), a plasma glycoprotein multimer that initiates platelet adhesion at sites of vascular injury. Under high shear stress, vWF unravels and binds to platelets and collagen to create a plug to stop bleeding. The recombinant multimeric vWF has three A domains connected by flexible linkers. The A3 domain binds to collagen exposed during vascular injury; the A2 domain contains a proteolytic site that helps to regulate clot size, and the A1 domain binds to platelets via the GP1Bα receptor. We assessed

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Biochemistry XXXX, XXX, XXX−XXX

the kinetic stability of the WT vWF triple A domain using chaperonin BLI denaturation pulse procedures. In this instance, the A3 domain contains a His tag that can be attached to the Ni-NTA biosensor surface and mimics the attachment that naturally occurs when the triple A domain protein binds collagen. This same attachment scheme was used to attach the His-tagged triple A domain onto Cu^{2+}-coated surfaces for rheodynamic analysis of platelet adherence and pause time measurements.[24] The GroEL binding signal, plotted as a function of the increasing denaturant pulse concentration, increased with an increasing denaturant concentration and resulted in a clear kinetically controlled denaturation isotherm (Figure 9A). Via comparison of the kinetically controlled denaturant profile with the equilibrium denaturation profile, the equilibrium profiles show transitions associated with the A1 and A2 domains were not readily observed by the chaperonin BLI detection platform after a 10 min denaturant pulse and 10 s wash. Only at higher denaturant concentrations (~5 M urea) do the GroEL binding amplitudes approach the equilibrium denaturation profiles that are attributed to domain A3 unfolding (Figure 9B).[32] As the denaturation pulse times increase, the kinetically controlled denaturation isotherm derived from the GroEL binding amplitude changes shifts toward the circular dichroism (CD)-derived equilibrium isotherms.[32]

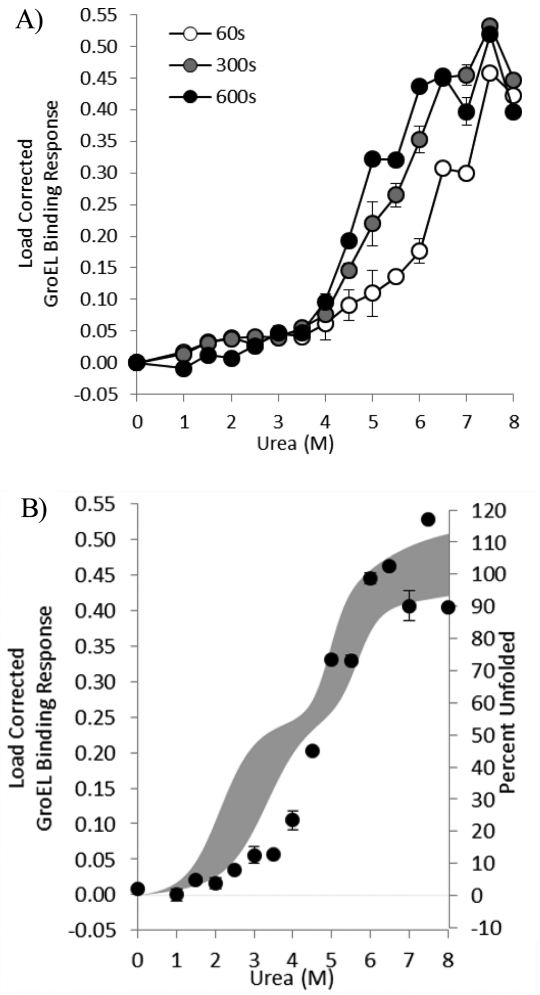

Figure 9. Kinetic stability isotherm of von Willebrand factor triple A domain generated with the BLI denaturant pulse assay. (A) Time-dependent left shift of the load-corrected GroEL binding response as a function of denaturant concentration. The automated kinetically controlled denaturation isotherm consisted of two separate runs with three runs per data point and error bars of one standard deviation. To record points every 0.5 M urea, two separate automated runs were conducted with eight biosensors followed by regeneration. Increasing the denaturant pulse time from 1 to 10 min results in a leftward shift in the denaturation isotherms. (B) An overlay of the kinetically controlled denaturation profile generated under a 10 min kinetic denaturant pulse (●) compared with the previously generated equilibrium denaturation profile with a 95% confidence interval (gray shaded region) shows a better fit at higher denaturant concentrations.

Tetanus Neurotoxin (TeNT) Kinetic Denaturant Pulse. TeNT is a bacterial protein toxin produced by *Clostridium tetani* that induces spastic paralysis in mammals following receptor-mediated endocytosis and subsequent escape from endosomes. To accomplish this end, the middle domain of the toxin undergoes an acid-induced refolding and membrane insertion event to translocate the enzymatically active portion of the toxin into the cell cytosol. The intrinsic acid-triggered partial unfolding of the toxin at the membrane interface is a crucial pretransition necessary for membrane insertion. Predictably, this protein aggregates in solution when it is subjected to denaturing conditions, and it has been difficult to produce reproducible cooperative transitions to evaluate the thermodynamic parameters of this protein during global unfolding. In addition, this protein is somewhat metastable even at low temperatures because it begins to aggregate at physiological temperature (37 °C), further complicating one's ability to study the mechanisms of membrane insertion. Thus, it is of interest to determine if this aggregation-prone protein can generate kinetically controlled denaturation transitions while immobilized on a BLI biosensor.

To evaluate the kinetic stability of the soluble TeNT, the protein was immobilized onto Ni-NTA biosensors through the N-terminal His tag. In addition to the standard denaturant pulse, the kinetically controlled denaturation assay was conducted in the presence of GT1b to assess if this natural TeNT ligand would stabilize the toxin. GT1b is a ganglioside, a glycolipid enriched in neuronal membranes, which binds two distinct sites on the receptor binding domain of TeNT that recognizes either the conserved carbohydrate backbone of gangliosides or the terminally linked sialic acid residues.[71] The concentration used for the experiments was above the K_ds (nanomolar range) for the receptor binding sites but below the critical micelle concentration (CMC at ~10 μM) for the ganglioside.[72] Previous observations with the immobilized anthrax toxin protective antigen prepore indicate that the large conformational transitions to the membrane insertion competent pore generated by physiological temperatures (37 °C) coupled with 1 M urea incubations[68] could be easily observed using BLI technologies.[31] Therefore, it is conceivable that the soluble TeNT toxin may undergo relevant preinsertion transitions from soluble to membrane-inserted species under low-denaturant conditions. Understandably, this kind of transition is predicted to expose hydrophobic patches that can bind the promiscuous chaperonin, GroEL.

In Figure 10, the kinetic denaturant pulse isotherms with and without GT1b overlapped and showed a large denaturation event (increased level of GroEL binding) occurring at urea pulse concentrations of 3 and 4 M. No recognizable transitions occurred under the low-denaturant pulse conditions, although there was a small noticeable rise in progressive chaperonin

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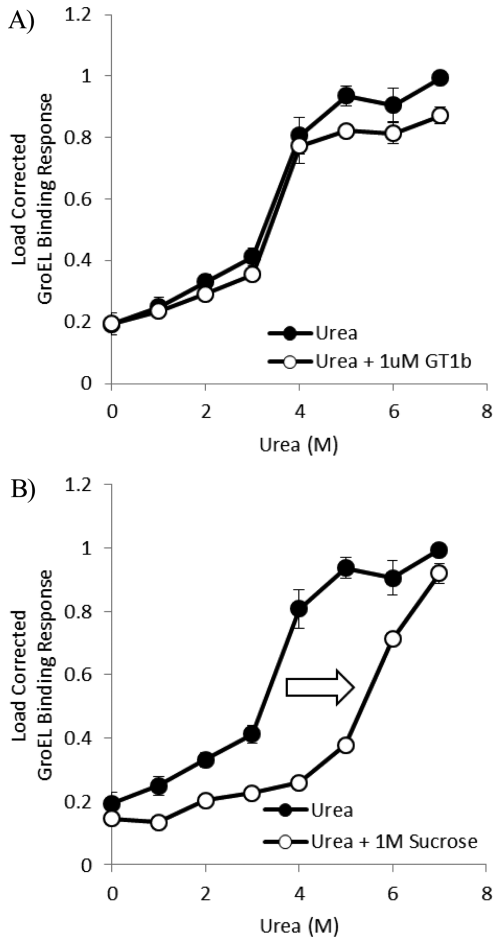

Figure 10. *C. tetani* tetanus neurotoxin (TeNT) denaturation profile. His-tagged TeNT (through the N-terminal light chain catalytic domain) was attached onto Ni-NTA tips at a concentration of 50 nM for 5 min (1 nm loading deflection), and a denaturant pulse procedure was performed. Each GroEL binding amplitude data point represents three separate automated Octet RED96 runs, and the errors bars (barely visible behind the point) represent one standard deviation following a 10 s wash step. (A) The kinetic isotherms were generated in the absence (●) and presence (○) of 1 μM GT1b. Control sensorgrams (no GroEL) exhibiting no contributions from GT1b dissociation were observed over the time course of the GroEL binding phase. A small amount of GroEL binding to the immobilized TeNT was detected under nondenaturing conditions (0 M urea). (B) The inclusion of the folding osmolyte 1 M sucrose in the denaturant pulse step delays the onset of the increased level of GroEL binding. The Z' factor at 5 M urea is 0.77 (Table S1).

binding from 0 to 3 M urea. Additionally and perhaps importantly, there was a clear binding of GroEL to the immobilized TeNT under the zero denaturant condition, indicating that this protein is inherently metastable. The transition that was observed was evidenced by the signal amplitude increasing from roughly 30 to 70% of the maximal signal amplitude. The kinetic isotherms of the attached toxin show clear reproducible transitions in the absence and presence of GT1b. Although a rightward shift was expected if a strong stabilizer was present, this was not readily observed at the concentration of GT1b tested. Instead, the addition of the presumed stabilizer led to a consistently reproducible (three runs overlaid) yet small decrease in binding amplitude. This decline in GroEL binding amplitude was not as dramatic as in the case with p53 or CFTR-NBD1. This indicated the presence of GT1b at this concentration did not confer a substantial delay or decrease in the GroEL binding amplitude during the denaturant pulse of TeNT immobilized through the His-tagged catalytic domain.

A more substantial stabilization occurred when the denaturant pulse steps included 1 M sucrose as an osmolyte stabilizer. A comparison of the kinetically controlled denaturation isotherms indicates that the osmolyte delays the unfolding of the TeNT toxin leading to a lower level of GroEL binding until higher denaturant concentrations are present in the denaturant pulse. The Z' factors between urea alone and urea with 1 M sucrose isotherms reach values of 0.60 and 0.77 in 3 and 4 M urea denaturant pulse solutions, respectively (Table S1).

DISCUSSION

Eisenstein, Yoshita, and others pioneered the use of the nucleotide-free GroEL chaperonin as a system for detecting the folding status of attached protein substrates with surface plasmon resonance (SPR). They monitored binding of chaperonin to partially folded protein substrates attached on immobilized surfaces to eventually follow the kinetics of the ATP/GroES-induced release of the GroEL chaperonin from the surface.[73,74] If the dynamic folding equilibria of a protein result in substantial populations of metastable folded states, this particular immobilization approach can also be used to examine a possible ligand- or solution-induced stabilization shift away from the metastable population.[14]

This work describes the successful development of a broad-based method for assessing the protein kinetic stability of inherently stable, metastable, and aggregation-prone proteins using a chemical denaturation pulse method coupled with a BLI biosensor surface-based chaperonin detection system. The automated platform facilitates the acquisition of highly reproducible chaperonin-dependent detection of partially folded protein populations within physiological temperature ranges. This platform can rapidly evaluate the protein stability of minute protein samples immobilized on biosensor surfaces. Detection of partially destabilized proteins is accomplished with a high-affinity nucleotide-free chaperonin that promiscuously binds to any protein with exposed hydrophobic patches. Most importantly, this platform technology can identify stabilizers of the native fold that diminishes the level of formation of hydrophobic partially unfolded populations prior to aggregation. The assay principle is simple as it is based on and is an extension of previous solution-based methods in which a reversal of misfolded/partially folded states by global ligand stabilizers results in a diminished level of chaperonin binding.[19,21] The method outlined herein accelerates the detection process because the level of binding of chaperonin to the target protein immobilized on the BLI biosensor predictably decreases in the presence of protein stabilizers. This is a particularly useful approach for identifying and validating correctors/stabilizers of difficult, aggregation-prone misfolding disease proteins.

Previous work from this laboratory utilized a reverse approach in which the chaperonin is directly coupled to the biosensor to detect and capture transient preaggregate species present within concentrated therapeutic protein solutions.[75] These transient preaggregates exist at low concentrations prior to the formation of larger dimeric or other subsequently formed aggregate populations and are thought to represent the "seeds of destruction" that degrade therapeutic protein formulations.

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Biochemistry XXXX, XXX, XXX−XXX

Currently, protein formulation integrity is frequently evaluated by particle size-dependent methods in which small or large aggregate populations are detected after they form. One drawback of using the approach in which GroEL is immobilized on BLI biosensors is when significant protein aggregation rapidly forms or is present before the chaperonin biosensor is dipped into the protein solution. In particular, extensive aggregation prior to probing the solution with the biosensor diminishes the biosensor signal amplitude and correlates with decreases in the extent of chaperonin biosensor capture of large aggregates. The simple fix for avoiding large scale solution-based aggregation interference is to immobilize the target protein to the BLI biosensor surfaces and to evaluate the presence of partially folded populations with chaperonin binding from solution with or without the denaturant pulse procedure.

The BLI chaperonin denaturant pulse system uses rationally designed affinity tags that immobilize and orient the target protein or target protein domain away from the biosensor surface to augment chaperonin binding, while precision loading avoids protein aggregation. Properly loaded proteins that are homogeneous and specifically oriented present adequate populations of immobilized proteins on the BLI biosensor tip surface that minimize or abolish intrasurface protein aggregation prior to GroEL binding. In this work, the robust, broad-based applicability of the BLI chaperonin detection platform was tested with a host of monomeric, dimeric, multidomain, and metastable proteins rather than focusing on just one class of target proteins.

The most common linkage for immobilization of the target proteins used in this approach is through engineered His tags that can be immobilized onto Ni-NTA biosensor tips. The denaturant pulse approach can be used to perturb the folded protein without diminishing Ni chelate binding affinities. A simple PubMed search indicates that this is a common methodology for capturing and sometimes refolding proteins on Ni-NTA surfaces from inclusion bodies (119 hits since 1996). For the BLI approach, this attachment is also advantageous because one can regenerate the biosensor tip nickel surface and automate the regeneration process to obtain precise replicates with highly reproducible signal responses. The denaturant pulse chaperonin BLI assay is routinely accomplished with small sample sizes (often micromole to nanomole quantities), provided that the amount of target protein loaded onto the 600 μm tip remains constant. Importantly, very little nonspecific binding of our highly purified chaperonin preparations is observed with the Ni-NTA biosensors. To avoid nonspecific binding, optimal chaperonin purity and tetradecameric stability are absolutely necessary for this assay. For instance, we observed that impure or structurally defective chaperonin samples containing protein impurities (bound peptides) or monomeric GroEL subunits showed significant nonspecific background binding for a wide variety of BLI biosensor surfaces. It is also important to use target proteins that are also highly pure. The most common method for ensuring reliable loading of the target protein is to include common protein stabilization conditions (e.g., sucrose, glycerol or other antiaggregation osmolytes). Optimized sample loading also decreases the relatively low level of nonspecific binding of GroEL (~0.1 nm) to undetectable signal levels. When the target protein loading is maintained at the lower portion of the linear response of the biosensor signal (Figure S1), considering the size of the GroEL binding site (~45 Å), the assumption that GroEL will bind a single immobilized polypeptide for partially folded protein domains is valid. The GroEL signal can be corrected for slight differences in loading of the target protein (load-corrected GroEL binding response).

To obtain reproducible data using the automated platforms, the timing of the loading, denaturant pulse, wash times to replace buffers and immersion in the GroEL binding buffers are precisely controlled. For example, the nanometer amplitude shifts that result from protein loading and GroEL binding reaction are reliably reproduced with timed data collection. Within the linear response region, the GroEL binding amplitudes are directly proportional to protein loading and are further corrected by protein loading (i.e., GroEL nanometer amplitude/protein load nanometer amplitude). Additionally, binding the high-affinity GroEL chaperonin to partially folded populations arrests refolding provided that the K_d of GroEL is in the nanomolar range or below (reviewed by Fenton and Horwich[18]) . As the denaturant concentration in the denaturant pulse reaction increases, the highest GroEL binding signals occur when the entire population of the attached target protein is completely denatured. Because this is a pulsatile denaturant exposure rather than an equilibrium process, this method can be used to assess only the kinetic stability of proteins.

The success of this approach depends on a number of crucial parameters that result in reproducible kinetically controlled denaturation isotherms. One element that must be maintained, particularly as the biosensor surfaces are regenerated multiple times, is that the loading of the target protein (reported as nanometer shift amplitudes) remains constant. The channel to channel reproducibility data presented were very good [signal variation of an ~3% standard deviation (SD) over nine separate regeneration cycles (Figure S2)] for stable proteins. Instances of poor reproducibility were observed only if the target protein sample degraded (e.g., aggregated) over time in the sample loading wells. There are multiple solutions to this problem. In some instances (protein is only stable for a short period of time and cannot be stabilized), fresh biosensor tips along with freshly prepared protein samples were used, forgoing regeneration. In a number of situations, the regeneration process itself may fail because the protein is not completely removed from the biosensor surface. In this work, the MalZ and TeNT proteins were not regenerated but rather were loaded onto freshly hydrated biosensor tips. For these two proteins, the standard deviation for each data point is very reasonable and had acceptable Z' factor values. In most instances, the standard deviations on the data points that make up the kinetically controlled denaturant isotherms are smaller than the symbols used to depict the data point.

Another viable alternative relies on stabilizing the target protein in the loading wells by including polyol osmolytes (glycerol or sucrose) in those samples to avoid or slow aggregation and/or misfolding [p53 loading (Figure S4B)]. It is noteworthy that the use of folding osmolyte solutions is a common commercial stabilizer for preventing misfolding and aggregation during long-term storage and has often been the additive of choice for preventing misfolding of metastable proteins (both soluble and membrane-bound) involved in various folding diseases.[76−80] The ability to rapidly detect the rescue of incorrect folding and stabilization of metastable proteins by folding osmolytes with the chaperonin BLI denaturant pulse system is a useful prerequisite in identifying and evaluating mutants for disease therapeutic development. The addition of osmolyte stabilizers into the denaturant pulse

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Biochemistry XXXX, XXX, XXX−XXX

solutions apparently slows the unfolding rates. This strategy allows us to establish a lower stabilization baseline for comparison with the unstabilized denaturation isotherm where optimization of time steps for denaturant and buffer washing can be evaluated. The demonstration that osmolytes can rescue misfolding is an important primary assay in which positive reversal of misfolding of the target protein makes it a candidate for reversing misfolding through small molecule stabilizer approaches. Hence, the ability to evaluate metastable proteins and even protein fragments (e.g., mimicking metastable folds that appear during protein translation such as the CFTR-NBD1 fragment) with this method while avoiding aggregation significantly broadens the applicability of this method.

There are some limits to the denaturant pulse BLI chaperonin biosensor method. For instance, the evaluation of the entire global stability of multidomain proteins is diminished because the orientation of particular domains at the biosensor surface restricts the interaction of GroEL with the unfolding protein. For example, in this work, the p53 DNA binding domain was immobilized to the BLI biosensor surface through a His-tagged MBP. While this orientation results in preferred interactions of the exposed p53 core DNA binding domain with the chaperonin, the stability of the attached MBP domain is refractory to GroEL binding (i.e., no effect on p53 stability when the MBP is stabilized by maltose binding). The immobilization of proteins with three domains such as large antibodies, the von Willebrand factor, and the tetani neurotoxin allows us to evaluate only those domains that are oriented in a way that allows them to be accessible to GroEL binding. In these examples, the GroEL binding denaturant pulse approach yielded single kinetic transitions. Interestingly and unexpectedly, however, the multidomain vWF A1−A2−A3 protein that is attached to the Ni-NTA surface through a His tag on the A3 domain shows a kinetic transition profile that is more complex, complete with a transition that correlates with the unfolding of the A3 domain that is attached to the biosensor surface (Figure 9). It is possible that flexible linkers among all three of these integrin-like domains may allow for interactions of domain A1 with domain A3 even while the protein is immobilized. Transitions that associate with A1 and A2 domain unfolding do not dominate the initial kinetic isotherms even during longer denaturant pulse times. Compared with the equilibrium denaturation isotherm, the small amount of chaperonin binding at low denaturant concentrations may result from either slow unfolding kinetics (perhaps due to domain−domain interactions within the A1−A2−A3 linkages) or fast refolding of the extended A1 domain. This can be determined by extending the denaturant pulse times beyond those used in the experiments presented here (Figure 9) and shortening the wash times between the denaturant pulse and GroEL binding steps. If the domains that interact with GroEL do rapidly refold during the short wash steps (10−30 s), one may not be able to observe any progressive increases in the magnitudes of GroEL binding signals in the denaturant pulse step at lower denaturant concentrations.

Provided that the immobilized target protein partially folded domains are accessible to the GroEL binding site, this type of analysis (i.e., varying denaturation times) is useful for evaluating potential kinetic stability changes for mutant proteins. Missense or single-amino acid changes that globally destabilize the target protein and/or a particular domain will result in a higher initial level of GroEL binding following exposure to lower denaturant pulse concentrations. In fact, the two metastable proteins, the CFTR-NBD1 fragment and p53 DNA binding domain, show reproducible GroEL binding in the absence of a denaturant pulse at 25 °C (Figures 6 and 7 at zero denaturant). As expected, the level of GroEL binding under zero-denaturant conditions increases if the BLI probe solution temperatures are elevated to 37 °C (data not shown).

The kinetic transition of the aggregation-prone TeNT is a perfect example in which this method is useful in evaluating toxin stabilization conditions, potentially leading to the development and validation of small molecules that prevent toxin-dependent transitions (novel anti-toxins) prior to cell insertion and toxin translocation. Curiously, with the TeNT toxin, a small amount of specific chaperonin binding to the attached toxin is observed prior to implementation of the denaturant pulse assay in the absence and presence of low concentrations of GT1b (Figure 10). Although osmolyte addition lowers the GroEL binding amplitude throughout the denaturant concentration ranges, the osmolyte does not impart conformational memory (i.e., longer-term stabilization) on the metastable form of the TeNT toxin because GroEL binding remains virtually unchanged after incubation in the polyol stabilizer for 5 min. Because this toxin slowly aggregates at room temperature without activation, the chaperonin apparently binds to a hydrophobic population of the protein even before any extensive denaturation occurs (Figure 10). Previous differential scanning calorimetry studies indicate that there are two distinct transitions that can be observed during thermal denaturation, but these transitions were not reversible.[81]

In solution, the TeNT toxin will slowly partition onto the chaperonin under physiological-temperature conditions, and distinct chaperonin (800 kDa)−TeNT toxin (150 kDa) complexes can be readily observed using electron microscopy (image data will be presented in a later publication). The TeNT toxin is immobilized onto Ni-NTA tips through the N-terminal light chain domain (active protease) that orients the translocation domain (heavy chain) and the GT1b receptor binding domain toward interacting with GroEL in solution. At this time, it is impossible to determine which of these latter two domains GroEL binds. Because the interaction of partially folded or hydrophobic proteins with GroEL is promiscuous, it is possible that both of these domains may be interacting with GroEL.

The versatility of this platform technology will help researchers determine how ligand interactions influence the kinetic stability of target proteins while avoiding deleterious aggregation side reactions. This approach is of considerable interest to the protein stability field. For example, the presence of tight binding ligands will both delay and even weaken the chaperonin binding after the denaturant pulse phase (Figure 1). Including high concentrations of weak binding ligands in the denaturant pulse phase appears to slow the general protein unfolding reaction as evidenced by the weakened chaperonin binding or shifted kinetic isotherms. This effect is readily apparent when folding osmolytes are included in the denaturant pulse steps (Figures 2, 7, 8, and 10). If the chaperonin binds to the partially folded mutant protein in the absence of a denaturant pulse (e.g., CFTR-NBD1 with a low T_m), one can directly incubate protein with stabilizer candidates (before and during the GroEL binding step) and evaluate the chaperonin binding directly, provided that the added ligand does not interfere with chaperonin binding interaction (Figure 6).

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Biochemistry XXXX, XXX, XXX−XXX

Future developments for this methodology will expand ligand discovery platforms. In particular, the sensitivity of the automated Octet BLI systems will allow one to perform ligand titrations prior to the stabilization assessment by the denaturant pulse. This approach can dramatically accelerate the throughput of protein−ligand binding/stabilization evaluations. With the current available Octet system, 16 biosensors can operate simultaneously to process 384 samples with kinetic characterization in a few hours. A commonly evaluated factor in high-throughput screening is the Z' factor, a dimensionless parameter that reflects the assay window and data variation.[82] The measured Z' factors obtained upon comparing the maximal differences between denaturation isotherms generated in the absence and presence of osmolyte stabilizers for MalZ, p53, CFTR, immobilized IgG, and TeNT [ranging from 0.4 to 0.88 (Table S1)] provide a working baseline for optimizing high-throughput protein stability screens. Because one can adjust the loading of target protein, the denaturant pulse times, and the wash times (to minimize refolding), this signal separation between a more stabilized protein (osmolyte stabilizer baseline, e.g., Figures 2, 7A, 8D−F, and 10) and the maximal chaperonin binding signal amplitude can be further optimized.

In practice, the chaperonin detection of dynamic partially folded states that are immobilized using label-free surface approaches can also be applicable using an SPR-based approach. However, SPR is a microfluidic flow-based system with most common throughputs of ∼20 complete reactions (titration analysis) per day. In contrast to the SPR system, BLI does not involve microfluidics, and the sensor surfaces are inexpensive compared with SPR. Employing dip and read solution changes rather than relying on microfluidic approaches greatly simplifies the experimental setup, particularly in the precision of changing denaturant pulse times, changing wash solutions, and obtaining reproducible signals following multiple regeneration procedures or when replicating precise loading conditions on separate biosensors. The Octet RED384 system BLI throughput can accommodate 16 protein-immobilized biosensors simultaneously in 384-well plates while monitoring interactions in real time and extracting kinetic parameters, such as k_{on}, k_{off}, and K_d. Newer BLI instruments such as the Octet HTX series can sample up to 32, 48, or 96 biosensor channels with one reading. In many instances, the BLI and SPR results are nearly identical. However, there have been numerous instances in which denatured proteins interact with the activated carboxymethyldextran SPR surfaces particularly during pH-dependent denaturation.[83] In addition, the integrity and function of the microfluidic flow system channels of the SPR instrumentation are compromised by protein aggregation within the microfluidic chambers. Unlike the BLI system, this particular flaw in the SPR system interferes with the evaluation of the protein loading reproducibility. Also, unlike the SPR system, BLI biosensors are useful for probing the contents of whole cell extracts. Furthermore, it is much easier to recover, evaluate, and actually visualize macromolecular complexes that form on the BLI surfaces if one can reverse the attachment chemistries. For example, large complexes, as well as GroEL−protein complexes, can be readily observed via electron microscopy by reversing the attachment on the biosensor surface.[31,75]

The approach outlined in this work is amenable to robotic integration and high-throughput screening. Using the newer state-of-the-art robotic 96-pin biosensor Octet HTX systems with the described 2 min chaperonin binding window (enough time for single-point measurement in an assay window with a previously determined optimal Z' factor vs osmolyte stabilizers) can allow one to process between 1500 and 3000 samples over an 8 h time period. If larger amounts of protein are available along with larger ligand libraries, it is useful to implement aggregation-based prescreens to pare down *in silico* candidates prior to performing actual titration and stabilization assessments. It is important to note that small molecule prevention of large scale aggregation may not always imply that the native fold has been stabilized.[84,85] Once a select set of test compounds that prevent large scale aggregation have been identified, the hit compounds can be evaluated to determine direct binding and stabilization of the target protein by using single-platform denaturant pulse chaperonin BLI biosensors. This type of analysis could sort these compounds into different categories based on their binding affinity and kinetics, such as strong binders and weak binders (slow k_{on} and k_{off} vs fast k_{on} and k_{off}). Subsequently, the denaturant pulse conditions (the strength of the denaturant, time of denaturant pulse, or including the candidate compound in GroEL solution) and wash times could be varied to classify strong and weak kinetic stabilizers. Lower-affinity ligands could be further developed through rational chemical modification approaches to develop stronger ligand interactions, particularly if the protein binding site is known. Performing small molecule titrations followed by protein stabilization assessments on the same biosensor surface will significantly accelerate small molecule drug discovery pipelines.

Finally, as the scope of screening procedures has been expanded to examine large chemical libraries, there have been numerous instances in which ligand binding results in a destabilization of the target protein rather than stabilization.[86] This was easily shown to be the case in our current work when the superoxide dismutase dimer was destabilized after Cu^{2+} was removed from the protein (Figure 5A). Using the target protein titration and chaperonin-detected stabilization approaches with this single platform can help determine if ligand binding events lead to protein stabilization or destabilization under physiological conditions. The latter phenomenon will be valuable for designing specific drugs that target metastable oncogenic proteins to destabilize target proteins, which may in turn accelerate clearance rates *in vivo*.

It is of interest to note that this method may be used to examine the kinetic stability of proteins that are very thermally stable. For example, the kinetic stability of small protein samples can be examined in the presence of increasingly stronger denaturants (urea to GuHCl to GuSCN) or tight binding ligands (e.g., increased protein stability due to binding of a reduced heme in some cytochrome proteins).[87] The differences in denaturant strength can be useful for generating reasonable denaturation isotherms through a denaturant concentration range. With the IgG denaturation profiles, it is clear that GuHCl was much more effective at denaturing IgG compared with the urea-generated profile. In the latter case, the unfolding was incomplete within the denaturant pulse time ranges even at high urea concentrations. For extremely stable proteins, one has the unique ability to transfer the biosensor with the attached protein from the BLI instrumentation to incubate the biosensor tips separately in solutions for long-term denaturation and reevaluating the chaperonin binding detection at later time intervals provided that solution evaporation is minimized and sample dissociation from the biosensor is negligible. This latter approach is relevant provided that the

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Biochemistry XXXX, XXX, XXX−XXX

linkage of the protein to the biosensor surface remains stable or involves covalent attachment.[31]

In this work, we have primarily focused on examining the GroEL binding amplitudes at a specific time point to generate the kinetically controlled denaturation isotherms. Because of the complexity of unfolding at various denaturant concentrations, coupled with the heterogeneity in protein folding intermediates that are observed to bind to GroEL,[88] the kinetics of binding of GroEL to and dissociation of GroEL from the species generated at lower GuHCl concentrations (smaller amplitudes) appears to be different from the kinetics of binding of GroEL to the species that was more substantially denatured (larger end point amplitudes). This may indicate that the species that are being recognized by GroEL after the denaturant is washed away may be intrinsically different at low and high denaturant pulse exposures. Thus, there is the real possibility that the initial species generated after low-denaturant concentration pulses may certainly be exposing slightly different (i.e., smaller) hydrophobic surfaces compared with those that are generated after higher-denaturant concentration pulses. Indeed, preliminary evaluation of the binding and dissociation kinetics of binding of GroEL (GroEL binding is not rate-limiting) for the multidomain protein vWF shows that the kinetic traces have to be fit to different kinetic models when the protein is exposed to low versus high denaturant concentrations. This latter situation may be more common for multidomain proteins that exhibit non-two-state global unfolding behavior. It is clear, however; that this method should be able to uncover kinetic stability differences (in the form of different kinetically controlled denaturation isotherms) of mutant proteins compared to their wild type versions, particularly those that result in misfolding diseases.

In summary, the chaperonin-based BLI denaturant pulse procedure is a highly versatile platform that can be used to readily assess and validate ligand stabilization or destabilization for stable monomers, small oligomers, and aggregation-prone metastable proteins. Ultimately, this approach has the capability of identifying compounds that can reverse or even enhance partial folding or misfolding under near physiological conditions. It is useful to evaluate the efficacy of small molecule stabilizers under physiological conditions, especially for missense protein folding mutants because one may be able to avoid excessive interactions with or partitioning onto chaperone proteins. Alternatively, an increased number of chaperone interactions with mutants that bind specific small molecule destabilizers may result in rapid clearance. In both instances, discovering and validating specific small molecule interactors with target proteins may enhance our ability to modulate the lifetime of these folded mutants *in vivo*, which, in turn, may have profound effects on folding disease outcomes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00293.

Additional data highlighting the reproducibility of the loading procedures and some additional data that illustrate the functional titration and reversibility of stabilization of SOD immobilized on biosensor tips using the BLItz system (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BLI, biolayer interferometry; CDB3, binding loop of p53 binding protein (NH_2-REDEDEIEW-COOH); CFTR-NBD1, cystic fibrosis transmembrane regulator nucleotide binding domain 1; EA, ethacrynic acid; MalZ, maltodextrin glucosidase; GuHCl, guanidine hydrochloride; GST, glutathione *S*-transferase; GSH, glutathione; GT1b, trisialoganglioside-GT1b; SOD1, superoxide dismutase; TeNT, tetanus neurotoxin; TTR, transthyretin; vWF, von Willebrand factor.

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