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pH Regulation of the Kinetic Stability of the Lipase from *Thermomyces lanuginosus*

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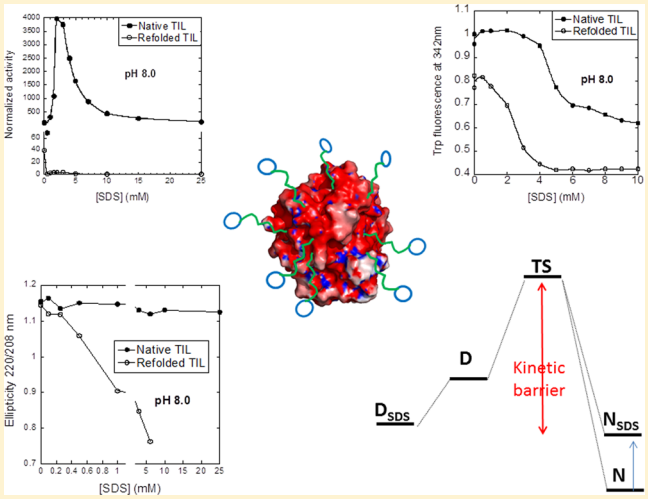
ABSTRACT: *Thermomyces lanuginosus* lipase (TlL) is a kinetically stable protein, resistant toward both denaturation and refolding in the presence of the ionic surfactant sodium dodecyl sulfate (SDS) and the nonionic surfactant decyl maltoside (DecM). We investigate the pH dependence of this kinetic stability. At pH 8, TlL remains folded and enzymatically active at multimillimolar surfactant concentrations but fails to refold from the acid urea-denatured state at submillimolar concentrations of SDS and DecM, indicating a broad concentration range of kinetic trapping or hysteresis. At pH 8, very few SDS molecules bind to TlL. The hysteresis SDS concentration range shrinks when moving to pH 4−6; in this pH range, SDS binds as micellelike clusters. Although hysteresis can be eliminated by reducing disulfide bonds, destabilizing the native state, and lowering the unfolding activation barrier, SDS sensitivity is not directly linked to intrinsic kinetic stability [its resistance to the general chemical denaturant guanidinium chloride (GdmCl)], because TlL unfolds more slowly in GdmCl at pH 6.0 than at pH 8.0. However, the estimated net charge drops from approximately −12 to approximately −5 between pH 8 and 6. SDS denatures TlL at pH 6.0 by nucleating via a critical number of bound SDS molecules on the surface of native TlL to form clusters. These results imply that SDS sensitivity is connected to the availability of appropriately charged regions on the protein. We suggest that conformational rigidity is a necessary but not sufficient feature of SDS resistance, because this has to be combined with sufficient negative electrostatic potential to avoid extensive SDS binding.



Most proteins are able to unfold and refold reversibly during a short time span, normally from fractions of a second to a few minutes. This means that the activation barrier separating the folded and denatured state is relatively low, allowing the protein to rapidly access the state that is thermodynamically most stable under the given conditions. However, some proteins are able to retain their native structure and activity if exposed to denaturing conditions after folding, despite an inability to fold from the denatured to the native state under these same conditions. This phenomenon is termed kinetic stability. A recent review of kinetic stability lists a large number of protein systems for which evidence of kinetic stabilization has been reported.[1] Kinetically stable proteins are trapped in their native conformations by a high energy barrier and consequently tend to be more resistant to unfolding under extreme solvent conditions, such as charged surfactant, extremes of pH, mechanical forces, and the presence of chemical denaturants, even though the overall change in the Gibbs free energy (ΔG) favors the unfolded state. At sufficiently slow conversion rates, the protein is for all intents and purposes completely trapped in the native state, which allow proteins to maintain activity under the extreme conditions they may encounter in vivo.[2] Many kinetically stable proteins can withstand exposure to a denaturing surfactant such as sodium dodecyl sulfate (SDS) unless they are boiled and may therefore be detected by comparing their migration on an SDS−polyacrylamide gel electrophoresis (PAGE) gel before and after boiling.[3] However, kinetic stability is not an all-or-none property and simply entails a relatively high barrier to unfolding and refolding that may or may not

Received: September 14, 2012
Revised: December 13, 2012
Published: December 18, 2012

include resistance to SDS unfolding; thus, not all proteins will necessarily be detected by the SDS boiling approach.

Much effort has been devoted to a physical−structural explanation of kinetic stability. Hydrophobic residues on the protein surface,[4] disulfide bonds,[5] metal-binding sites,[6] and solvation barriers[7] increase kinetic stability. A connection between kinetic stability and oligomeric (β-sheet rich) quaternary structure has also been proposed.[8] For some hyperthermophilic proteins, electrostatic interactions have been suggested to be a major factor in their slow unfolding because of the formation of ion pairs.[9,10] Long-range interactions as well as rigidity may play an important role.[11] The majority of kinetically stable proteins reported so far have a high content of β-sheet structure. α-Helix interactions tend to be local, i.e., close in the primary sequence, and isolated α-helical structure can in some cases be retained despite a loss of tertiary interactions. In contrast, β-sheet interactions are more global and tend to form only late in the folding process; conversely, their unraveling requires extensive unfolding. All these may play a role in the way in which denaturants are able to bind and denature the proteins. For example, proteins denatured in surfactants such as SDS tend to adopt α-helical structures,[12] and individual α-helices have been identified as the initial site of attack in a protein engineering study of the denaturation of the mixed α/β-protein S6.[13] Nevertheless, secondary structure per se is not linked in a simple fashion to kinetic stability: some all-α-proteins are known to be kinetically stable,[14] and the kinetic stability of α-lytic protease appears to be determined by the unfolding of a β-hairpin.[15] However, no monomeric α-helical proteins have been identified. Long-range interactions, e.g., between individual subunits, therefore seem to play a central role in protein kinetic stability. Furthermore, not all proteins with β-structure are kinetically stable, as illustrated by the two β-sandwich proteins TII27 and TnfN3.[16] Despite the fact that they share the same fold, TII27 could be denatured by SDS monomers while Tnfn3 was denatured only in the presence of SDS micelles. This was ascribed to differences in surface electrostatic potential that affect protein−SDS interactions. The underlying effect of β-structure was, however, evident in that the unfolding kinetics were significantly slower than those of the α-helix-containing proteins we have studied, namely, the mixed α/β-proteins S6 and CI2[13,17] and the all-α-helix myoglobin.[18]

Here we systematically investigate the kinetic stability of the 269-residue lipase (triacylglycerol acylhydrolase) from *Thermomyces lanuginosus* (TlL). This enzyme has been shown to be kinetically stable by Sanchez-Ruiz and co-workers; a detailed analysis that combined experimental analyses of different mutants with computational simulations led to the proposal that the kinetic stability of TlL arises from a large solvation barrier, caused by the lack of synchronicity between water penetration and the severing of intramolecular interactions.[7] TlL finds widespread use in the detergent industry because of its resistance to inactivation under harsh washing conditions. Kinetic stability is a likely prerequisite for TlL's ability to remain active at a surfactant concentration that normally denatures most other proteins. We have previously shown that it can be activated as well as inhibited by surfactants,[19] reflecting the presence of a helix forming a flexible lid over the active site. The lid needs to be opened to allow substrate access, and this opening, which is triggered by adsorption onto an interface or substrate, clearly alters the overall flexibility of the protein and thus the susceptibility to surfactants. TlL has a mixed α/β-fold and a catalytic triad composed of Ser, Asp, and His, similar to what is found in serine proteases. It also contains three disulfide bridges (Cys36−Cys41, Cys104−Cys107, and Cys22−Cys268) that stabilize the tertiary structure and four tryptophans that are useful probes of changes in tertiary structure.

We show that TlL is a kinetically stable protein that resists SDS denaturation at pH 8 yet is unable to refold even at very low concentrations of SDS and the nonionic surfactant decyl maltoside (DecM). However, the SDS concentration range over which this hysteresis is observed shrinks when the pH is decreased to ≤6. This shrinkage coincides with the ability of SDS to form micellelike clusters on TlL. These micellelike clusters do not form under hysteresis conditions. Unfolding in buffer, extrapolated from high concentrations of the denaturant guanidinium chloride, shows a half-life of ∼14 days at pH 10, ∼77 days at pH 8, and 2.3 years at pH 6 but only 0.6 h at pH 4. This indicates a very high and very pH-dependent activation barrier associated with unfolding. As a consequence, TlL is trapped in the native state around neutral pH. Thus, pH plays a key role in the kinetic stability of TlL. Lowering the pH promotes SDS binding, lowering the activation barrier to unfolding at pH 6 despite the protein's intrinsic slow folding at this pH. Thus, SDS clusters on the protein surface are key agents in surfactant-mediated denaturation.

MATERIALS AND METHODS

Chemicals. Tris(hydroxymethyl)aminomethane (Tris), SDS, and glycine were from AppliChem (Darmstadt, Germany). Sodium acetate was from Merck. Decyl maltoside was from Anatrace. All other chemicals were from Sigma-Aldrich (St. Louis, MO). All chemicals were of the highest grade available. Experiments were performed at 25 °C unless otherwise stated.

TlL Preparation. TlL was provided by Novozymes A/S as a liquid formulation, diluted with water, dialyzed extensively against water at 5 °C for 24 h, centrifuged to remove small amounts of precipitate, and stored at −80 °C. The protein concentration was determined using a molar extinction coefficient of 37275 M^{-1} cm^{-1}.

Unfolding Kinetics in GdmHCl. TlL unfolding kinetics were investigated using a Cary Eclipse fluorescence spectrophotometer (Varian). Different concentrations of GdmHCl in 10 mM buffer were mixed with 20 μL of 50 μM TlL (final volume of 1 mL) in a 1.5 mL cuvette using magnetic stirring. Fluorescence was recorded using excitation at 295 nm and emission at 338 nm. Slit widths of 5 nm were used. The final TlL concentration was 1 μM, and the temperature was held constant at 25 °C unless specified. Rate constants were obtained by fitting fluorescence data to a single-exponential decay.

Interaction of Native TlL with Surfactants or GdmCl. TlL was equilibrated with varying concentrations of SDS or GdmCl in 10 mM buffer at appropriate pH values. Fluorescence spectra were recorded on an LS-55 luminescence spectrometer (Perkin-Elmer Instruments) using 1.0 μM TlL with excitation at 295 nm and slit widths of 10 nm. CD spectra were recorded on a JASCO J-715 spectropolarimeter (Jasco Spectroscopic Co. Ltd.) with 5.0 μM TlL using a data pitch of 0.2 nm, a scan speed of 50 nm/min, a response time of 2 s, a bandwidth of 1 nm, and three accumulations. Temperature scans were measured use a scan speed of 90 °C/h from 20 to 100 °C.

Refolding of TlL in a Surfactant or GdmCl. TlL (25 μM) was initially unfolded for 1 h in 10 mM glycine (pH 2) and 6 M urea (for SDS refolding experiments) or in 8 M GdmCl at the appropriate pH (for GdmCl refolding experiments). Refolding was initiated by a 25-fold dilution with 10 mM buffer at the appropriate pH to 1 μM TlL. Folding was monitored by CD, fluorescence, or activity at different times. Equilibrium folding of TlL in the absence or presence of 5 mM reducing agent TCEP was conducted using the same approach described above. In unfolding experiments, 25 μM TlL was incubated with 5 mM TCEP in 10 mM Tris (pH 8) for 1 h prior to being mixed with SDS. In refolding experiments, 25 μM TlL was incubated with 5 mM TCEP in 10 mM glycine (pH 2) and 6 M urea for 1 h prior to being mixed with SDS in 10 mM Tris (pH 8).

Pyrene. Pyrene is a highly hydrophobic molecule with low solubility in water (2−3 μM). The ratio of the emission peaks at 372.5 nm (I_1) and 383.5 nm (I_3) can be used to evaluate the polarity of its environment.[20] TlL was mixed with appropriate amounts of SDS and buffer to a final concentration of 1 μM. After equilibration for 30 min, pyrene was added from a 50 μM stock in ethanol to a final concentration of 1 μM. Scans were performed from 360 to 410 nm using an excitation wavelength of 335 nm and excitation and emission slit widths of 5 and 2.5 nm, respectively.

Isothermal Titration Calorimetry. Calorimetric measurements were conducted on an ITC apparatus (MicroCal Inc., Northampton, MA). The reference cell was filled with water, and the sample cell was loaded with a solution of 0.2−4 mg/mL (6.7−135 μM) TlL. The cell solution was titrated with aliquots of 3 or 5 μL of 100 mM SDS in buffer [10 mM Tris (pH 8.0), 50 mM MES (pH 6.0), or 50 mM NaOAc (pH 4.0)]. All experiments were conducted at 25 °C. Heat signals were integrated using Origin.

Activity Assays. Activity assays in the presence of SDS and DecM were performed using 866 nM TlL in 50 mM buffer at the appropriate pH and 2 μM 4-(trifluoromethyl)umbelliferyl butyrate (TFUB) (diluted from a 10 mM stock in DMSO). Immediately after the TlL and substrate had been mixed, the mixture was transferred to a cuvette and the emission at 500 nm measured over 2 min using an excitation wavelength of 356 nm and excitation and emission slit widths of 2.5 nm, giving rise to linear plots over this time period. The relative activity was obtained as the slope of these plots. For refolding experiments, TlL was first denatured in 6 M urea and 10 mM glycine (pH 2.0), diluted 100-fold in the presence of different concentrations of surfactant, and then equilibrated for 1 h, after which activity was measured as described previously. The activity assays in the presence of SDS and GdmCl at different temperatures were measured using a concentration of 100 nM TlL in 50 mM Tris (pH 8.0) and 360 μM *p*-nitrophenyl butyrate (diluted from a 100 mM stock in ethanol). TlL and *p*-nitrophenyl butyrate were incubated with SDS or GdmCl at the appropriate temperature for 5 min before being mixed. Immediately after the TlL and substrate had been mixed, the mixture was transferred to a cuvette and the absorption at 400 nm measured over 2 min, giving rise to linear plots over this time period. The slope of these plots is used as a relative measure of activity.

Protease Assay. Protease assays were conducted using final concentrations of 240 μg/mL TlL and 0−30 μg/mL trypsin. At different time points, solutions were taken out and mixed with 2× loading buffer (containing SDS and DTT), boiled for 10 min, and subjected to SDS−PAGE.

Visualization of the Electrostatic Potential on the Solvent Accessible Surface. Possible interaction sites with surfactants were investigated by calculating and visualizing the electrostatic potential on the solvent accessible surface of TlL. Protein Data Bank (PDB) entry 1TIB[21] was used in the investigation, and PDB2PQR[22,23] was used to assign correct side chain pK_a values at pH 8, 6, and 4. Electrostatics were calculated using ABPS[24] under default parameters (Protein Dielectric, 2.0 Solvent dielectric, 80.0). Visualization of the potential was performed in PyMOL[25] where maps were contoured at ±5 kT/e.

RESULTS

TlL Is Globally Unfolded in High Concentrations of Urea at pH 2. In this study, we attempt to elucidate the properties that allow *T. lanuginosus* lipase (TlL) to remain active in the presence of surfactants that normally denature most proteins. We first need to identify conditions for unfolding TlL to a state that can be used as a starting point for refolding studies. Complete unfolding of TlL cannot be accomplished by high concentrations of urea at neutral pH or by lowering the pH, although a partial loss of secondary structure is observed at pH 2 (Figure 1). However, a combination of the two [urea in 10 mM glycine (pH 2.0)] leads to the formation of a random coil structure. Titration with increasing concentrations of urea reveals a denaturation midpoint of 3.24 M urea (Figure 1). We therefore use a combination of 10 mM Gly (pH 2) and 6 M urea to unfold TlL.

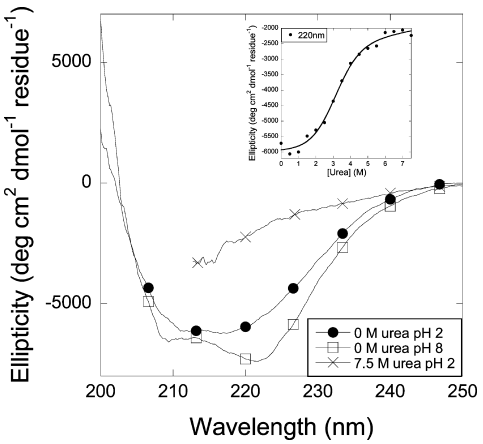


Figure 1. pH and urea unfolding of TlL. Spectra at pH 8 show native secondary structure, while some structure has been lost at pH 2. Addition of 7.5 M urea at pH 2 results in a random coil conformation. The transition to random coil is illustrated in the inset where the ellipticity at 220 nm is plotted as a function of urea. The data are fit to a two-state unfolding curve[45] to yield a denaturation midpoint of 3.2 ± 0.1 M urea.

TlL Is Trapped in Different Conformations in the Presence of Surfactants, Depending on the Starting Conditions, pH, and Surfactant Concentration. When a protein has reached equilibrium, the rates of folding and unfolding will be equal, according to the principle of microscopic reversibility. For kinetically stable proteins, the underlying rate constants will be so slow (with half-lives on the order of months to years) that this equilibrium may not be

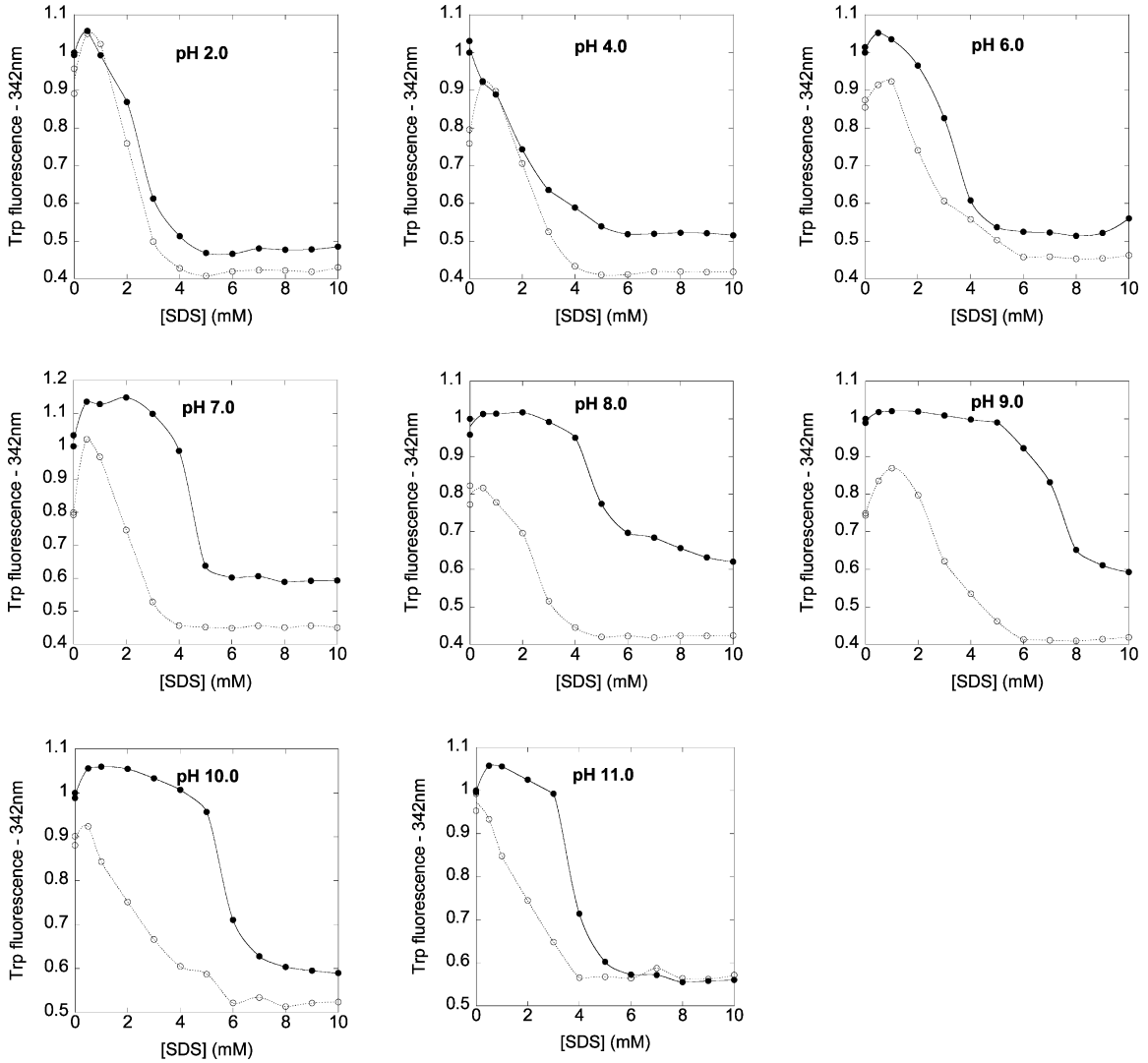


Figure 2. TlL–SDS interactions measured with Trp fluorescence at different pH values as a function of SDS concentration. Lines are interpolations to guide the eye. Filled circles with solid lines indicate data for samples in which SDS had been added to native TlL, while empty circles with dotted lines indicate data for samples in which TlL is refolded from 6 M urea (pH 2) in the presence of SDS. Solutions were incubated for 24 h prior to measurement. Trp fluorescence units were normalized so they are all 1 at 0 mM SDS for the native (unfolding) state at different pH values.

established under normal experimental conditions. Thus, these proteins may not reach the same structural and functional state in a manner independent of the starting conditions on the experimentally accessible time scale. An analysis of kinetic stability requires us to compare the structure and activity of TlL over a range of surfactant concentrations, starting out from conditions where the protein is initially either in the native state or in the denatured state. As surfactants, we use anionic SDS and nonionic decyl maltoside (DecM). We have titrated native TlL with SDS in 10 mM buffer at different pH values and monitored the reaction using Trp fluorescence (Figure 2). At pH 2 and 4, the tryptophan fluorescence of native TlL decreases with an increasing SDS concentration without any native baseline region, indicating a low level of resistance against SDS denaturation. At pH 6–11, there is a small but significant increase in tryptophan fluorescence around 0–0.5 mM SDS, which then levels out at different concentrations of SDS, followed by a major decrease in fluorescence. Far-UV CD spectra at pH 8.0 (Figure 3) of TlL_{native}^{SDS} reveal no significant change in secondary content in SDS.

A comparison of the enzymatic activity (Figure 4) and fluorescence data (Figure 2) of native TlL reveals a marked increase in activity at low SDS concentrations. SDS and other surfactants are known to increase TlL activity at concentrations below the cmc, most likely by inducing local conformational changes that promote opening of the lid covering the active site.[19] This activation occurs during the native state baseline region in the fluorescence titration; i.e., the activity increase does not lead to changes in the Trp environment. The decrease in activity at higher SDS concentrations is accompanied by a decrease in Trp fluorescence. However, at pH 8, native TlL remains nativelike in the presence of 10 mM SDS, but the melting temperature (t_m) decreases from 72 to 40 °C (Figure 5 and ref 26). At pH 2–7 and 9–11, native TlL denatures in the presence of 10 mM SDS because we observed clear changes in secondary structure between 0 and 10 mM SDS (cf. data shown for pH 9 in Figure 5). Furthermore, native TlL in 10 mM SDS loses the ability to unfold cooperatively in thermal scans (Figure 5). Note that the regions characterized by nativelike fluorescence and enzymatic activity shrink with a decrease in pH, which means that the ability of native TlL to resist SDS

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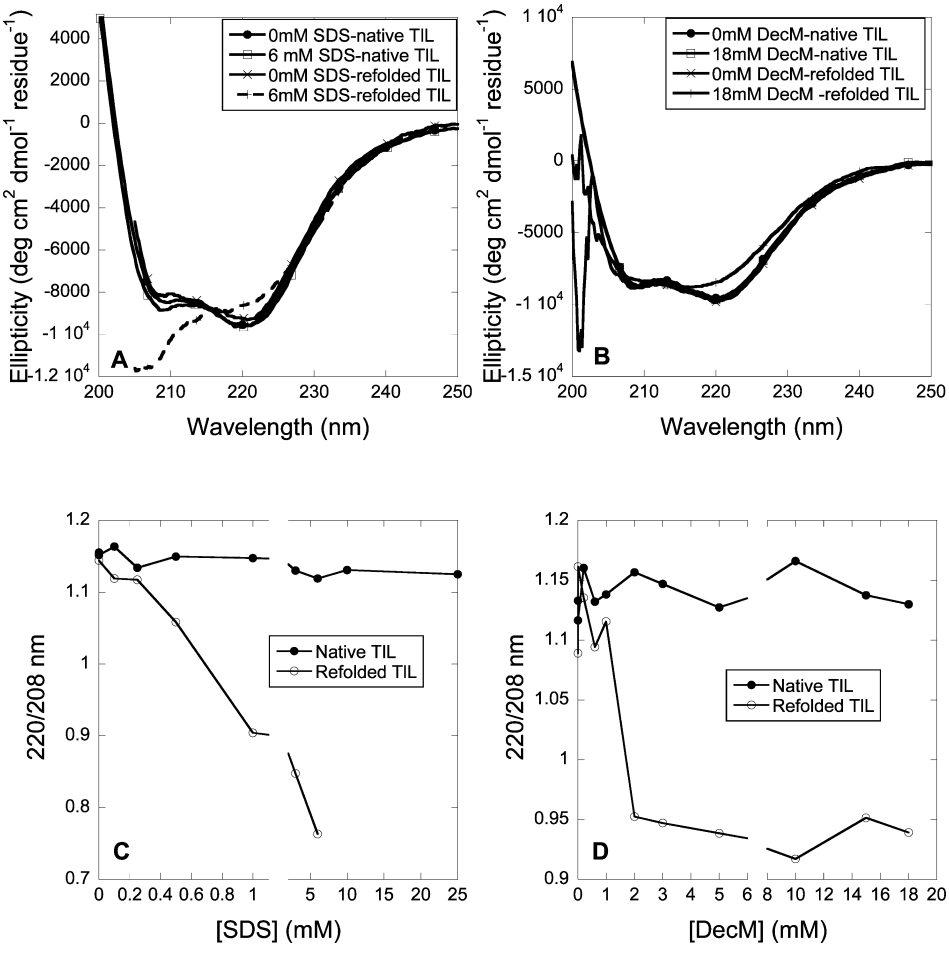

Figure 3. Effect of SDS and DecM on TlL folding and refolding at pH 8. (A) Far-UV CD spectra of native TlL with and without 6 mM SDS and unfolded TlL refolded in the absence and presence of 6 mM SDS. (B) Far-UV CD spectra of native TlL with and without 18 mM DecM and unfolded TlL refolded in the absence and presence of 18 mM DecM. The 220 nm/208 nm ratio reports on the interference of SDS (C) and DecM (D) in the refolding process.

denaturation weakens with a decrease in pH. A similar shrinkage is seen in activity assays when we keep the pH at 8.0 but increase the temperature from 20 to 60 °C, although limited activation is observed even at 60 °C (Figure 4C).

We now turn to the refolding of TlL from the pH- and urea-denatured state (pH 2.0 and 6 M urea) to pH 4−8 and low urea concentrations in the presence of surfactant. In the absence of surfactant, the fluorescence and CD spectra of refolded and native TlL are almost the same at pH 8.0 (Figures 2 and 3), showing that it is possible to refold TlL. Furthermore, activity is largely restored (Figure 4). However, in the presence of SDS, the refolding efficiency is markedly diminished. This effect is most dramatic in activity measurements where a sharp decrease in activity of refolded TlL is observed as the SDS concentration increases to 1 mM; above 1 mM SDS, no activity can be detected (Figure 4A−C). Above pH 6, we also observed clear differences between the fluorescence of native and refolded TlL upon titration with SDS (Figure 2), although the hysteresis in fluorescence intensity is not so pronounced as for activity. CD spectra also indicate a difference between the secondary structure of native and refolded TlL at pH 8.0: CD spectra of TlL refolded in the presence of higher concentrations of SDS (e.g., 6 mM in Figure 3A) are typical of SDS-denatured proteins, where a shift in the relative intensities at the two minima (220 and 208 nm) is often observed.[27] This makes the 220 nm/208 nm ratio a good indicator of the degree of denaturation (Figure 3C,D).

When refolding takes place in the presence of the nonionic surfactant decyl maltoside (DecM), the change in CD spectra is most profound at concentrations above the cmc (~2 mM) (Figure 3B,D). The activity of TlL refolded into DecM decreases with the same monotonic decline as in SDS, while the activity of native TlL initially decreases but then increases to a high plateau level at high DecM concentrations (Figure 4), also at pH values as low as 4. Clearly, DecM−lipase interactions are not nearly as pH-dependent as SDS−lipase interactions. The pH-dependent activation of TlL by SDS is most probably related to titration of charged side chains.

We also decided to investigate how much TlL's three disulfide bonds contribute to kinetic stability at pH 8. Both native and unfolded TlL were incubated with 5 mM reducing agent TCEP prior to exposure to SDS. Clearly, reduction of the disulfide bonds destabilizes TlL (Figure 6). While the fluorescence intensity for nonreduced TlL is almost constant up to 4 mM SDS, for reduced TlL the fluorescence decreases sharply already around 1 mM SDS. Importantly and in strong contrast to the case for nonreduced TlL, there was no hysteresis between the refolding and unfolding curves for reduced TlL. Refolding curves for reduced and nonreduced TlL are essentially identical, but this is probably fortuitous as the

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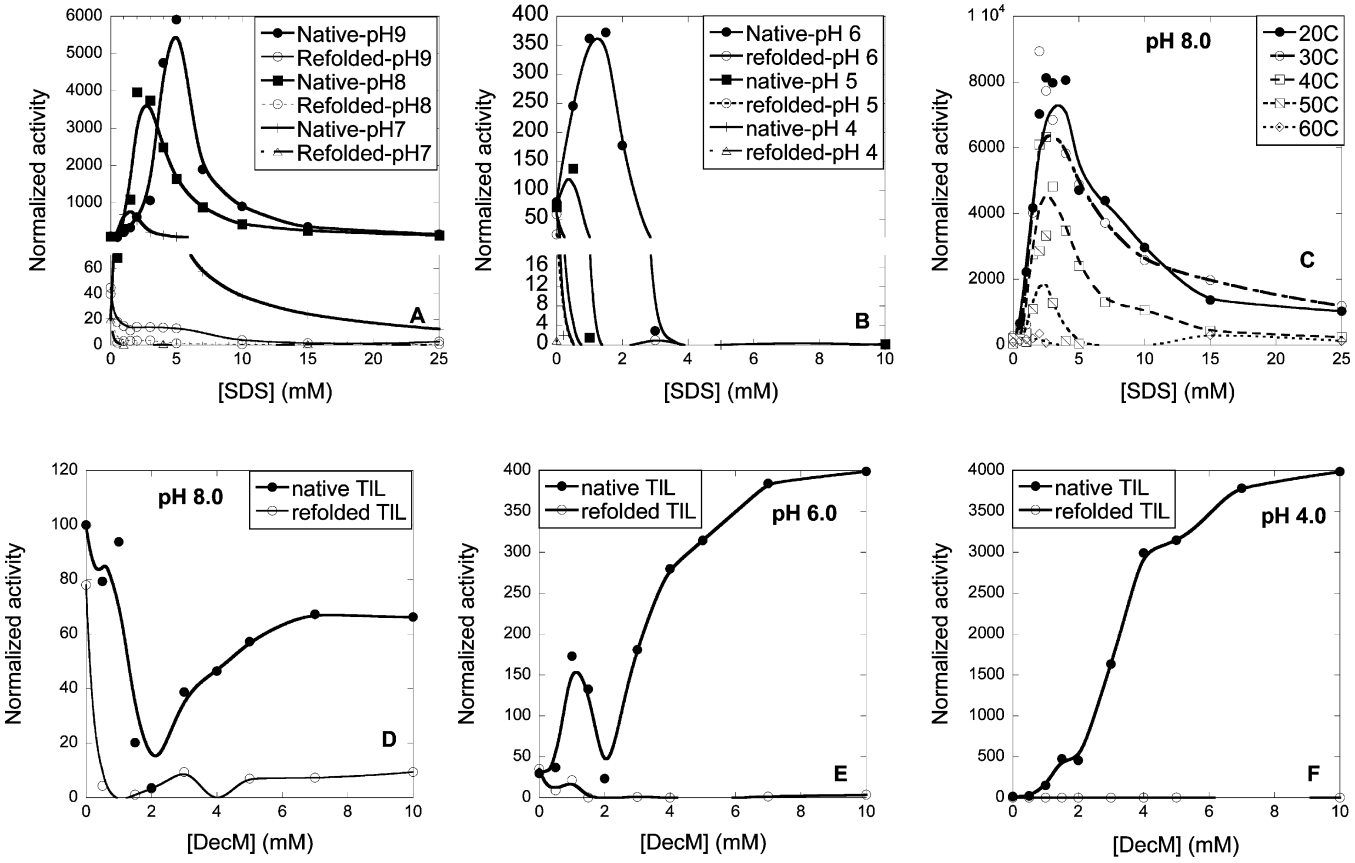

Figure 4. Relative enzymatic activity of native and refolded TlL toward TFUB at different pH values in the presence of SDS (A and B) or DecM (D–F). Activity units were normalized to pH 8.0 in buffer, so TlL has 100% activity at 0 mM SDS or DecM for the native (unfolding) state at pH 8.0 and 25 °C. We cannot measure activity at pH 10 and 11 because of the fast autohydrolysis of TFUB at these two pH values. Note that the maximum of the *Y* axis in panel A is 6000 units but only 400 units in panel B, reflecting the marked decline in activity with lowered pH. (C) Relative enzymatic activity of native TlL at different temperatures in the presence of SDS. Activity units were normalized, so native TlL has 100% activity at 0 mM SDS (pH 8.0) and 20 °C. All lines were constructed by interpolation to guide the eye.

denatured and native states are very different in the two experiments.

Isothermal Titration Calorimetry Shows Enhanced Binding of SDS to TlL at pH 6 and 4. Clearly, pH affects TlL–SDS interactions, and this is likely to be reflected in the number of SDS molecules binding to TlL. Stoichiometric and thermodynamic information about the interactions between TlL and SDS is provided by isothermal titration calorimetry (ITC). Different concentrations of TlL were titrated with SDS at pH 4, 6, and 8, leading to enthalpograms with several characteristic points (Figure 7). The enthalpograms can be described by a number of exothermic processes (Figure 7): two peaks (points 1 and 3) at pH 8, three peaks (points 1, 3, and 4) at pH 6, and four peaks (points 1, 2, 4, and 5) at pH 4. It is clear that the decrease in pH leads to more transitions and a much greater amount of heat release in the ITC profile. After the last peak, the signal merges with that of SDS titrated into buffer. From this point onward, the concentration of monomeric SDS does not increase further and no further interaction between TlL and SDS is registered.

Previously, we have described the stoichiometry between SDS and proteins at different points in the enthalpogram using a mass balance approach.

$$[\mathrm{SDS}]_{\mathrm{X}}^{[\mathrm{protein}]} = [\mathrm{SDS}]_{\mathrm{free}} + N[\mathrm{protein}] \quad (1)$$

where $[\mathrm{SDS}]_{\mathrm{X}}^{[\mathrm{protein}]}$ is the (total) SDS concentration at which transition X occurs at a given protein concentration, $[\mathrm{SDS}]_{\mathrm{free}}$ is the concentration of unbound SDS, and N is the number of SDS molecules bound per protein molecule at the given transition.[28−32] This equation allowed us to determine both binding numbers and $[\mathrm{SDS}]_{\mathrm{free}}$ at the different transition points (Table 1). However, point 1 at pH 8 (with an apparent binding number of −14.17) and point 1 at pH 6 (with an apparent binding number of 0) in the enthalpogram appear to be anomalous. These transition points do not shift to higher SDS concentrations when the TlL concentration is increased; point 6 remains constant, while point 1 actually shifts to lower SDS concentrations. A possible explanation is that the enthalpies of peak 1 at pH 8 and 6 could be the sum of different processes that overlap in such a way that when they are shifted at higher protein concentrations, an apparently anomalous displacement takes place. Such an overlap could be a combination of lid opening and activation at point 1. Such an overlap is not expected to take place at pH 4 where TlL starts to denature as indicated by activity and fluorescence data.

Upon comparison of the binding numbers at pH 4–8, it is clear that TlL binds more SDS at low pH while it shows an unusually low degree of SDS binding at pH 8, especially at the cmc. At the final stage of titration, TlL binds ∼108 SDS molecules (equivalent to 1.08 g of SDS/g of TlL) at pH 6 and 4, while it binds only 23 SDS molecules (0.23 g of SDS/g of

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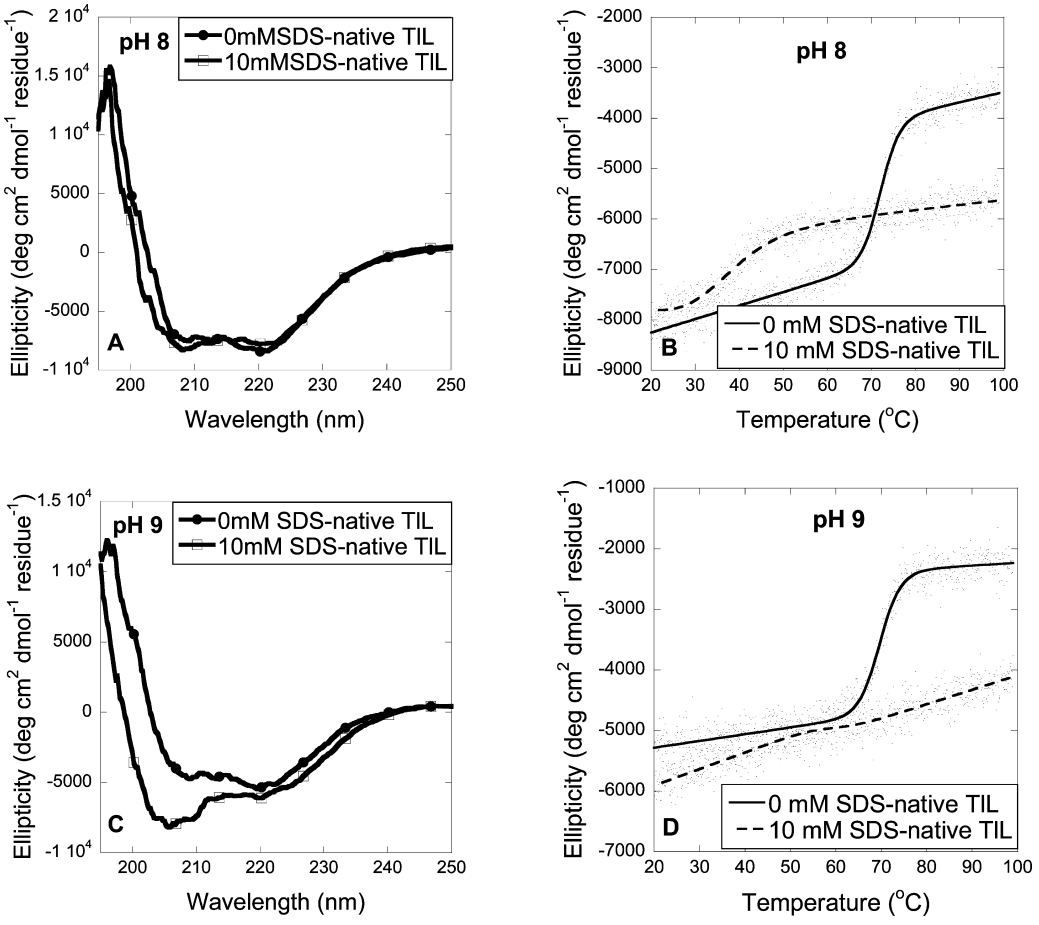

Figure 5. TlL−SDS interactions measured with far-UV CD. (A and C) Spectra of native TlL and native TlL to which 10 mM SDS had been added at pH 8 (A) and pH 9 (C). (B and D) Temperature scans in which we follow ellipticity at 220 nm for native TlL and native TlL to which 10 mM SDS had been added at pH 8 (B) and pH 9 (D).

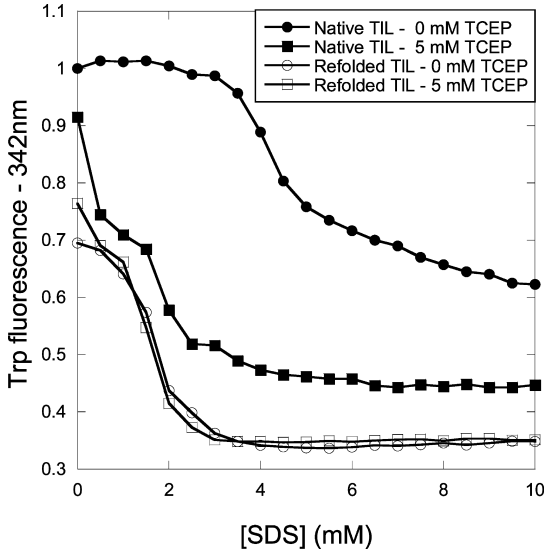

Figure 6. Effect of reducing disulfide bonds on the unfolding and refolding of TlL in SDS at pH 8.0. Native or denatured TlL was incubated with different concentrations of SDS in the absence or presence of 5 mM TCEP and the Trp fluorescence recorded. Fluorescence intensity is normalized to that of the native nonreduced state in the absence of SDS.

TlL) at pH 8 where TlL is stable against SDS denaturation. Proteins with no or reduced disulfide bonds bind ∼1.4 g of SDS/g of protein, while proteins with intact disulfide bonds typically bind 0.6−1.0 g of SDS/g of protein.[33] Electrostatic interactions are expected to change with pH. TlL (pI 5.2) has a total estimated charge of approximately +18 at pH 4.0, approximately −5 at pH 6.0, and approximately −12 at pH 8.0. This difference in charge appears to affect only the very first binding step (inflection point 1 at pH 4.0), which is absent at pH 6.0 and 8.0.

Pyrene Fluorescence Demonstrates the Formation of SDS Clusters on the TlL Surface at pH 6 and 4. We next address whether the increased level of binding at lower pH leads to higher-order structures of SDS on the TlL surface. As pyrene fluorescence can be used to determine the cmc of surfactant and formation of hemimicelles on proteins,[34] we measured pyrene fluorescence with and without TlL in the presence of SDS at pH 8, 6, and 4 (Figure 8). In the absence of TlL, the pyrene I_3/I_1 ratio changes from ∼0.6 to ∼0.93 when pyrene partitions into SDS micelles, indicating micelle formation in the concentration ranges of 4−6 mM SDS at pH 8, 2.5−4 mM SDS at pH 6, and 1.5−3 mM SDS at pH 4. At pH 8, this formation of SDS micelles as shown by pyrene fluorescence correlates very well with the Trp fluorescence decrease in the titration experiment of TlL with SDS (Figure 2), indicating that no clusters are formed below the cmc at this pH. In contrast, micellelike clusters clearly form on the TlL

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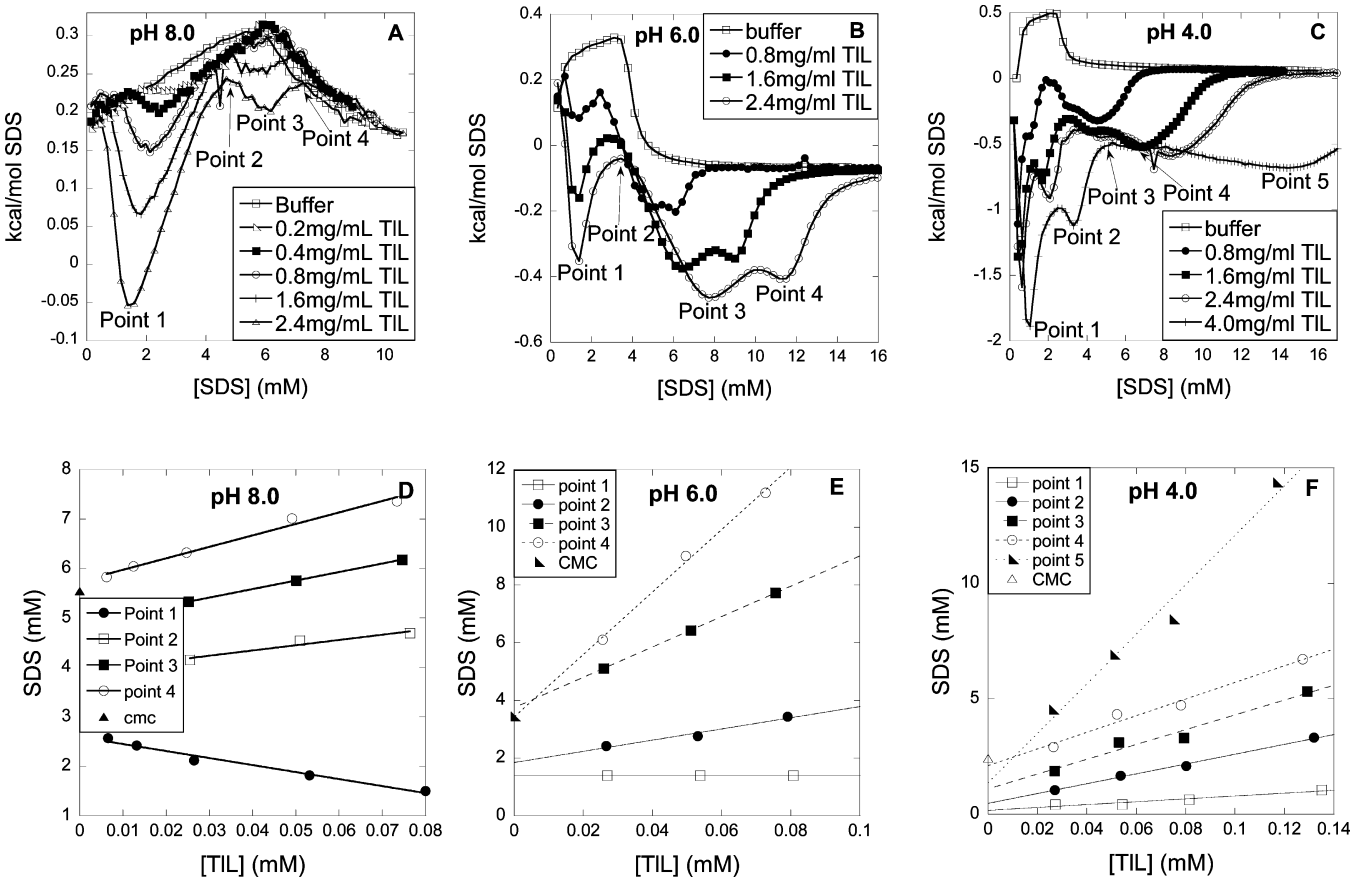

Figure 7. ITC enthalpograms of TlL upon titration with SDS at pH 4, 6, and 8. (A–C) SDS is titrated into buffer and into different concentrations of TlL. Discussed transition points are indicated in the graph. Lines were added by interpolation to guide the eye. (D–F) Total concentrations of SDS, at the different transition points indicated in panels A–C, respectively, plotted as a function of TlL concentration. Binding numbers and free SDS concentrations were derived from eq 1 and are summarized in Table 1.

Table 1. Binding Parameters for SDS–TlL Interactions at pH 8, 6, and 4

	binding number[b]			$[SDS]_{free}$ (mM)[b]		
point[a]	pH 8.0	pH 6.0	pH 4.0	pH 8.0	pH 6.0	pH 4.0
1	−14.17 ± 1.23	0 ± 0	6.19 ± 1.18	2.59 ± 0.06	1.39 ± 0	0.16 ± 0.1
2	10.61 ± 2.71	19.40 ± 3.8	21.2 ± 1.07	3.92 ± 0.15	1.85 ± 0.22	0.46 ± 0.08
3	17.20 ± 0.05	52.6 ± 0.2	32.0 ± 4.39	4.90 ± 0.01	3.75 ± 0.011	1.09 ± 0.35
4	23.18 ± 1.71	108.2 ± 7.4	36.03 ± 4.0	5.74 ± 0.07	3.42 ± 0.39	2.10 ± 0.32
5			107.2 ± 12.0			1.35 ± 0.9

[a]Transition points indicated in Figure 6A–C. [b]Parameters defined in eq 1.

surface at pH 4 and 6 (where there is no kinetic stability), suggesting a close link between cluster formation and the lack of kinetic stability. At pH 4, the sharp increase in the pyrene I_3/I_1 ratio at low SDS concentrations suggests that micellelike clusters form at very low SDS concentrations in the presence of TlL, while micellelike clusters only start to form in 1.5 mM SDS at pH 6. This difference rationalizes the appearance of hysteresis below 1.5 mM SDS at pH 6.

pH- and Temperature-Regulated Equilibrium Denaturation–Renaturation Transition and Unfolding Kinetics. To ascertain whether TlL's kinetic stability is a phenomenon that is strictly related to interactions with ionic and nonionic surfactants or reflects a more general property of the protein, we measured the equilibrium denaturation–renaturation transition in GdmCl and unfolding kinetics in GdmCl at different pH values and temperatures by Trp fluorescence. Given that GdmCl reacts only weakly and unspecifically with proteins,[35] the stability of TlL obtained using different GdmCl concentrations will reflect intrinsic protein properties rather than the presence of specific binding sites. Refolding and unfolding transitions do not coincide at pH 6 and 8, even after incubation for several weeks, while they overlap after 1 h at pH 4 (Figure 9). This extremely slow approach toward equilibrium is further substantiated by measuring the kinetics of unfolding in GdmCl. These can be fit to single-exponential decays, whose unfolding half-lives scale logarithmically with GdmHCl concentrations (Figure 10A). The unfolding half-life in 0 M GdmHCl is extrapolated to 14 days at pH 10, 77 days at pH 8, and 2.3 years at pH 6, but only 0.6 h at pH 4. This trend agrees well with the titration data in Figure 9. The long half-lives are comparable to those obtained for other kinetically stable proteins.[11] However, the slow unfolding at pH 6.0 in GdmCl

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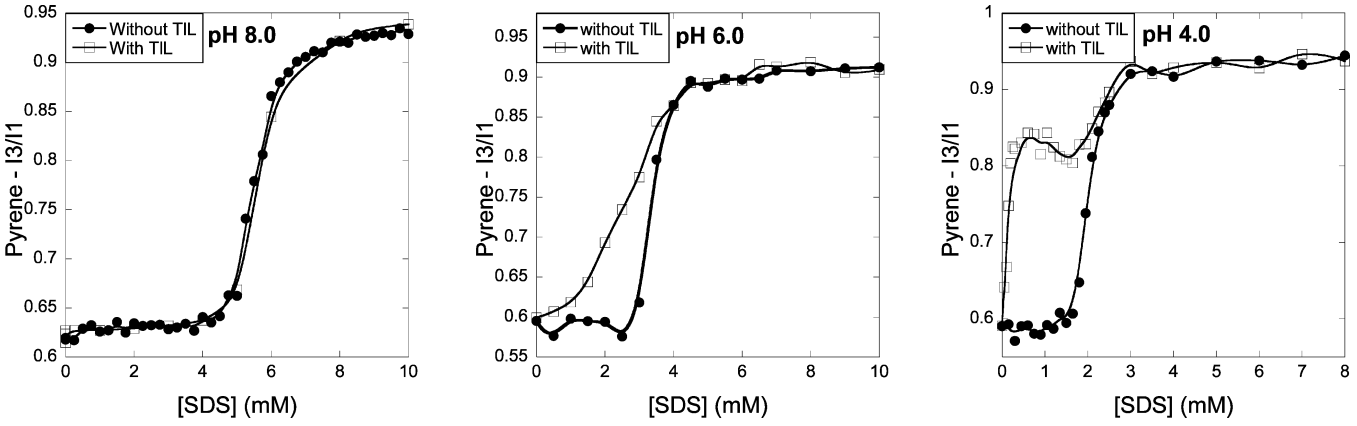

Figure 8. TlL−SDS interactions measured with fluorescence at pH 8, 6, and 4. The pyrene I_3/I_1 ratio reports on the formation of micelles in the absence and presence of 1 μM TlL. Lines were added by interpolation to guide the eye.

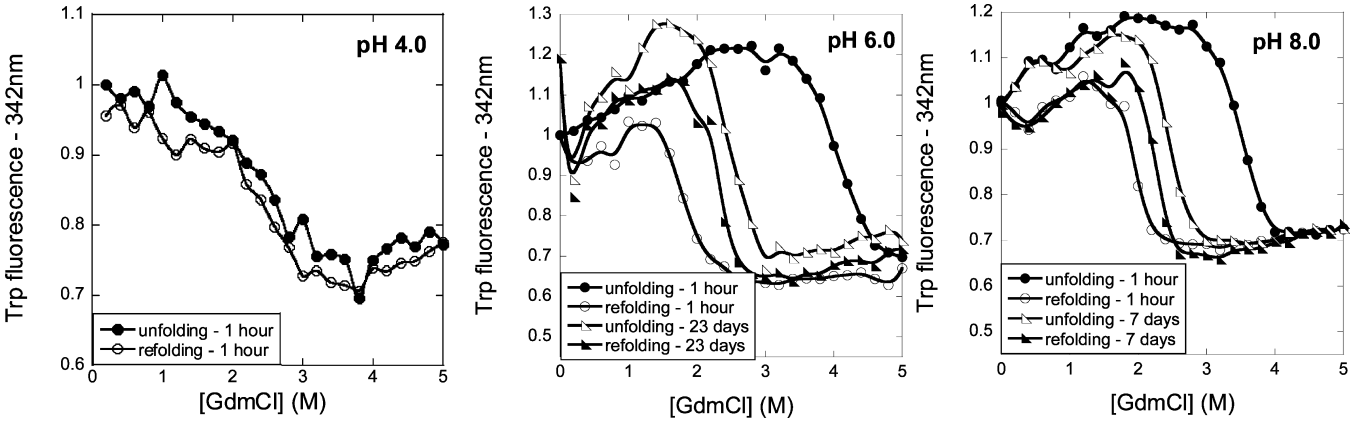

Figure 9. Apparent equilibrium unfolding and refolding transitions of TlL in 0−5 M GdmCl at pH 4, 6, and 8. GdmCl-induced denaturation and renaturation transition curves. Unfolding transition curves were made by incubating TlL in various concentrations of GdmCl and measuring at different time points. We conducted the refolding reaction by denaturing TlL in 8 M GdmCl for 1 h, diluting it in various final concentrations of GdmCl, and measuring at different time points. Trp fluorescence units were normalized so they are all 1 at 0 mM GdmCl for the native (unfolding) state. Lines were added by interpolation to guide the eye.

contrasts with the lack of kinetic stability against SDS at this pH value.

Unfolding rates at pH 8.0 at different temperatures extrapolated to 0 M GdmCl (Figure 10B) show an increase in unfolding rates at high temperatures (Figure 10C), while a leveling off is observed at lower temperatures, which may be attributed to the change in heat capacity associated with unfolding.[36] Importantly, GdmCl cannot activate TlL (Figure 10D), because activity simply follows the amount of folded protein; activation is not linked to simple denaturation of the protein. Thus, TlL behaves as a "normal" protein with regard to unfolding in GdmCl, where thermodynamic stability is linked directly to enzyme kinetics.

The Electrostatic Potential of Tll Undergoes Major Changes between pH 6 and 4. SDS-induced denaturation of proteins usually occurs by the formation of small clusters on the surface of proteins. To obtain information about possible sites where SDS can bind on the surface of TlL, we calculated the electrostatic potential on the surface accessible surface at varying pH values. The potential between pH 10 and 7 (data shown for pH 8.0 in Figure 11) was essentially constant and dominated by a negative potential in which small patches and/or areas of positive potential could be observed, in line with a net charge of −12 at pH 8. Only a small patch situated around the N-terminus showed a somewhat larger area of positive potential. Decreasing the pH to 6 leads to an only small positive increase in potential, although this decrease in pH was sufficient to induce sensitivity to SDS denaturation. The changes did not involve new patches of positive potential on the surface of TlL. Rather, regions changed from negative potential to neutral (Figure 11). A further decrease in the pH to 4 resulted in major global changes to the surface potential, consistent with the fact that TlL has an isoelectric point (pI) of 5.0.[19] We found no obvious large patches with positive potential, constituting potential SDS binding sites. However, there were many small patches with positive potential well distributed all over the enzyme.

■ DISCUSSION

TlL Is Kinetically Stable. Kinetically stable proteins have limited access to partially and globally unfolded conformations under both native and denaturing conditions. Under both industrial and physiological conditions, kinetic stability can be a more useful parameter than thermodynamic stability, because the former value will be the actual determinant of the amount of active protein under the given conditions. Enhancing kinetic stability is also considered a viable strategy for inhibiting amyloidogenesis, which typically requires access to flexible and partially unfolded conformations.[37−39]

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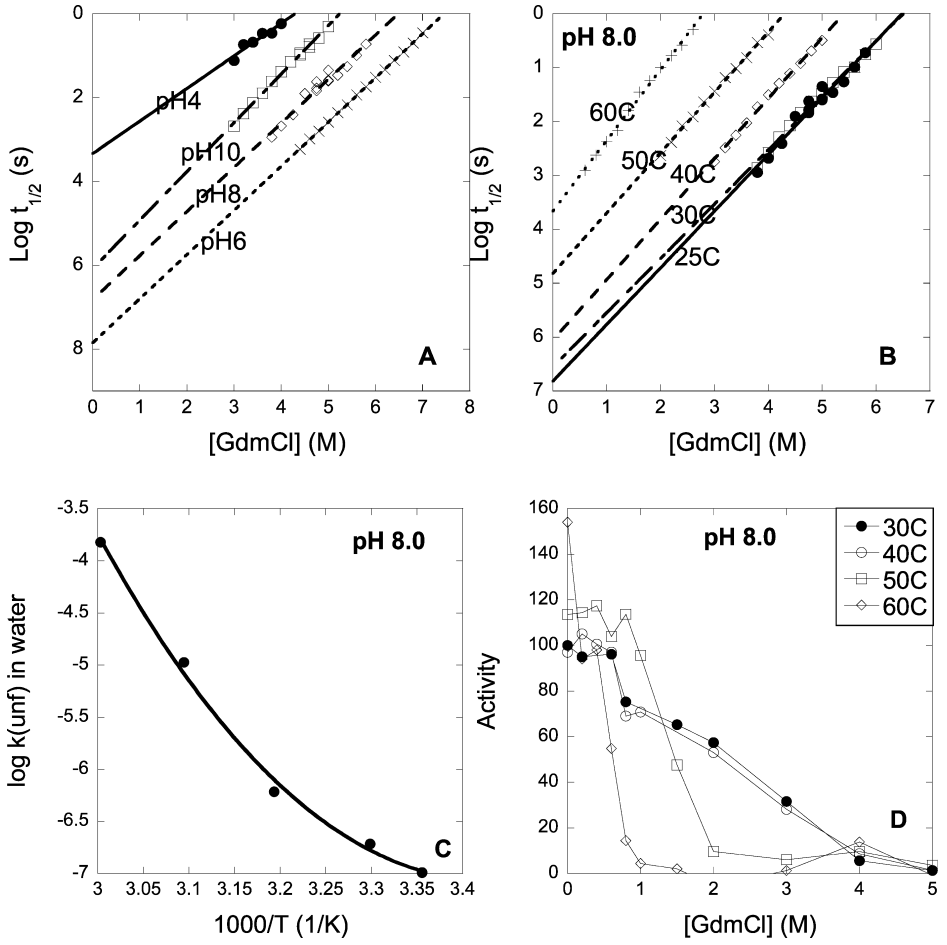

Figure 10. (A) TlL unfolding half-lives as a function of GdmCl concentration. Linear fits extrapolated to 0 M GdmCl indicate the unfolding half-lives in buffer at pH 10−4. (B) TlL unfolding half-lives as a function of GdmCl at pH 8.0 and different temperatures. Note the inversion of the *Y* axis in panels A and B. (C) Extrapolated unfolding rates in buffer at pH 8.0 and different temperatures. (D) Relative activity of native TlL as a function of GdmCl concentration at pH 8.0 and different temperatures. Activity units were normalized so TlL has 100% activity at 0 M GdmCl, pH 8.0, and 30 °C. Lines in panels C and D were added by interpolation to guide the eye.

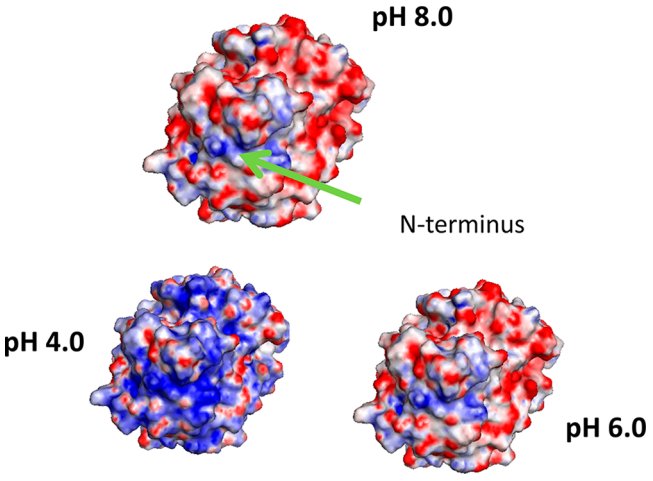

Figure 11. Electrostatic potential of TlL at pH 8, 6, and 4. Red indicates negative potential, white neutral, and blue positive potential.

Probably the most well-characterized kinetically stable protein is α-lytic protease (αLP).[2,40] In its native and active state, it is actually thermodynamically unstable. The native structure is reached with the help of the proregion, which is is cleaved off upon activation of the protease, leaving it trapped in a conformation that is thermodynamically unfavorable. The energy barrier associated with unfolding, however, prevents the protein from unfolding. Furthermore, αLP also has rigidity well beyond that of traditionally thermodynamically stabilized proteins.[40] Hydrogen−deuterium exchange monitored by NMR showed very slow exchange over the entire protein, confirming high rigidity not only for the core but also throughout the protein.[40]

While TlL is synthesized with a signal sequence to export it outside the cell, TlL does not have a folding catalyst similar to the proregion found in αLP. Unlike αLP, the native state of TlL must thus be thermodynamically favored over the denatured state. This is confirmed by our studies of the regain of TlL activity from the low-pH- and urea-denatured state (D), which show that the native state is readily accessible in the absence of surfactant, and the unfolding rate is extremely slow. Thus, under native conditions, the native structure of TlL (N) is both kinetically and thermodynamically favored over the unfolded state, indicating a small barrier between D and the transition state (TS) but a large barrier between TS and N (which can be lowered by reducing the three disulfide bonds). However, even small amounts of surfactant dramatically increase the D−TS barrier and keep the N−TS barrier high. This higher D−TS barrier, which we have also seen for refolding of the lipolytic enzyme cutinase,[41] could be caused by a dramatic stabilization

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of D or the formation of a very stable non-native state when TlL forms a complex with SDS during the refolding process. This is consistent with CD spectra that show a structured but non-native state when we attempt to refold TlL in the presence of SDS.

The Kinetic Stability of TlL Is Most Likely Linked to β-Sheet Formation, Disulfide Bonds, and the Solvation Barrier. A central element in kinetic stability is long-range interactions that are prevalent in β-sheet structure. TlL has mixed α/β-structure, like many other kinetically stable proteins.[14] The dominant secondary structure in TlL consists of a central β-sheet spanning eight individual β-strands oriented in both parallel and antiparallel directions. It is possible that the β-sheet in TlL in combination with disulfide bonds is the key element in the structure that keeps TlL from unfolding. More generally, the decreased rate of local and global unfolding of kinetically stable proteins may account for their resistance to SDS-induced denaturation. The rest of the TlL structure, especially the α-helical lid region, is probably somewhat more flexible, although TlL is overall rigid enough at pH 6−8 to completely withstand proteolytic attacks by up to 30 μg/mL trypsin at 37 °C (data not shown). Other elements proposed to be important for rigidity include tight turns that prevent flexibility, because of a high content of glycine residues that facilitates tight packing in the short turns and loops.[40] The glycine content of TlL is only ∼10% as opposed to 18% in αLP, but glycine is still the most common amino acid in TlL. In addition to the large β-sheet, three disufide bridges also play an important role in its rigidity. Reduction of the disulfide bonds at pH 8 clearly increases TlL's sensitivity to SDS and removes hysteresis, indicating a loss of kinetic stability. This illustrates that reduction of the stability of the native state of TlL by removal of the disulfide bonds is sufficient to reduce the activation barrier of unfolding enough to allow the different species to equilibrate and reach the same level of folded or unfolded structure on the experimentally accessible time scale. Reduced disulfide bridges increase the level of conformational freedom of TlL, providing easier access for SDS to bind and denature TlL. Two disulfide bridges are located in loop regions (Cys36−Cys41 and Cys104−Cys107), and the third (Cys22−Cys268) is associated with the C-terminus. We suggest that rigidity in loops and flexible regions is important for kinetic stability.

Furthermore, a detailed experimental and computational analysis of TlL kinetic stability indicated that a solvation barrier (caused by the asynchrony between penetration of water molecules into the protein core and the breakage of internal interactions) may contribute to the kinetic stability of the lipase from *T. lanuginosus*.[7] The authors found very large effects of mutation on activation enthalpy and entropy, but these were not accompanied by correspondingly large changes in kinetic urea *m*-values, indicating that there were no large changes in the solvent exposure of the transition state. Similar conclusions were drawn from a study of triosephosphate isomerases (TIMs) from three different species.[42] Thus, there is a clear trend that the high solvation barrier (likely a result of natural evolution) contributes to protein kinetic stability.[42]

Kinetic Stability Is Compromised at Lower pH Values, Which Promotes SDS Binding. pH plays a very important role in the kinetic stability of TlL. The degree of hysteresis between unfolding and refolding in surfactants is diminished with increasing distance from pH 8.0. In GdmCl, TlL has the slowest unfolding and refolding rate at pH 6 where TlL takes 23 days to reach equilibrium, but the incubation time needed for reaching equilibrium decreases as we move away from pH 6.0. pH changes can affect protein rigidity by titrating out salt bridges and thus lowering the activation barrier for unfolding. Electrostatic interactions have been suggested to be a major factor in slow unfolding of some protein because of the formation of ion pairs.[9,10] For example, hen egg white lysozyme has a much higher barrier to unfolding than its homologue α-lactalbumin because of strong local electrostatic fields that perturb the pK_a values of ionizable residues and stabilize the native state at the expense of partially unfolded states.[43] Nevertheless, it may be difficult to predict folding barriers entirely from the electrostatic properties of the native state, because the transition state (whose energy governs the height of the activation barrier of unfolding) is more plastic than the native state and can therefore form more nonspecific and even non-native electrostatic contacts.[44] SDS-induced denaturation is clearly related to titration of charged side chains on the surface and hydrophobic interaction with the hydrophobic core of protein, while GdmCl unfolds protein by weak interactions with the peptide backbone.

However, it appears that only a small increase in positive electrostatic potential between pH 8.0 and 6.0, where the theoretical net charge decreases from −12 to −5, is needed to tip the balance from kinetic stability toward SDS sensitivity. TlL contains six His (H110, H135, H145, H198, H215, and H258) residues that protonate in this pH range. While only His135 is directly exposed on the surface of TlL, it is possible that this small increase allows a sufficient number of SDS molecules to bind to trigger a subsequent cooperative change, leading to cluster formation and conventional protein denaturation. An alternative explanation for the role of His protonation at low pH is that protonation leads to a loss of rigidity and increased flexibility and therefore also access to SDS binding. In this light, it is tempting to speculate that SDS denatures by nucleating via a critical number of bound SDS molecules on the surface of a protein to form clusters. Furthermore, this transition requires the ability to bind SDS (i.e., needs an appropriate electrostatic potential with positive patches and neighboring hydrophobic regions) but is not impeded by intrinsic protein stability. TlL is actually more stable kinetically and thermodynamically at pH 6.0 than at pH 8.0 according to GdmCl unfolding experiments, though it is more susceptible to SDS denaturation at pH 6. Thus, under certain conditions, a protein's sensitivity to SDS will not necessarily be a good indication of kinetic stability. We suggest that β-sheet rigidity and disulfide bonds are necessary but not sufficient features of kinetic stability, because they have to be combined with sufficient negative electrostatic potential to avoid extensive SDS binding.

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Funding

D.E.O., K.K.A., and P.S. were supported by the innovation consortium BIOPRO. H.W. was funded by the Danish Research Training Council, Solvay Pharmaceuticals (now Abbott), and Novozymes A/S. D.E.O. is supported by the Danish Research Foundation (inSPIN) and the Lundbeck foundation (BioNET 2).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Professors Jan Skov Pedersen and Cristiano Oliveira for helpful and insightful discussions about lipase structures. We thank Dr. Allan Svendsen for help with electrostatic calculations.

■ ABBREVIATIONS

αLP, α-lytic protease; cmc, critical micelle concentration; DecM, decyl maltoside; GdmCl, guanidinium chloride; pI, isoelectric point; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; pNPB, *p*-nitrophenyl butyrate; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TII27, 27th immunoglobulin domain from human cardiac titin; TlL, *T. lanuginosus* lipase; TlL_{native}^{SDS}, native TlL to which SDS had been added; TlL_{refold}^{SDS}, TlL that we attempted to refold from low pH and high urea concentrations to pH 4−10 and low urea concentrations in the presence of SDS; Tnfn3, third fibronectin type III domain from human tenascin; TFUB, 4-(trifluoromethyl)umbelliferyl butyrate.

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