Structural Basis of Protein Kinetic Stability: Resistance to Sodium Dodecyl Sulfate Suggests a Central Role for Rigidity and a Bias Toward β-Sheet Structure[†]

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Received April 22, 2004; Revised Manuscript Received June 30, 2004

ABSTRACT: The term kinetic stability is used to describe proteins that are trapped in a specific conformation because of an unusually high-unfolding barrier that results in very slow unfolding rates. Motivated by the observation that some proteins are resistant to sodium dodecyl sulfate (SDS)-induced denaturation, an attempt was made to determine whether this property is a result of kinetic stability. We studied many proteins, including a few kinetically stable proteins known to be resistant to SDS. The resistance to SDS-induced denaturation was investigated by comparing the migration on polyacrylamide gels of identical boiled and unboiled protein samples containing SDS. On the basis of the different migration of these samples, eight proteins emerged as being resistant to SDS. The kinetic stability of these proteins was confirmed by their slow unfolding rate upon incubation in guanidine hydrochloride. Further studies showed that these proteins were also extremely resistant to proteolysis by proteinase K, suggesting that a common mechanism may account for their resistance to SDS and proteolytic cleavage. Together, these observations suggest that a rigid protein structure may be the physical basis for kinetic stability and that resistance to SDS may serve as a simple assay for identifying proteins whose native conformations are kinetically trapped. Remarkably, most of the kinetically stable SDS-resistant proteins in this study are oligomeric β-sheet proteins, suggesting a bias of these types of structures toward kinetic stability.

Kinetic stability is a poorly understood property of a select group of naturally occurring proteins that are trapped in their native conformations by an energy barrier and consequently are resistant to unfolding. Kinetic stability can be best explained by illustrating the unfolding process as a simple equilibrium reaction between two protein conformations, the native folded state (N) and the unfolded state (U), separated by a higher energy transition state (TS) (Figure 1). Because the height of the TS barrier determines the rate of folding and unfolding, kinetically stable proteins possess an unusually high energy TS, which results in extremely slow unfolding rates that virtually trap the protein in its native state (Figure 1A). Even though the overall change in Gibbs free energy (ΔG) may be favorable for unfolding under extreme solvent conditions, such as high concentrations of denaturant, the high activation energy of the TS significantly slows down the unfolding rate (Figure 1B). It has been suggested that the presence of a high kinetic energy barrier separating the folded and unfolded states is an evolutionary feature intended to allow proteins to maintain activity in the extreme conditions they may encounter in vivo (*1*). The examples of the kinetically stable proteins α-lytic protease (extracellular enzyme) (*1*), *Escherichia coli* OmpA (bacterial membrane protein) (*2*), and pyrrolidone carboxyl peptidase (hyperthermophilic protein) (*3*) illustrate the kinetic adaptation of proteins that must retain enzymatic function in conditions where degradation might easily take place. In addition, thermodynamic stability alone does not fully protect proteins that are susceptible to irreversible denaturation and aggregation arising from partially denatured states that become transiently populated under physiological conditions (*4*). Therefore, the development of a high kinetic energy barrier to unfolding may serve to protect susceptible proteins against such harmful conformational "side-effects".

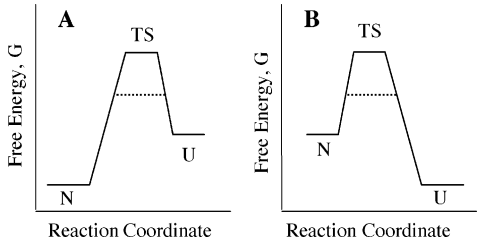

FIGURE 1: Free-energy diagram to illustrate the higher unfolding energy barrier for a kinetically stable protein under native (A) and denaturing (B) conditions as compared to that of a normal protein (represented by the dashed line).

The physical basis for kinetic stability is poorly understood and no structural consensus has been found to explain this phenomenon. In previous studies, the addition of hydrophobic residues on the protein surface (*5*), the engineering of disulfide bonds (*6*), and the introduction of metal-binding sites (*7*) have been shown to increase kinetic stability. A connection between kinetic stability and oligomeric quaternary structure has also been proposed (*8*). In the case of some hyperthermophilic proteins, electrostatic interactions have

[†] This work was supported by grants from the National Science Foundation (NSF MCB-9984913 to W.C.). M.M. was partially supported by a Rensselaer Polytechnic Institute Undergraduate Summer Research Fellowship.

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10.1021/bi0491898 CCC: $27.50
Published on Web 08/13/2004

been suggested to be a major factor in their slow unfolding because of the formation of ion pairs (*9*, *10*). However, there is evidence that some kinetically stable proteins retain their slow unfolding rate even at low pH, where electrostatic interactions should be significantly weakened (*3*, *11*). Thus, it appears that no common structural feature exists to explain kinetic stability, and perhaps this property may be achieved by different means, depending on the individual protein.

Under native conditions, kinetically stable proteins have limited access to partially and globally unfolded conformations (*12*). These properties impart a strong proteolytic resistance by reducing the occurrence of accessible conformations susceptible to proteolytic attack (*12*, *13*). Some kinetically stable proteins have also been found to be resistant to denaturation by sodium dodecyl sulfate (SDS).[1] Among them are the β-sheet proteins streptavidin (*14*), transthyretin (*15*), P22 tailspike protein (*16*), and the *E. coli* membrane protein, OmpA (*2*). We hypothesized that the decreased rate of local and global unfolding of kinetically stable proteins also accounts for their resistance to SDS-induced denaturation and that this property may be common to all kinetically stable proteins. Therefore, to further probe the relationship between kinetic stability and SDS resistance, over 30 proteins, including some known to be SDS-resistant and/or kinetically stable, were studied. Eight proteins were found to resist denaturation by SDS, and these were shown or confirmed to be kinetically stable and resistant to proteolysis by proteinase K. Interestingly, these proteins are mostly oligomeric with a very high content of β-sheet structure. The results below suggest that a rigid three-dimensional structure may be the physical basis for kinetic stability and that the resistance of certain proteins to denaturation by SDS may be used as the basis for a simple assay to identify kinetically stable proteins, with the potential of being adaptable for various high-throughput applications.

MATERIALS AND METHODS

SDS−Polyacrylamide Gel Eectrophoresis (PAGE) Assay. Lyophilized proteins were obtained from Sigma [papain (PAP), chymopapain (CPAP), avidin (AVD), and superoxide dismutase (SOD)] and Calbiochem [streptavidin (SVD), serum amyloid P (SAP), and transthyretin (TTR)]. Salmonella phage P22 tailspike protein (TSP) was a gift from J. King (MIT). All of the remaining proteins (Table 1) were obtained from Sigma with the exception of catalase, which was purchased from Calbiochem. Stock solutions (1 mg/mL) of all proteins studied except PAP, CPAP, and TSP were made using 10 mM sodium phosphate buffer (PB) (pH 7.0). Stock solutions (1 mg/mL) of PAP and CPAP were prepared with 25 mM 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) and 1 mM EDTA (pH 5.3). The stock solution of the TSP was 0.8 mg/mL in 50 mM Tris and 2 mM EDTA (pH 7.6). All electrophoresis samples contained ~5 μg protein and 1% SDS in 0.125 M Tris (pH 6.8). Protein samples were unheated or boiled for 10 min prior to analysis by SDS−PAGE, using 15% Acrylamide Pager Gold precast gels (Cambrex) and 0.1% SDS in Tris/Glycine buffer (pH 8.3) as the running buffer. The gels were then stained using Coomassie blue.

Table 1: List of Proteins that Were Analyzed by SDS−PAGE to Assay for SDS-Resistance

protein	2° structure	number of subunits	SDS stable
ADH	mixed	1	no
AVD	β	2	yes
β amylase	α	1	no
carbonic anhydrase	mixed	1	no
catalase	α	1	no
CPAP	mixed	1	yes
chymotrypsin	mixed	2	no
ConA	β	2	no
γ crystallin	β	1	no
β glucoronidase	mixed	2	no
GAPDH	α	6	no
hemocyanin	mixed	6	no
hemoglobin	α	2	no
hyaluronidase	α	1	no
insulin	mixed	1	no
BLA	mixed	1	no
luciferase	α	2	no
lysozyme	mixed	1	no
β2M	β	1	no
neuraminidase	β	1	no
PAP	mixed	1	yes
TSP	β	3	yes
pectin lyase A	β	2	no
rhodanese	mixed	1	no
ribonuclease A	mixed	1	no
rubredoxin	β	1	no
SAP	β	5	yes
SVD	β	4	yes
SOD	β	2	yes
TTR	β	4	yes
TIM	α	2	no
trypsin	β	1	no
urease	mixed	2	no

Proteolysis. For limited proteolysis experiments, the concentration of the proteins was determined by weighing the lyophilized protein on a microbalance. Each proteolysis reaction mixture contained about 0.5 mg/mL protein and 5 μg/mL proteinase K (Fisher Scientific**)** in 25 mM Tris and 1 μM EDTA (pH 8.3) and was incubated at 25 °C for 48 h. The reaction was stopped with a solution of 2.5 μM phenylmethylsulfonyl fluoride and 4% SDS in 0.125 M Tris and 3.4 μM 1,4-dithio-DL-threitol (pH 6.8). Samples were boiled and gel electrophoresis was performed using 16% acrylamide Novex precast gels (Invitrogen). Running buffer was 0.1% SDS in Tris/tricine buffer (pH 8.1).

Fluorescence. Unfolding kinetics induced by guanidine hydrochloride (GdnHCl) were monitored using an F-4500 fluorescence spectrophotometer (Hitachi, Danbury, CT). The concentration of GdnHCl was determined using an Abbe Mark II refractometer (Leica, Buffalo, NY). Protein solutions (0.05 mg/mL) in 25 mM PB and 0.20 M sodium chloride (pH 7.2) were treated with GdnHCl solution made using the same buffer, to a final concentration of 6.6 M. The excitation/emission wavelengths used were 275/350 nm (β2M), 275/360 nm (BLA, ConA, GAPDH, and TIM), 280/320 nm (ADH), 295/360 nm (CPAP and TTR), 295/350 nm (PAP), 295/340 nm (SAP and TSP), 280/330 nm (SOD), 295/333

[1] Abbreviations: ADH, yeast alcohol dehydrogenase; AVD, avidin; β2M, β2-microglobulin; BLA, bovine α-lactalbumin; ConA, concanavalin A; CPAP, chymopapain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GdnHCl, guanidine hydrochloride; PAP, papain; PB, phosphate buffer; SAP, serum amyloid P; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SOD, copper/zinc superoxide dismutase; SVD, streptavidin; TSP, P22 tailspike protein; TIM, triosephosphate isomerase from porcine muscle; TTR, transthyretin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

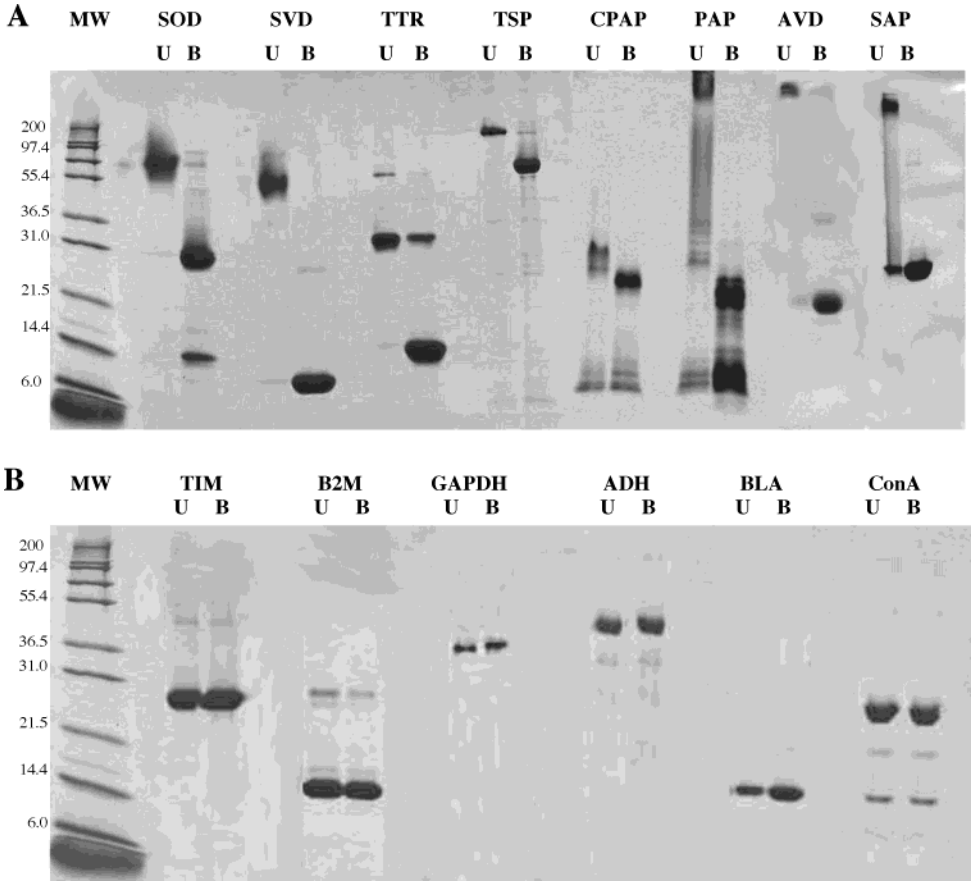

FIGURE 2: SDS−PAGE assay of SDS-resistant proteins (A) and the non-SDS-resistant control group (B). Identical protein samples were either unheated (U) or boiled (B) for 10 min immediately prior to loading onto the gel.

nm (SVD), and 280/340 nm (AVD). Kinetic traces were analyzed by fitting to a sum of exponentials.

RESULTS AND DISCUSSION

SDS-Resistant Proteins: An Assay for Kinetic Stability. The initial step of our study involved the identification of SDS resistance from a group of 33 proteins (Table 1). Among these were a few control proteins (SVD, TTR, and TSP) that were known to be both kinetically stable and SDS-resistant. SDS resistance was assayed by comparing the migration on a gel of boiled and unboiled protein samples containing SDS (Figure 2). Proteins that migrated to the same location on the gel regardless of whether the sample was boiled were classified as not being stable to SDS (Figure 2B). Those proteins that exhibited a slower migration when the sample was not boiled were classified as being at least partially resistant to SDS-induced denaturation (Figure 2A). The slower migration is a sign of decreased SDS binding and consequently of a lesser overall negative charge of the SDS−protein complex compared to the fully SDS-bound proteins. Of the proteins tested, eight were found or confirmed to exhibit resistance to SDS, including SOD, SVD, TTR, TSP, CPAP, PAP, AVD, and SAP (Table 1 and Figure 2A).

To probe the kinetic stability of our SDS-resistant proteins, we used fluorescence spectroscopy (Figure 3) and demonstrated their slow unfolding rates even in 6.6 M GdnHCl at 20 °C. To gather further evidence of the kinetic stability exhibited by these proteins under native conditions, their unfolding rate constants in the absence of the denaturant were obtained by measuring the unfolding rate at different GdnHCl concentrations and extrapolating to 0 M (Figure 4A). The native state unfolding rate constants for TTR (*17*) and SVD (*14*) were obtained from the literature. The unfolding rate in the absence of denaturants for all of the SDS-resistant proteins was found to be very slow (Table 2), with protein half-lives ranging from 79 days to 270 years. Thus, the fact that all of the SDS-resistant proteins are also kinetically stable, suggest that the latter property may be responsible for the former.

To further test the correlation between kinetic stability and SDS resistance, we selected a group of six proteins that did not exhibit resistance to SDS and analyzed their unfolding behavior in varying concentrations of GdnHCl. The group was chosen to represent a variety of structural characteristics, and consisted of porcine TIM, GAPDH, β2M, BLA, ConA, and ADH. At 6.6 M, the unfolding of these proteins was too fast to detect with a standard fluorescence spectrophotometer (data not shown). The lack of kinetic stability exhibited by these proteins was further demonstrated by their native unfolding rates, which ranged from 14 min to 19 h (Figure 4B and Table 2).

The above results support the existence of a correlation between kinetic stability and resistance to SDS-induced denaturation. Therefore, SDS−PAGE could serve as a simple method for identifying and selecting kinetically stable proteins. This method has the advantage that proteins can be easily tested for kinetic stability without having to carry out unfolding experiments. Also, only microgram amounts of sample are needed, and the method is potentially suitable for identifying kinetically stable proteins present in cell

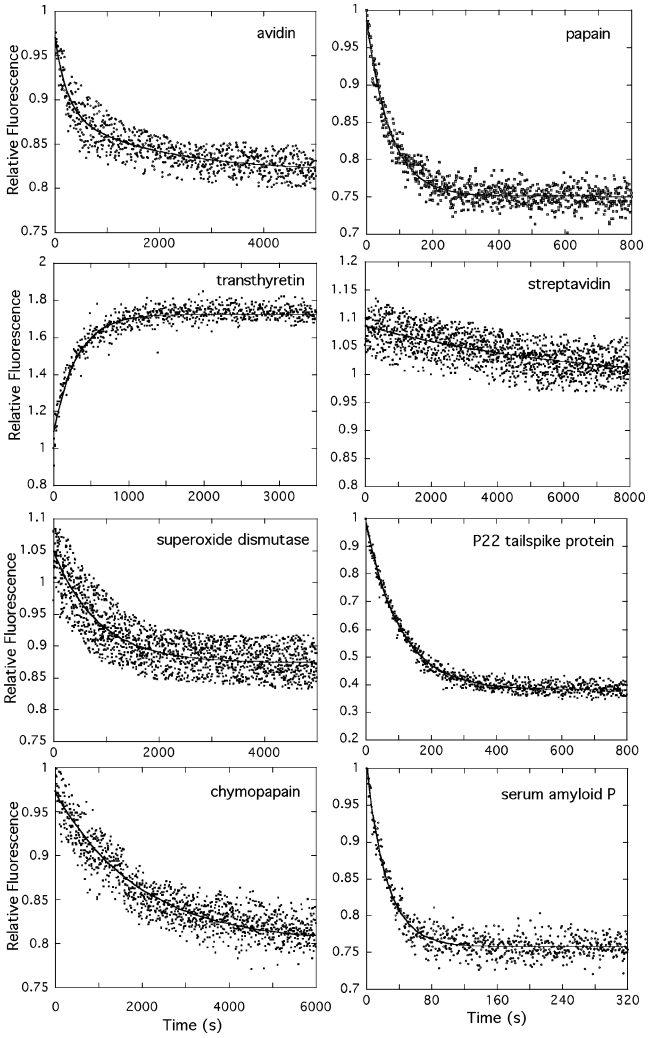

FIGURE 3: Fluorescence-detected unfolding kinetics of the SDS-resistant proteins upon incubation in 6.6 M GdnHCl at 20 °C. Relative fluorescence was obtained by dividing each data point by the first point of the native protein baseline. No missing amplitude was observed, indicating that the observed kinetic trace accounts for the full-unfolding transition.

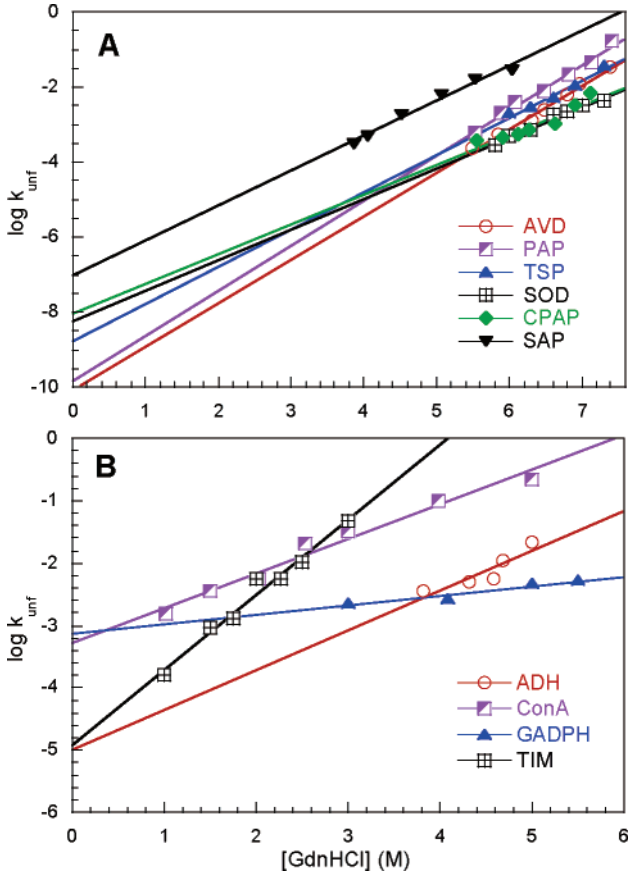

FIGURE 4: Unfolding rates of the SDS-resistant proteins (A) and non-SDS-resistant control group (B) under nativelike conditions are shown by extrapolating the unfolding rate determined at different concentrations of GdnHCl to 0 M. The *y* intercept of each extrapolation curve indicates the unfolding rate of the native protein (Table 2).

extracts without need for purification. From an application perspective, this assay has the potential of being adaptable for high-throughput applications to enhance the kinetic stability of proteins of interest. This could lead to proteins with greater shelf life and/or decreased tendency to aggregate, consistent with the suggestion that the deterioration of an energy barrier between native and pathogenic states as a result of mutation may be a key factor in the misfolding and aggregation of some proteins linked to amyloid diseases (*4*, *18*).

Why Are Kinetically Stable Proteins Resistant to SDS? SDS is an anionic detergent and a strong denaturant of proteins when present at concentrations above its critical micelle concentration (~7 mM in water) (*19*, *20*). Although there are examples in the literature of proteins that are not susceptible to denaturation by SDS, it is not clear what chemical−physical property is responsible for this resistance. It has been shown that there is no correlation between thermodynamic stability and SDS resistance (*21*). Although it has been suggested that surface charges in a protein can modulate SDS resistance, there is no general effect. For example, very acidic proteins, such as pepsin, will not interact with SDS because of charge repulsion (*21*). However, proteins with a high ratio of basic to acidic residues on the surface are not necessarily more susceptible to being denatured by SDS. We calculated the ratio of basic to acidic residues on the eight SDS-resistant proteins in our study and obtained a value of 1.2 ± 0.2, which is slightly higher than the ratio (0.95) that we calculated for the average protein based on amino acid composition.

To explore the correlation between SDS resistance and the structural rigidity that makes some proteins resistant to proteolytic cleavage, our SDS-resistant and non-SDS-resistant proteins were subjected to a proteolytic susceptibility test using the nonspecific and aggressive protease, proteinase K, at a protease to protein ratio of 1:100. As shown in Figure 5A, the SDS-resistant proteins remained largely intact after 48 h of incubation with proteinase K at 25 °C. Only TTR and SAP exhibited a small degree of degradation by proteinase K (Figure 5). The remarkable degree of resistance to proteolysis exhibited by the kinetically stable/SDS-resistant proteins is uncommon among proteins and hints at the unusual degree of structural rigidity that they possess. In contrast, of the 25 non-SDS-resistant proteins that we studied, 18 were completely degraded and 7 proteins exhibited some degree of proteolytic resistance. A representative group (same as in Figure 2B) is shown in Figure 5B. These results show that, whereas kinetically stable proteins are resistant to proteolytic cleavage, kinetic stability is not a requirement

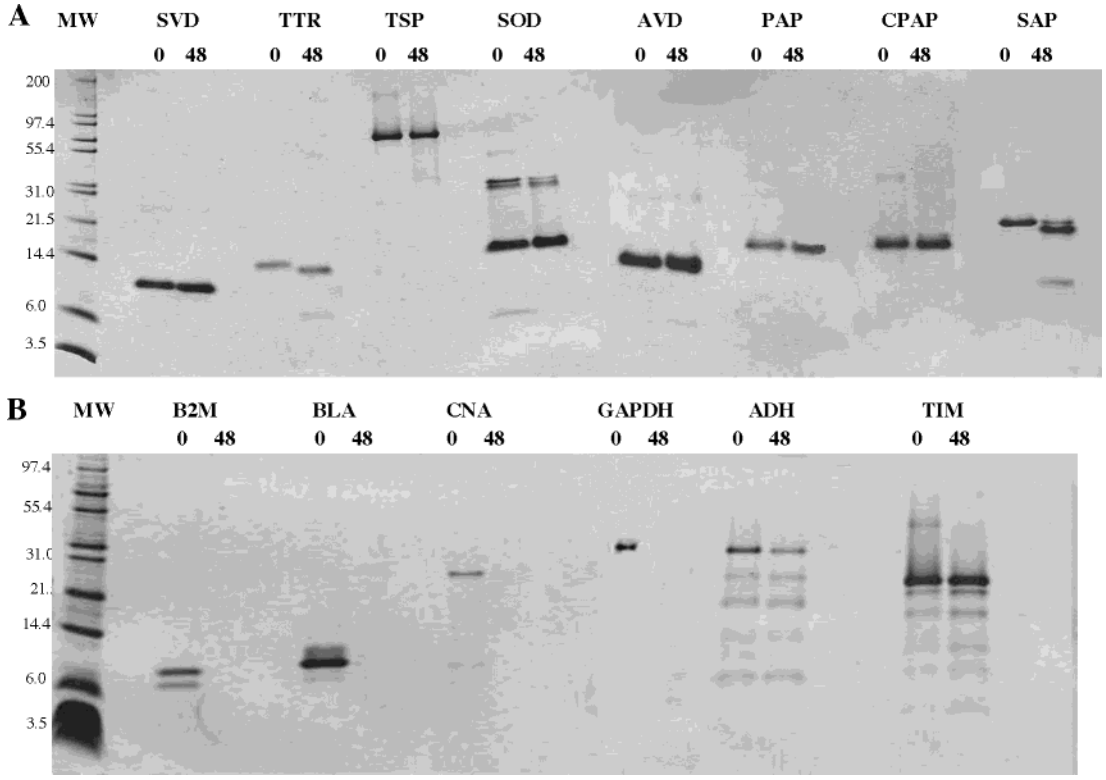

FIGURE 5: Proteolytic resistance of the SDS-resistant proteins (A) and proteolytic susceptibility of the nonresistant control group (B) are determined by incubating them for 48 h with proteinase K using a protease/protein ratio of 1:100 (w/w) and boiling the samples prior to loading onto the gel.

Table 2: Unfolding Rate Constant and Half-Lives of Proteins Resistant and Not Resistant to SDS

SDS-Resistant			
protein	k_{unf} (sec^{-1}) in 6.6 M GdnHCl	k_{unf} (sec^{-1}) in 0 M GdnHCl[a]	unfolding half-life
AVD	2.1×10^{-3}	8.1×10^{-11}	270 years
TTR	3.2×10^{-3}	9.0×10^{-11}	244 years[b]
PAP	1.5×10^{-2}	1.3×10^{-10}	165 years
TSP	1.0×10^{-2}	1.6×10^{-9}	13 years
SOD	1.7×10^{-3}	6.0×10^{-9}	3.7 years
CPAP	1.6×10^{-4}	8.8×10^{-9}	2.5 years
SVD	1.0×10^{-4}	2.5×10^{-8}	318 days[c]
SAP	4.0×10^{-2}	1.0×10^{-7}	79 days
Not SDS-Resistant			
protein	k_{unf} (sec^{-1}) in 6.6 M GdnHCl	k_{unf} (sec^{-1}) in 0 M GdnHCl[a]	unfolding half-life
ADH	unobservable[d]	1.0×10^{-5}	19 h
TIM	unobservable	2.8×10^{-5}	15 h
BLA	unobservable	1.6×10^{-5}	12 h[e]
β2M	unobservable	4.9×10^{-4}	24 min[f]
ConA	unobservable	5.3×10^{-4}	22 min
GAPDH	unobservable	8.2×10^{-4}	14 min

[a] These values were obtained by extrapolating the linear plots in Figure 4 to 0 M GdnHCl. Because of the long-range extrapolation, these values may have errors greater than 25%. [b] Value obtained from ref *17*. [c] Value obtained from ref *14*. [d] Rate was too fast (>1 s^{-1}) to observe by conventional manual mixing fluorescence spectroscopy. [e] Value obtained from ref *28*. [f] Value obtained from ref *29*.

for proteolytic resistance. The different requirements for SDS and protease resistance may be due to the nonspecific binding of SDS to unfolded proteins in contrast to the specific binding requirement of proteases. While a protease requires the binding of specific structural elements to its active site to initiate protein cleavage, SDS appears to only require access to the interior of the protein. It has been shown that protease resistance is influenced not only by the exposure of the unfolded chain, but also by sequence determinants within the unfolded protein that may caused these proteins to be poor substrates for proteolytic degradation (*22*). Thus, SDS resistance is a much more effective probe for identifying proteins with high kinetic stability.

Our results point to kinetic stability as the molecular basis for the resistance of some proteins to SDS. Because each of the SDS-resistant proteins that we studied was also found to be resistant to proteolysis by proteinase K, we propose that, like in the case of proteolytic susceptibility, resistance to SDS is linked to the reduced occurrence of both local- and global-unfolding transitions in these proteins. Like proteases, SDS binding appears to rely on transitions between protein conformations, moments of weakness in which the protein is susceptible to SDS binding, and thereby entrapment. Kinetically stable proteins are characterized by unusually low structural flexibility (*12*, *23*). The structural rigidity of kinetically stable proteins results in suppression of partial unfolding. Furthermore, Truhlar et al. have shown that it is not only the barrier toward global unfolding, but also the high cooperativity of the unfolding transition of kinetically stable proteins that results in its protease resistance (and presumably SDS resistance) by limiting partial-unfolding transitions (*24*). Thus, this may explain why unless provided with energy in the form of heat (e.g., through boiling), kinetically stable proteins infrequently assume such open conformations under native conditions and are therefore resistant to SDS.

Implications for the Physical Basis of Kinetic Stability. A key to understanding kinetic stability in proteins may lie in determining the physical basis for their structural rigidity, because this appears to be a common property of kinetically

stable proteins (*12*, *23*). Arguably, the most compelling evidence that rigidity may be the key physical requirement for protein kinetic stability comes from the observation that proteins become highly rigid and kinetically stable when incubated in very high concentrations of organic solvent (*25*, *26*). In such an environment, the absence of bulk water presumably reduces the energetic driving force for partial and global unfolding, thereby increasing rigidity (*26*). Under a normal aqueous environment, kinetically stable proteins may owe their rigidity to the lack of weak points on its surface where bulk water could penetrate to induce local and global unfolding. Consistent with this idea, recent work by Machius et al. has shown that kinetic stability can be increased by introducing hydrophobic mutations at the surface of the protein to stabilize and rigidify regions that may be involved in local unfolding (*5*). Furthermore, strategically located metal ions, disulfide bonds, salt bridges, and hydrophobic residues at the surface may be useful for enhancing the kinetic stability of a given protein by serving as molecular "clips" or "staples" to avoid water penetration, resulting in rigid structures where partially unfolded states are not sampled under nativelike conditions.

Are there any structural features that may bias a protein toward possessing kinetic stability? In an attempt to better understand the structural basis for kinetic stability, we performed a rudimentary structural analysis on our pool of SDS-resistant proteins based on their structure coordinates obtained from the Protein Data Bank (PDB) (Figure 6). Each protein was found to exhibit specific stabilizing characteristics, including disulfide bonds (PAP, CPAP, and SOD), oligomeric interfaces (all except PAP and CPAP), and bound metals (SOD). Amino acid composition calculations based on the PDB coordinates likewise yielded no common trend in the amino acid content and no consistent deviation from the amino acid composition found in natural proteins, implying that no correlation exists between kinetic stability and primary structure. However, the presence of predominantly β-sheet and oligomeric structures emerged as a common characteristic of most of the kinetically stable proteins that we studied. It is plausible that the higher content of nonlocal interactions in β-sheet proteins may allow for higher rigidity than in α-helical proteins. Although clearly not all oligomeric β-sheet proteins are kinetically stable/SDS-resistant (see Table 1), the apparent bias for kinetic stability in oligomeric β-sheet proteins may serve to prevent them from aggregation. When induced to assume a partially or globally unfolded conformation, β-sheet proteins are particularly susceptible to misfolding and aggregation, potentially leading to protein misfolding diseases (*18*). It has been shown that to avoid aggregation natural β-sheet proteins use various negative design strategies, such as the placement of loops, β bulges, prolines, and charged residues at the end of β sheets (*27*). Perhaps kinetic stability may be another strategy used by nature to minimize the potential for protein misassembly. It will be interesting to determine how widespread this property is among other oligomeric and/or β-sheet proteins and whether it correlates with their tendency toward aggregation. Despite the apparent bias toward β-sheet proteins, the observation of kinetic stability and SDS resistance in PAP and CPAP, which are monomeric proteins containing an α and a β domain, shows that these properties are also accessible to other proteins.

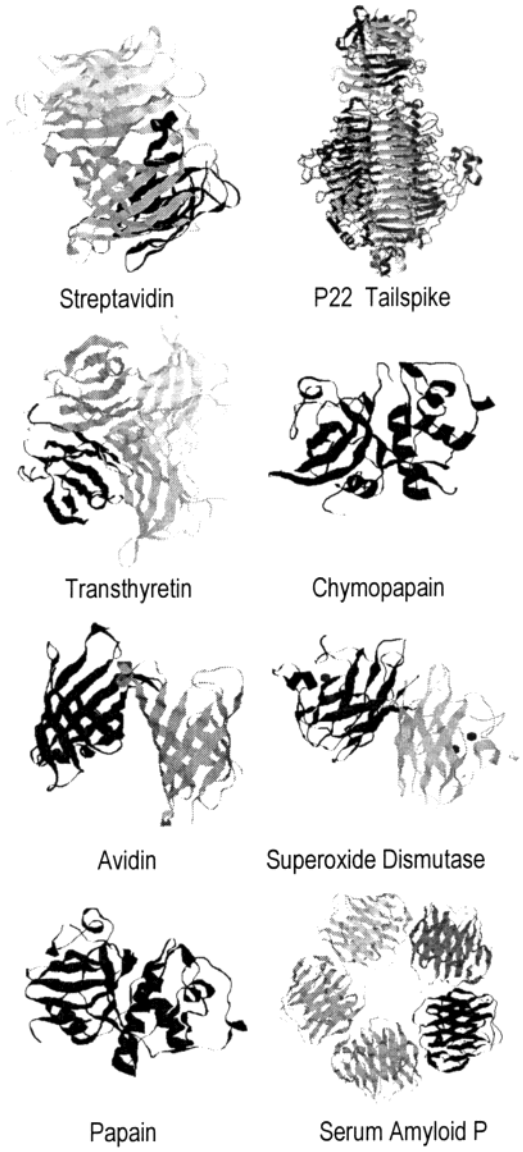

FIGURE 6: Ribbon diagrams of the SDS-stable proteins reveal a high content of oligomeric β-sheet structures. Coordinates were obtained from the Protein Data Bank using the following PDB codes: SOD, 1SPD; SVD, 1SWU; TTR, 1GKE; TSP, 1TYU; CPAP, 1YAL; PAP, 1PPN; AVD, 1RAV; and SAP, 1SAC.

CONCLUSION

In summary, our results suggest that SDS-resistance is a common property of kinetically stable proteins and that SDS−PAGE may be used as a simple assay to probe for kinetic stability in purified proteins or protein extracts. We propose that, analogous to proteolytic susceptibility, proteins become vulnerable to denaturation by SDS during their partial- and global-unfolding transitions. However, because of the nonspecific nature of SDS denaturation, it is highly effective in detecting proteins that possess high kinetic stability. Together, these results provide compelling support for the idea that kinetic stability is a common property of rigid protein structures. We anticipate that our kinetic stability assay may allow us to build a larger database of kinetically stable proteins, thus paving the way for further studies geared toward understanding the relationship between kinetic stability and protein structure.

ACKNOWLEDGMENT

We are grateful to Jonathan King for providing the P22 tailspike protein sample, to Anne Robinson for the picture of the P22 tailspike trimer, and to Jinhyuk Chung for critical reading of the manuscript.

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BI0491898