BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Quantifying the Kinetic Stability of Hyperstable Proteins via Time-Dependent SDS Trapping

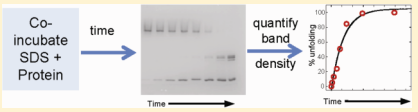
Ke Xia,[†] Songjie Zhang,[†] Brendan Bathrick,[†] Shuangqi Liu,[†] Yeidaliz Garcia,[‡] and Wilfredo Colón*[,†]

[†]Department of Chemistry and Chemical Biology, and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States

[‡]Industrial Biotechnology Program, University of Puerto Rico at Mayagüez, Mayagüez, Puerto Rico 00681-9000

Supporting Information

ABSTRACT: Globular proteins are usually in equilibrium with unfolded conformations, whereas kinetically stable proteins (KSPs) are conformationally trapped by their high unfolding transition state energy. Kinetic stability (KS) could allow proteins to maintain their activity under harsh conditions, increase a protein's half-life, or protect against misfolding−aggregation. Here we show the development of a simple method for quantifying a protein's KS that involves incubating a protein in SDS at high temperature as a function of time, running the unheated samples on SDS-PAGE, and quantifying the bands to determine the time-dependent loss of a protein's SDS resistance. Six diverse proteins, including two monomer, two dimers, and two tetramers, were studied by this method, and the kinetics of the loss of SDS resistance correlated linearly with their unfolding rate determined by circular dichroism. These results imply that the mechanism by which SDS denatures proteins involves conformational trapping, with a trapping rate that is determined and limited by the rate of protein unfolding. We applied the SDS trapping of proteins (S-TraP) method to superoxide dismutase (SOD) and transthyretin (TTR), which are highly KSPs with native unfolding rates that are difficult to measure by conventional spectroscopic methods. A combination of S-TraP experiments between 75 and 90 °C combined with Eyring plot analysis yielded an unfolding half-life of 70 ± 37 and 18 ± 6 days at 37 °C for SOD and TTR, respectively. The S-TraP method shown here is extremely accessible, sample-efficient, cost-effective, compatible with impure or complex samples, and will be useful for exploring the biological and pathological roles of kinetic stability.



Despite their remarkable functional capabilities, proteins usually fold into conformations that are marginally stable to allow their efficient degradation and functional regulation. However, with this conformational frailty also comes the risk of unwanted degradation and misfolding into potentially toxic conformations. Since the 1960s when it was realized that most small globular proteins exist in equilibrium with unfolded conformations, the stability of proteins has been usually determined by its equilibrium partitioning between native and unfolded states, yielding an equilibrium constant, K, from which the free energy of unfolding is determined.[1] Although this thermodynamic treatment of stability as an equilibrium reaction has been extremely valuable, it does not account well for proteins that are extremely resistant to degradation or harsh conditions.[2] Long-lived proteins appear to rely on kinetic stability (KS) for their physical resilience. The concept of KS as an alternative explanation for protein stability was introduced in the early 1990s.[3] Since KSPs are characterized by having a large transition state energy barrier for unfolding that traps them in their native state, determining the unfolding rate of a protein under native conditions provides a quantitative assessment of KS, regardless of its thermodynamic stability.

Unlike the well-established and straightforward theoretical and experimental approach for determining the thermodynamic stability of a protein,[1] the methods available for quantifying the KS of proteins have significant limitations. The most common approach involves the use of optical spectroscopy (e.g., fluorimeter) to measure the rate of protein unfolding at different concentrations of denaturant (e.g., urea). The log of the unfolding rate is then plotted against the denaturant concentration, and the long extrapolation of the linear plot to 0 M denaturant yields the unfolding rate under native conditions.[4] A serious limitation of this approach is that the denaturants urea and guanidine hydrochloride (GuHCl) have serious drawbacks for determining the unfolding half-life of KSPs. Urea is not able to denature many KSPs, whereas the ionic compound GuHCl often yields inaccurate unfolding half-lives as high as hundreds of years,[5,6] presumably by interfering with the protein's surface charges.[7] Other more recent methods to analyze the KS of proteins include scan-rate-dependent scanning calorimetry,[8] pulse proteolysis,[9−11] real-time fluorescent labeling,[12] and temperature-jump fluorescence microscopy.[13] Although these methods have their advantages and unique applications, most have limitations related to sensitivity, convenience, and/or accessibility. An ideal assay for quantifying the KS of proteins should be simple, accessible, and protein-efficient and allow the analysis of impure proteins.

In this study, we take advantage of our initial discovery of a correlation between protein KS and SDS resistance[14] to develop a simple assay involving the time-dependent SDS

Received: August 29, 2011
Revised: November 20, 2011
Published: November 22, 2011

   dx.doi.org/10.1021/bi201362z | *Biochemistry* 2012, 51, 100−107

trapping of proteins (S-TraP) at elevated temperatures to quantify the KS of proteins. Our results demonstrate that the time-dependent loss of a protein's SDS resistance correlates linearly with its unfolding rate (i.e., KS), thereby implying that the mechanism by which SDS denatures a protein involves conformational trapping. Thus, the rate by which SDS traps KSPs is determined and limited by their slow unfolding rate. This simple, accessible, and flexible assay has unique advantages over current methods and shows promise for studying the kinetic stability of proteins in its biological context.

EXPERIMENTAL PROCEDURES

Materials. All proteins (trypsin inhibitor-TRI, bromelain-BRO, glucose oxidase-GO, β-glucosidase-GLC, catalase-CAT, and streptavidin-SVD) and SDS were purchased from Sigma-Aldrich (St. Louis, MO). GO is from *Aspergillus niger*, SVD is from streptomyces avidinii, TTR is from human plasma, SOD is from bovine, GLC is from almonds, BRO is from pineapple stem, CAT is from *Aspergillus niger*, and TRI is from *Glycine max* soybean. These proteins were chosen because they were known from the literature to be kinetically stable or because we found them to be SDS-resistant. It should be noted that, unlike the catalase assayed in our previous study,[14] the catalase from *Aspergillus niger* used in this study is SDS-resistant. All the other chemicals and reagents required for the S-TraP assay were purchased from Thermo Fisher Scientific Inc. (Pittsburgh, PA).

Protein Sample Preparation and Incubation for S-Trap. Each protein was dissolved in 20 mM sodium phosphate buffer (pH 7.0) containing 0.3 M Tris base to a protein concentration of ∼1 mg/mL. Stock protein solution was diluted with SDS buffer (0.3 M Tris base, 30% glycerol, 5% SDS, 0.05% bromophenol blue, pH 6.8) so that the final concentrations of protein and SDS were ∼0.2 mg/mL and 1% (∼35 mM), respectively. Before the protein at RT was mixed with SDS, SDS buffer was incubated at a higher than the target temperature (T_h) to quickly achieve the desired temperature after mixing. The T_h was calculated using the equation T_h (K) × mass(SDS buffer) + 293.15 (K) × mass(protein buffer) = T_{target} × total mass. After incubation for different time periods, the samples were quickly placed in iced water for 3−5 s to "quench" the trapping and then centrifuged at 3000 rpm for 5 s at RT. Since bromelain is a member of the family of cysteine proteases, which are inhibited by zinc, zinc sulfate (800 μM) was added to bromelain to slow down self-digestion.[15]

1D SDS-PAGE. Protein samples were loaded onto the wells of a 12% acrylamide gel (8.3 cm × 8 cm × 0.75 mm). Running buffer contained 25 mM Tris base, 0.2 M glycine, and 0.1% SDS. The electrophoresis separation was performed on a Biorad mini-gel apparatus connected to an EC 135-90 power supply at 200 V. The gels were stained with Coomassie blue R250.

Gel Picture Analysis. Destained gels were imaged by a Biorad Gel Doc XR+ system and then analyzed by the Biorad program Image Lab, which has a feature to subtract the gel background from each band. The ratio between the amount of unfolded and total protein was used to determine the unfolding fraction (r_u). r_u for the first lane (0 s, RT incubation control) should in principle be 0, but this was not always the case (e.g., BRO). The fraction of unfolded protein relative to the total amount of proteins, R_{ui}, at different incubation times was defined as $R_{ui} = (r_{ui} - r_{u0})/(r_{u\infty} - r_{u0})$, where r_{ui} and r_{u0} are the fraction unfolded at time point i and 0, respectively, and $r_{u\infty}$ is the fraction unfolded at the highest time point when unfolding is complete. For e.g., if r_{u0} is 0.3 and r_{u1} is 0.4 after 10 s incubation at 70 °C, and $r_{u\infty}$ is 0.8, then $R_{u1} = (0.4 - 0.3)/(0.8 - 0.3) = 0.20$ or 20% protein unfolding. The R_u vs incubation time was fitted to a one-exponential equation by KaleidaGraph (Synergy, PA) to obtain the unfolding rate (k_u) at the incubation temperature. Compared to calculating k_u, based only on native band intensity, this relative quantification method avoids errors caused by the inevitably slightly different sample loading amounts between different lanes.

Circular Dichroism Spectroscopy (CD). CD spectra were measured with a Jasco spectropolarimeter (JASCO Inc., MD). Bromelain was dissolved in 50 mM Tris and 800 μM zinc sulfate. Other proteins were dissolved in 20 mM sodium phosphate buffer to form a 1 mg/mL solution. Far-UV CD spectra were recorded between 200 and 250 nm under a constant nitrogen flush, using a 1 mm path length quartz cells. The spectra were derived from averages of four scans recorded at 50 nm/min along with a 1 s response time. For each protein, the scans of native solutions and the unfolded proteins at high temperature were compared. The wavelength at which the two scans had the largest difference was selected for the time course scan for that protein. The selected wavelength for GO was 220 nm, GLY was 221 nm, SVD was 227 nm, BRO was 220 nm, CAT was 235 nm, and TRI was 225 nm. The bandwidth was 1 nm, response time was 1 s, sensitivity was standard, and data were recorded at every 0.2 s.

Obtaining k_u from CD Data via Eyring Plot. The CD data were graphed as ln k_u/T vs $1/T$, better known as an Eyring plot, which is linear and can be extrapolated to the desired temperature to obtain k_u. To obtain the error associated with the k_u values obtained by the Eyring plot, the Eyring plot was fitted using Graphpad Prism (GraphPad Software, CA) to obtain the simple linear regression formula for estimating the protein unfolding half-life at the target temperature. Graphpad Prism also calculates the 95% confidence interval boundaries for the true best-fit linear regression line.[16] Errors of the unfolding half-lives obtained from the CD data were calculated based on 95% confidence interval boundaries.

Obtaining k_u from S-TraP Data via Eyring Plot. Since S-TraP does not yield accurate data at 37 °C for most KSPs due to the faster k_f relative to k_u (Figure 1S), it is necessary to carry out the S-TraP experiments at higher temperatures. The unfolding rates determined by S-TraP at the various temperatures are then graphed as ln k_u/T vs $1/T$. For every temperature we carried out each S-TraP experiment at least three times, and all replicates were considered as separate points (since their errors are independent) in the linear regression calculations of the statistical software Graphpad Prism. The software carries out a linear regression analysis to minimize the sum of the squares of the vertical distances of the points from the line. The software uses the resulting line and slope error to calculate the unfolding rate and standard deviation at the extrapolated temperature of interest.

RESULTS

Time-Dependent SDS Trapping of Proteins (S-TraP) Reveals Differences in Kinetic Stability. The SDS resistance of kinetically stable proteins (KSPs) suggests that SDS denature proteins irreversibly by trapping them when they transiently expose hydrophobic residues during their partial and global unfolding. If so, then a protein's time-dependent denaturation by SDS should directly probe its rate of unfolding. Therefore, we designed an experiment that involves incubating

dx.doi.org/10.1021/bi201362z | Biochemistry 2012, 51, 100−107

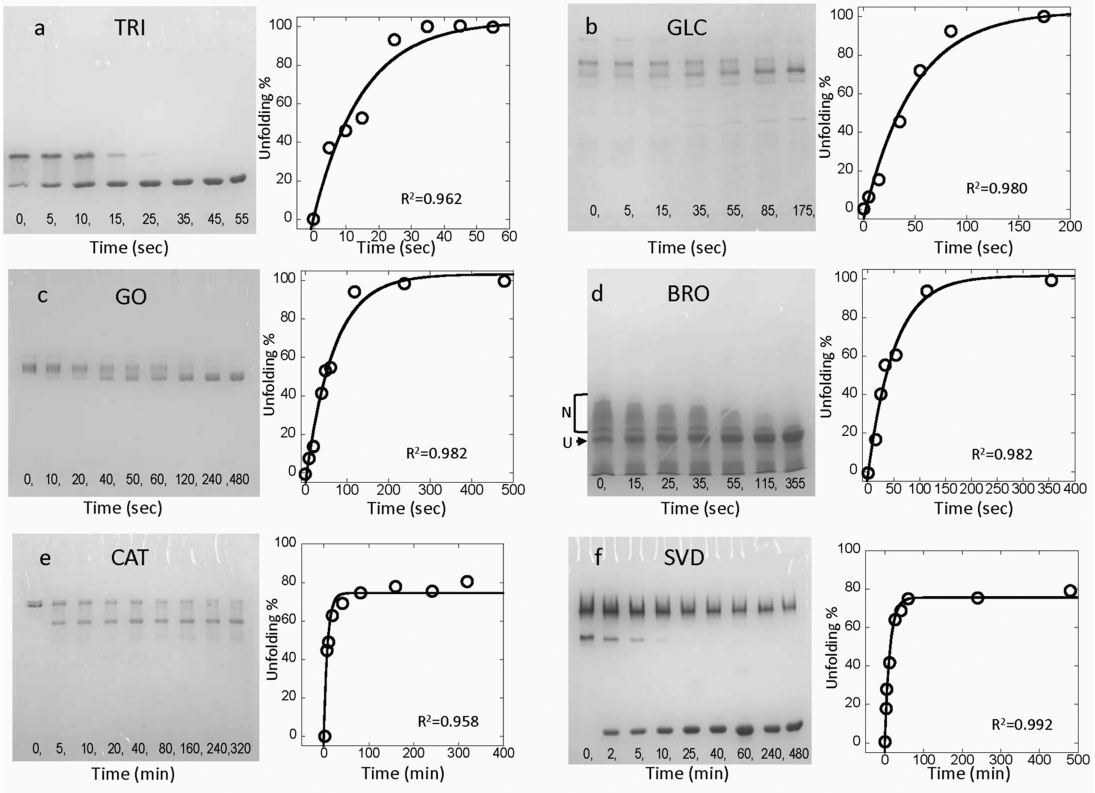


Figure 1. S-TraP experiment for proteins incubated in 1% SDS buffer at 70 °C. A diverse group of proteins was assayed, including monomers (trypsin inhibitor-TRI and bromelain-BRO), dimers (glucose oxidase-GO and *β*-glucosidase-GLC), and tetramers (catalase-CAT and streptavidin-SVD). Each gel lane shows the denaturation of the protein after a certain incubation time. Gel data were imaged and fitted to a single exponential to determine the unfolding rates. In panel d, the N indicates the native band while the U indicates the unfolding band.

Table 1. Structural, Thermodynamic, and Kinetic Parameters for Proteins Analyzed by S-TraP

protein name	oligomer	monomer MW (kDa)	T_m (°C)	$t_{1/2}$ at 70 °C S-TraP	$t_{1/2}$ at 70 °C from Eyring plot
trypsin inhibitor (TRI)	1	20	65[c]	10 ± 1 s	27 ± 2 s[a]
bromelain (BRO)	1	27	63[d]	37 ± 2 s	50 ± 1 s[a]
glucose oxidase (GO)	2	80	58[e]	46 ± 5 s	45 ± 1 s[a]
β-glucosidase (GLC)	2	65	40–50[f]	35 ± 2 s	43 ± 1 s[a]
streptavidin (SVD)	4	13	75[g]	11 ± 2 min	9 ± 1 min[a]
catalase (CAT)	4	97	70[h]	5.4 ± 1.4 min	4.6 ± 0.4 min[a]
superoxide dismutase (SOD)	2	16	89, 96[i]	37 ± 17 min	42 ± 6 min[b]
transthyretin (TTR)	4	14	97.8[j]	24 ± 5 min	15 ± 1 min[b]

[a]Calculations from the Eyring plot of unfolding rates from CD measurements at different temperatures. Errors were calculated based on 95% confidence interval boundaries, as described in Experimental Procedures. [b]Calculations from the Eyring plot of unfolding rates from S-TraP at different temperatures. Errors were calculated based on 95% confidence interval boundaries, as described in Experimental Procedures. [c]Reference 31. [d]Reference 32. [e]Reference 33. [f]Reference 34. [g]Reference 35. [h]Reference 36. [i]Reference 37. [j]Reference 38.

a KSP in SDS for various periods, followed by SDS-PAGE analysis to observe the transition from the SDS-resistant protein (upper band) to the unfolded SDS-trapped protein (lower band). Preliminary experiments revealed that SDS trapping at RT or 37 °C was extremely slow for some proteins. Therefore, we optimized the temperature and chose to coincubate the protein and SDS at 70 °C because at this temperature we could analyze proteins with a broad range of stability. We chose six proteins (trypsin inhibitor-TRI, bromelain-BRO, glucose oxidase-GO, *β*-glucosidase-GLC, catalase-CAT, and streptavidin-SVD) that were SDS-resistant, structurally diverse, and commercially available. Figure 1 shows the raw gel data for these six proteins incubated in SDS for different periods. Each gel exhibits a time-dependent transition from the SDS-resistant native state to the SDS-trapped denatured state. The difference in migration between these two bands depends mostly on the protein's oligomeric structure, molecular weight (MW), and the gel acrylamide composition. For example, GLC and GO are dimeric proteins of 65 and 80 kDa monomers, respectively, and migrate in the upper region of 12% acrylamide gels, nearby their respective dimers (Figures 1b and 1c). In contrast, the native and denatured states of the tetrameric protein SVD, which consists of 12 kDa monomers, migrate far apart from each other.

The six KSPs experienced gradual denaturation over different incubation times. At the extremes, TRI was completely denatured within 30 s of incubation in SDS at 70 °C, whereas the SVD transition was not completed after 8 h of incubation. Thus, these proteins exhibit different sensitivities to SDS, perhaps due to their different kinetic stabilities. To analyze the

dx.doi.org/10.1021/bi201362z | *Biochemistry* 2012, 51, 100–107

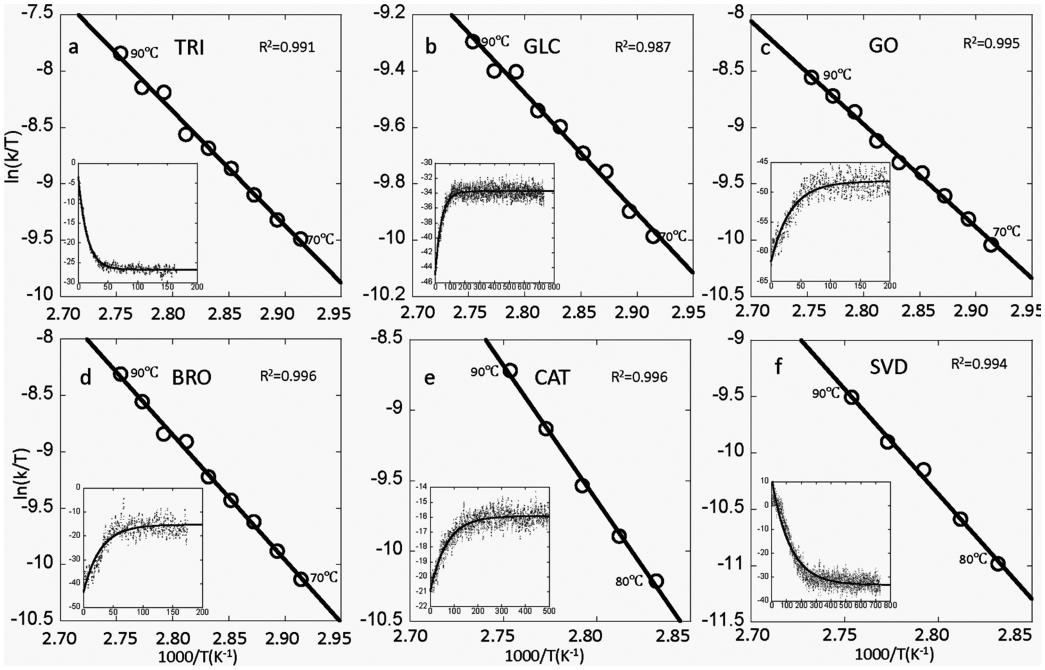


Figure 2. Eyring plot of temperature-induced unfolding rates in SDS-free buffer monitored by CD. Unfolding data for the six proteins used in Figure 1 are shown in panels a–f. The insets show unfolding kinetic traces monitored at 80 °C and fitted to a single-exponential equation. The *y*- and *x*-axes represent the CD signal and time (in seconds or minutes), respectively. See Experimental Procedures section for details of the Eyring plot. The minimum temperature tested for each protein was at least 5 °C higher than the melting temperature of that protein.

transitions, the gels were imaged and the bands were quantified using the software Image Lab. After gel background subtraction and normalizing the band intensity relative to the time "0" band, the data were plotted as "% unfolded" vs time and fitted to a single-exponential equation to obtain the rate of SDS trapping (i.e., the rate of protein unfolding) at 70 °C (Figure 1). The rate of SDS trapping at 70 °C yielded unfolding half-lives that varied from 10 s for TRI to 11 min for SVD (Table 1). Interestingly, SVD and CAT were not fully denatured even after hours of incubation (Figure 1e,f), suggesting the presence of a much slower unfolding phase, perhaps due to cross-linking or the presence of extremely stable complexes (e.g., SVD-biotin). The experiments in Figure 1 were repeated at least three times and exhibited good reproducibility, including for bromelain (BRO), which is a monomeric SDS-resistant[17] protease that is not sold in high purity. Even at time zero, the native state of BRO exhibits a broad band, perhaps due to autoproteolysis, that disappears over time upon incubation in SDS. Thus, it appears that coincubation of KSPs and SDS at a denaturing temperature allows SDS to trap the proteins in a time-dependent manner, presumably according to their inherent unfolding rates.

Rate of SDS Trapping Shows Linear Correlation with the Unfolding Rate Determined by Circular Dichroism (CD). To determine the accuracy of the unfolding rates determined by S-TraP, we used CD to monitor the temperature-induced unfolding rates of the proteins (Figure 2). We incubated the proteins at temperatures that were at least 5 °C above their T_m (the T_m values are shown in Table 1 for guidance) so that the CD-observable unfolding rate would be dominated by the microscopic unfolding rate (k_u), with minimal contribution from the microscopic folding rate (k_f). To confirm the quality of the CD data and to obtain extrapolated k_u values at 70 °C for CAT and SVD (Figure 2e,f), we plotted the ln k_u/T vs $1/T$ (i.e., Eyring plot). The linear Eyring plots confirmed the quality of the protein unfolding kinetics data. Remarkably, the unfolding half-lives ($t_{1/2}$) determined by S-Trap and CD are very similar (Table 1) and show an excellent linear correlation when plotted against each other (Figure 3). These results support the idea that the

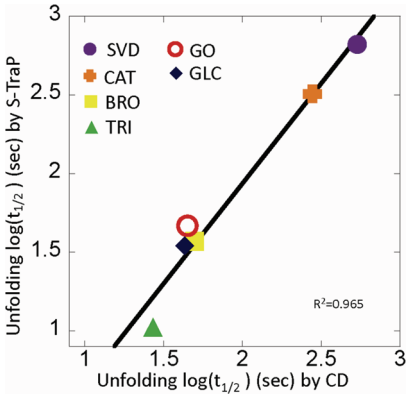


Figure 3. Correlation of the unfolding half-life ($t_{1/2}$) at 70 °C obtained from the CD-Eyring plot and S-TraP data. The unfolding $t_{1/2}$ of the proteins tested by the two different methods has a strong linear correlation. Therefore, S-TraP could be used to quantify the KS of a protein.

mechanism by which SDS denatures KSPs involves conformational trapping of unfolded states and that the rate of SDS trapping (k_t) is determined and limited by the unfolding rate of the protein.

In cases where protein unfolding is inherently irreversible or protein folding is very slow compared to SDS trapping, the incubation temperature will not have a major effect on the observed unfolding rate ($k_{u\text{-}obs}$) obtained by S-TraP. However, if the native and denatured states of the protein are in

dx.doi.org/10.1021/bi201362z | *Biochemistry* 2012, 51, 100–107

equilibrium, then the incubation temperature must allow k_u to dominate so that $k_u \sim k_{u\text{-}obs}$; otherwise, $k_{u\text{-}obs} < k_u$. We chose 70 °C for comparing S-TraP with CD because it was the highest single temperature we could use for S-TraP that was above the T_m in most cases and allowed us to monitor the unfolding rate for all six KSPs in the group. Interestingly, for the tetrameric proteins CAT and SVD, which have T_ms of 70 and 75 °C, respectively, S-TraP and CD results were very similar (Table 1), suggesting that their folding-oligomerization at temperatures near their T_ms do not compete with SDS trapping. Thus, we suspect that for most monomeric proteins S-TraP experiments should be carried out at temperatures >5 °C higher than their T_ms, although the highest temperature that is practical will be limited by how fast they unfold. In contrast, for oligomeric proteins, it should be possible to obtain accurate $k_{u\text{-}obs}$ with S-TraP at temperatures near the T_m, if the folding-oligomerization rate is significantly slower than SDS binding, which has been reported to occur within the 5 ms dead time (perhaps in microseconds) of a stopped-flow instrument.[18]

Using S-TraP To Determine a Protein's Unfolding Rate at Physiological Temperature. In most cases, the KS of a protein will be most relevant at physiological temperature. However, S-TraP will usually not be feasible at 37 °C because the faster k_f relative to k_u will cause $k_{u\text{-}obs}$ to be significantly underestimated or might not allow SDS trapping to occur for the most KSPs. We have observed both of these scenarios for the proteins studied here (Figure 1S). Therefore, to determine a protein's k_u using S-TraP at 37 °C, extrapolation of an Eyring plot is required.[19] To test this approach with S-TraP, we chose the proteins transthyretin (TTR) and superoxide dismutase, (SOD), whose high KS cannot be easily probed by current methods. Urea cannot unfold TTR and SOD, and guanidine hydrochloride (GuHCl) often yields overestimated unfolding half-lives of up to hundreds of years.[5,6]

TTR and SOD were incubated in 1% SDS solution at different temperatures from 75 to 90 °C, and their unfolding rate was determined by S-TraP. Representative data at 85 °C are shown in Figure 4. The k_u obtained at different temperatures were used to make an Eyring plot, and the linear fit was extrapolated to 37 °C (Figure 4). The linearity of the Eyring plot at lower temperature should not be compromised by SDS, since protein unfolding kinetic studies in the 15−45 °C range have shown a linear Eyring plot in the presence of 10−500 mM SDS.[20] Interestingly, the unfolding half-lives for TTR and SOD at 37 °C were 18 ± 6 and 70 ± 37 days, respectively. Since the high T_m for SOD and TTR (89 and 98 °C, respectively (Table 1)) and their tendency to aggregate at higher temperature did not allow us to obtain reliable CD data, we could not verify the accuracy of the S-TraP results. Because of the log-scale y-axis and the long extrapolation to 37 °C, even a small error in the slope of the Eyring plot could result in unfolding rates that are significantly lower or higher than the actual values. However, several observations suggest that the S-TraP data is reliable even though it was collected at temperatures below the T_m of the proteins. First, the similar k_u values obtained by the S-TraP and CD methods for both CAT and SVD show that it is possible to obtain accurate k_u values below the T_m for some proteins. Second, the Eyring plot shows no downward curvature, as would be expected if protein folding and SDS binding were competing (see eq 1). Third, since SOD and TTR are oligomeric β sheet proteins, their refolding-oligomerization rate at high temperature is likely to be relatively slow compared to the low milliseconds (or faster)

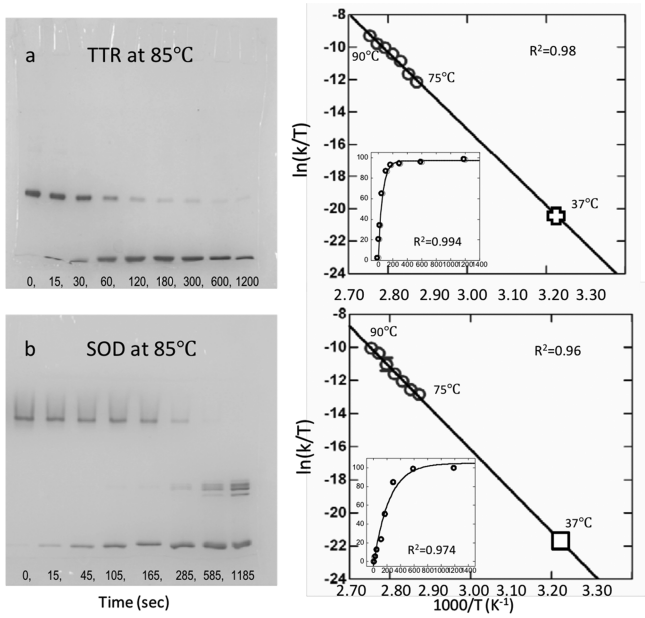


Figure 4. S-TraP analysis of the highly kinetically stable proteins TTR (a) and SOD (b). The unfolding rates of TTR and SOD at different temperatures were determined by S-TraP analysis, and representative S-TraP data at 85 °C are shown. The various unfolding rates were used for an Eyring plot, which showed the expected linear profile, and yielded via extrapolation to 37 °C an unfolding rate of 18 ± 6 and 70 ± 37 for TTR (open cross) and SOD (open square), respectively. Errors were calculated as described in the Experimental Procedures. Most error bars at each temperature point are within the size of the symbols. The gels were stained with Coomassie blue R250.

SDS binding.[18] Thus, our data suggests that S-TraP could be useful to probe the KS of extremely stable proteins that cannot be reliably studied by conventional methods.

DISCUSSION

SDS as a Trapping Agent for Denatured States. SDS-PAGE was a method introduced over 40 years ago for separating proteins.[21] Currently SDS-PAGE is arguably the most fundamental technique in protein science. The interaction between a protein and a charged surfactant such as SDS is complex and involves both hydrophobic and electrostatic interactions. Despite the widespread use of SDS, the mechanism by which it denatures proteins when present at concentrations above the critical micelle concentration (cmc) is unclear.[20] It has been proposed that below 100 mM, SDS (cmc ∼ 7 mM in water[22]) denature proteins by a mechanism involving ligand-binding-type unfolding kinetics, and it was shown that the activation barrier for unfolding is surprisingly similar to that in water.[20] The observation that SDS does not affect the activation energy for unfolding is consistent with our results and suggests that SDS binding to a protein's surface has minor effect on the structure and free energy of its native state.[20] However, in contrast to a binding-induced denaturation mechanism, our data suggest that SDS's role in the denaturation of KSPs (and perhaps most proteins) involves a binding-trapping mechanism that is largely determined and limited by the unfolding rate (i.e., kinetic stability) of the protein.

The dependence of SDS trapping on the exposure of hydrophobic residues is reminiscent of the mechanism of hydrogen−deuterium exchange in proteins.[23] Therefore, SDS

trapping could be described as

$$N \underset{k_f}{\overset{k_u}{\rightleftarrows}} U \xrightarrow{k_t} C \quad (1)$$

where k_u, k_f, and k_t are the microscopic rates of protein unfolding, protein folding, and SDS trapping, respectively. The equilibrium between the native (N) and unfolded (U) states is defined as $K_u = k_u/k_f$, but C represents the irreversible formation of the SDS−protein complex. At the SDS (1% w/v, 35 mM) and protein (0.3 mg/mL, low micromolar) concentrations used in this study, 96% of the SDS is free based on the known 1.4 g:1 g SDS:protein binding ratio.[24] Therefore, its concentration is virtually constant and can be combined into k_t. Thus, the observed rate constant ($k_{t,obs}$) for SDS-trapping is given by

$$k_{t,obs} = k_u k_t/(k_t + k_f) \quad (2)$$

which is analogous to the observed rate of H exchange.[23] At high temperature $k_t \gg k_f$, and the equation is reduced to $k_{t,obs} = k_u$, a condition known as the EX1 exchange limit.[23] The inefficient SDS trapping observed at 37 °C (Figure 1S) suggests that at low temperature $k_f \gg k_t$, thereby indicating that SDS trapping is not diffusion limited. Under these conditions, eq 1 reduces to $k_{t,obs} = k_u k_t/k_f$, known as the EX2 limit.[23] Therefore, for S-TraP to yield an accurate k_u, the experiment must be done at a temperature higher than the T_m, where the folding rate, k_f, is slow relative to k_t, and eq 1 is reduced to $k_{t,obs} = k_u$. In cases where protein unfolding is irreversible or protein folding is inherently slow, then a broader range of temperatures would be possible.

In the SDS-trapping mechanism described above (eqs 1 and 2), we assumed a two-state protein folding mechanism for simplicity purposes. However, since most KSPs identified to date appear to be oligomeric or larger than most two-state-folding proteins, it seems likely that their folding mechanism will be more complex. It should also be noted that kinetic stability and thermodynamic stability are not necessarily mutually exclusive properties, since a KSP that folds via a two-state mechanism would likely also be thermodynamically stable. Thus, regardless of the folding mechanism and thermodynamic stability, a protein that unfolds slowly due to a high unfolding energy barrier is kinetically stable, and this property can be quantified by S-TraP.

Limitations and Advantages of the S-TraP Method. Although the S-TraP method is only applicable to proteins that are SDS-resistant, it is unique and provides unprecedented convenience and access for exploring and quantifying the KS of the most stable proteins found in nature. Another gel-detection method that can be used to monitor the KS of protein is pulse proteolysis,[9−11] where the proteolytic cleavage of proteins is followed by SDS-PAGE as a function of time. Pulse proteolysis in the presence of urea is an effective method for probing the KS of proteins that are not SDS-resistant,[9] but it is not clear how useful it would be to probe the KS of SDS-resistant proteins, most of which are also resistant to proteolysis and urea.

A potential weakness of the S-TraP assay is that the 24% (v/v) glycerol used in the incubation buffer to prevent protein aggregation at high temperature might increase the unfolding energy barrier by stabilizing the native state. Interestingly, it does not appear that glycerol had a significant effect on the unfolding half-life (Figure 3, Table 1) of the proteins studied here, perhaps because for each KSP the native state and the unfolding transition state was similarly stabilized, resulting in minimal changes to the net activation energy for unfolding. Alternatively, the concentration of SDS used (1%) might somehow counteract the stabilizing effect of glycerol.[25] Since glycerol and osmolytes in general appear to stabilize proteins by shifting the equilibrium toward the native state to avoid unfavorable interactions with the protein backbone in the unfolded state, perhaps glycerol is less effective in stabilizing KSPs because they are trapped in their native state.[26] Regardless, more proteins will need to be studied, and the concentration of glycerol optimized before a conclusion can be reached about the effect of glycerol and SDS on the stability of KSPs. Despite these potential limitations, the S-TraP method is extremely accessible, sample-efficient, and cost-effective. Also, since GuHCl often yields inaccurate unfolding half-lives,[5,6] and most KSPs are resistant to urea, S-TraP provides a unique method to quantify the KS of hyperstable proteins, as we have shown for TTR and SOD (Figure 4). Furthermore, whereas conventional methods for studying the KS of proteins require purified proteins, S-TraP could be carried out in cultures containing overexpressed protein.

Potential Biological, Biomedical, and Practical Applications of S-TraP. The S-TraP method seems promising for studying the KS of proteins under various situations. For example, it might be desirable to know a protein's unfolding half-life at physiological conditions, while in storage at 4 °C, or during a commercial application at high temperature. The linear Eyring plot observed for TTR and SOD (Figure 4) suggests that the S-TraP method could be applied at convenient (e.g., $T > T_m$) temperatures to determine via extrapolation the unfolding rate of a protein at the temperature of interest.[19,20] S-TraP might be particularly useful to determine the unfolding half-life of proteins designed or engineered to function at high temperature. S-TraP might also be useful to probe the KS of proteins in biological fluids or cells. If the protein density is too high and interferes with 1D SDS-PAGE, S-TraP might be combined with diagonal 2D SDS-PAGE[6] or Western blot analysis.

From a biomedical perspective, perhaps the most significant application of the S-TraP method will be the ability to probe the KS of proteins linked to disease. Mutation-induced loss of protein KS has been linked to some protein misfolding diseases.[27] In familial amyloid polyneuropathy it is known that missense mutations can compromise the KS of transthyretin (TTR), facilitating its tetramer dissociation and subsequent aggregation into amyloid fibrils.[28] Mutations might also cause non-KSPs to misfold into toxic kinetically stable conformations that are resistant to cellular degradation. For instance, the prion protein, which is involved in various genetic and transmissible diseases lacks KS.[29] In contrast, the misfolded prion conformation is extremely resistant to degradation due to its remarkable KS,[30] which largely explains why infectious prions survive the GI track and are orally transmissible. It is increasingly appreciated that the unfolding rate (i.e., the KS) of proteins is the "gate keeper" for protecting native proteins against misfolding and premature degradation, as well as for potentially inhibiting the degradation of toxic conformations.[2,4] Thus, methods like S-TraP that can conveniently measure the KS of different proteins and mutants will be needed to advance our understanding of the extent to which KS contributes to the biology and pathology of an organism.

dx.doi.org/10.1021/bi201362z | Biochemistry 2012, 51, 100−107

■ ASSOCIATED CONTENT

Supporting Information

Figure 1S. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: (518) 276-2515. Fax: (518) 276-4887. E-mail: colonw@rpi.edu.

Funding

This work was supported by a grant (MCB-0848120) from the National Science Foundation to W.C.

■ ACKNOWLEDGMENTS

We thank Christopher Bystroff and George Makhatadze for their useful comments of our manuscript.

■ ABBREVIATIONS

CD, circular dichroism; KS, kinetic stability; KSP, kinetically stable protein; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; S-TraP, SDS trapping of proteins; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

■ REFERENCES

(1) Pace, C. N. (1975) The stability of globular proteins. *CRC Crit. Rev. Biochem. 3*, 1−43.

(2) Plaza del Pino, I. M., Ibarra-Molero, B., and Sanchez-Ruiz, J. M. (2000) Lower kinetic limit to protein thermal stability: a proposal regarding protein stability in vivo and its relation with misfolding diseases. *Proteins 40*, 58−70.

(3) Baker, D., and Agard, D. A. (1994) Kinetics versus thermodynamics in protein folding. *Biochemistry 33*, 7505−7509.

(4) Sanchez-Ruiz, J. M. (2010) Protein kinetic stability. *Biophys. Chem. 148*, 1−15.

(5) Lai, Z., McCulloch, J., Lashuel, H. A., and Kelly, J. W. (1997) Guanidine hydrochloride-induced denaturation and refolding of transthyretin exhibits a marked hysteresis: equilibria with high kinetic barriers. *Biochemistry 36*, 10230−10239.

(6) Xia, K., Manning, M., Hesham, H., Lin, Q., Bystroff, C., and Colon, W. (2007) Identifying the subproteome of kinetically stable proteins via diagonal 2D SDS/PAGE. *Proc. Natl. Acad. Sci. U. S. A. 104*, 17329−17334.

(7) Ibarra-Molero, B., Loladze, V. V., Makhatadze, G. I., and Sanchez-Ruiz, J. M. (1999) Thermal versus guanidine-induced unfolding of ubiquitin. An analysis in terms of the contributions from charge-charge interactions to protein stability. *Biochemistry 38*, 8138−8149.

(8) Tur-Arlandis, G., Rodriguez-Larrea, D., Ibarra-Molero, B., and Sanchez-Ruiz, J. M. (2010) Proteolytic scanning calorimetry: a novel methodology that probes the fundamental features of protein kinetic stability. *Biophys. J. 98*, L12−14.

(9) Na, Y. R., and Park, C. (2009) Investigating protein unfolding kinetics by pulse proteolysis. *Protein Sci. 18*, 268−276.

(10) Park, C., and Marqusee, S. (2005) Pulse proteolysis: a simple method for quantitative determination of protein stability and ligand binding. *Nature Methods 2*, 207−212.

(11) Park, C., Zhou, S., Gilmore, J., and Marqusee, S. (2007) Energetics-based protein profiling on a proteomic scale: identification of proteins resistant to proteolysis. *J. Mol. Biol. 368*, 1426−1437.

(12) Ignatova, Z., and Gierasch, L. M. (2004) Monitoring protein stability and aggregation in vivo by real-time fluorescent labeling. *Proc. Natl. Acad. Sci. U. S. A. 101*, 523−528.

(13) Ebbinghaus, S., Dhar, A., McDonald, J. D., and Gruebele, M. (2010) Protein folding stability and dynamics imaged in a living cell. *Nature Methods 7*, 319−323.

(14) Manning, M., and Colón, W. (2004) Structural basis of protein kinetic stability: Resistance to sodium dodecyl sulfate suggests a central role for rigidity and a bias towards beta sheet structure. *Biochemistry 43*, 11248−11254.

(15) Kezdy, F. J., and Kaiser, E. T. (1976) Active site titration of cysteine proteases. *Methods Enzymol. 45*, 3−12.

(16) Motulsky, H. (1995) *Intuitive Biostatistics*, p 386, Oxford University Press, Oxford.

(17) Bhattacharya, R., and Bhattacharyya, D. (2009) Resistance of bromelain to SDS binding. *Biochim. Biophys. Acta 1794*, 698−708.

(18) Nielsen, M. M., Andersen, K. K., Westh, P., and Otzen, D. E. (2007) Unfolding of beta-sheet proteins in SDS. *Biophys. J. 92*, 3674−3685.

(19) Jackson, S. E., and Fersht, A. R. (1991) Folding of chymotrypsin inhibitor 2. 2. Influence of proline isomerization on the folding kinetics and thermodynamic characterization of the transition state of folding. *Biochemistry 30*, 10436−10443.

(20) Otzen, D. E. (2002) Protein unfolding in detergents: effect of micelle structure, ionic strength, pH, and temperature. *Biophys. J. 83*, 2219−2230.

(21) Shapiro, A. L., Vinuela, E., and Maizel, J. V. Jr. (1967) Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Commun. 28*, 815−820.

(22) Reynolds, J. A., Herbert, S., Polet, H., and Steinhardt, J. (1967) The binding of divers detergent anions to bovine serum albumin. *Biochemistry 6*, 937−947.

(23) Englander, S. W., and Mayne, L. (1992) Protein folding studied using hydrogen-exchange labeling and two-dimensional NMR. *Annu. Rev. Biophys. Biomol. Struct. 21*, 243−265.

(24) Reynolds, J. A., and Tanford, C. (1970) Binding of dodecyl sulfate to proteins at high binding ratios. Possible implications for the state of proteins in biological membranes. *Proc. Natl. Acad. Sci. U. S. A. 66*, 1002−1007.

(25) Das, T. K., Mazumdar, S., and Mitra, S. (1998) Characterization of a partially unfolded structure of cytochrome c induced by sodium dodecyl sulphate and the kinetics of its refolding. *Eur. J. Biochem. 254*, 662−670.

(26) Bolen, D. W., and Baskakov, I. V. (2001) The osmophobic effect: natural selection of a thermodynamic force in protein folding. *J. Mol. Biol. 310*, 955−963.

(27) Sacchettini, J. C., and Kelly, J. W. (2002) Therapeutic strategies for human amyloid diseases. *Nat. Rev. Drug Discovery 1*, 267−275.

(28) Colón, W., and Kelly, J. W. (1992) Partial denaturation of transthyretin is sufficient for amyloid fibril formation in vitro. *Biochemistry 31*, 8654−8660.

(29) Hornemann, S., and Glockshuber, R. (1998) A scrapie-like unfolding intermediate of the prion protein domain PrP(121−231) induced by acidic pH. *Proc. Natl. Acad. Sci. U. S. A. 95*, 6010−6014.

(30) Prusiner, S. B., Groth, D., Serban, A., Stahl, N., and Gabizon, R. (1993) Attempts to restore scrapie prion infectivity after exposure to protein denaturants. *Proc. Natl. Acad. Sci. U. S. A. 90*, 2793−2797.

(31) Roychaudhuri, R., Sarath, G., Zeece, M., and Markwell, J. (2003) Reversible denaturation of the soybean Kunitz trypsin inhibitor. *Arch. Biochem. Biophys. 412*, 20−26.

(32) Habib, S., Khan, M. A., and Younus, H. (2007) Thermal destabilization of stem bromelain by trehalose. *Protein J. 26*, 117−124.

(33) Haouz, A., Glandieres, J. M., and Alpert, B. (2001) Involvement of protein dynamics in enzyme stability. The case of glucose oxidase. *FEBS Lett. 506*, 216−220.

(34) Yu, H. L., Xu, J. H., Lu, W. Y., and Lin, G. Q. (2007) Identification, purification and characterization of beta-glucosidase from apple seed as a novel catalyst for synthesis of O-glucosides. *Enzyme Microb. Technol. 40*, 354−361.

(35) Gonzalez, M., Argarana, C. E., and Fidelio, G. D. (1999) Extremely high thermal stability of streptavidin and avidin upon biotin binding. *Biomol. Eng. 16*, 67−72.

(36) Moosavimovahedi, A. A. (1994) Interaction of Aspergillus-Niger catalase with sodium N-dodecyl sulfate. *Pure Appl. Chem. 66*, 71−75.

(37) Roe, J. A., Butler, A., Scholler, D. M., Valentine, J. S., Marky, L., and Breslauer, K. J. (1988) Differential scanning calorimetry of Cu,Zn-

superoxide dismutase, the apoprotein, and its zinc-substituted derivatives. *Biochemistry 27*, 950−958.
(38) Lundberg, E., Olofsson, A., Westermark, G. T., and Sauer-Eriksson, A. E. (2009) Stability and fibril formation properties of human and fish transthyretin, and of the Escherichia coli transthyretin-related protein. *FEBS J. 276*, 1999−2011.