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Pulse proteolysis: A simple method for quantitative determination of protein stability and ligand binding

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Thermodynamic stability is fundamental to the biology of proteins. Information on protein stability is essential for studying protein structure and folding and can also be used indirectly to monitor protein-ligand or protein-protein interactions. While clearly valuable, the experimental determination of a protein's stability typically requires biophysical instrumentation and substantial quantities of purified protein, which has limited the use of this technique as a general laboratory method. We report here a simple new method for determining protein stability by using pulse proteolysis with varying concentrations of denaturant. Pulse proteolysis is designed to digest only the unfolded proteins in an equilibrium mixture of folded and unfolded proteins that relaxes on a time scale longer than the proteolytic pulse. We used this method to study the stabilities of *Escherichia coli* ribonuclease H and its variants, both in purified form and directly from cell lysates. The ΔG_{unf}° values obtained by this technique were in agreement with those determined by traditional methods. We also successfully used this method to monitor the binding of maltose-binding protein to maltose, as well as to rapidly screen cognate ligands for this protein. The simplicity of pulse proteolysis suggests that it is an excellent strategy for the high-throughput determination of protein stability in protein engineering and drug discovery applications.

The experimental determination of protein stability is an essential step in studies of protein structure, folding and engineering[1–3]. Biophysical techniques such as circular dichroism and fluorescence spectroscopy determine protein stability by monitoring conformational changes induced by thermal or chemical denaturation[4,5]. Though these methods have been well established, their requirements for substantial amounts of pure proteins and expensive biophysical instruments have prevented them from being broadly applied in biological research. Increasing interest in high-throughput determination of protein stabilities for drug discovery and protein engineering has prompted the development of new methods to meet these emerging needs.

In principle, any probe that can monitor the fraction of folded protein (f_{fold}) as a function of perturbant can be used to measure protein stability. To develop an easy, high-throughput method of determining protein stability, we sought a probe that is simple, sensitive and robust, so that the stability of a protein even in a crude sample can be studied. Proteolysis, coupled with electrophoresis, is a good candidate, as the enzymatic degradation of proteins has long been recognized as an effective method to distinguish between folded and unfolded conformations[6–11]. Effective proteolytic digestion of a protein requires unfolding of the substrate, either globally or locally[12], so folded and unfolded proteins have very different proteolytic susceptibilities. When proteolysis under native conditions occurs through global unfolding, protein stability can be estimated by comparing the proteolysis rates of folded proteins with those of unfolded proteins or peptide substrates[9,12]. Inferring global stability of a protein from proteolysis kinetics, however, can be misleading; proteolysis under native conditions can also occur through local fluctuation rather than global unfolding[12]. Moreover, determining proteolysis kinetics is a labor-intensive procedure and is not practical for high-throughput assays.

Here we present a new method for determining protein stability by measuring f_{fold} directly through the application of a pulse of proteolysis in varying concentrations of urea, rather than by measuring proteolysis kinetics. 'Pulse proteolysis' is designed to digest only unfolded proteins in equilibrium mixtures of folded and unfolded proteins. We assume that a protein is in equilibrium between a folded and an unfolded state in its transition zone, where both states have detectable populations. The equilibrium constant for unfolding (K_{unf}) is defined as:

$$K_{unf} = \frac{U_{eq}}{F_{eq}} = \frac{k_u}{k_f},$$

where U_{eq} and F_{eq} are the populations in the unfolded and folded states, respectively, and k_u and k_f are the kinetic constants for unfolding and folding, respectively (**Fig. 1**). When excess protease is added to this equilibrium mixture, unfolded proteins are digested rapidly with the rate constant of intrinsic proteolysis for unfolded proteins (k_p). Proteolysis of the folded proteins, however, is much slower, unless the folded proteins have intrinsically unstructured regions. To be digested effectively, folded proteins must first unfold (**Fig. 1**). Therefore, when intrinsic proteolysis is much faster than unfolding, a short incubation time (pulse proteolysis) will digest only unfolded proteins, leaving the folded proteins intact. Even if

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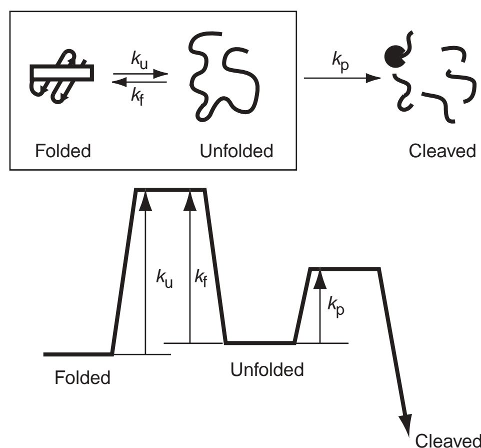

Figure 1 | Principle of pulse proteolysis. Equilibrium between folded and unfolded proteins is shown schematically in the box. k_u and k_f are the unfolding and folding kinetic constants, respectively. When a protease is added, unfolded protein is digested rapidly with an intrinsic proteolysis rate constant for unstructured peptides (k_p). The energy diagram shows kinetic conditions required for pulse proteolysis; k_p must be much greater than k_u and k_f.

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the folded protein can be digested by some high-energy local fluctuation, as long as this is slow compared to proteolysis of the unfolded protein, the folded protein should not be affected. Because only k_p, not k_u, is dependent on protease concentration, the use of excess amounts of protease increases even further the difference in the rates of proteolysis of folded and unfolded proteins. The population of folded protein is then determined by measuring the amount of remaining protein after the pulse, yielding f_{fold}, the fraction of folded proteins. This method allows one to take a 'snapshot' of the equilibrium by using a pulse of proteolysis with an excess amount of protease.

We show here that pulse proteolysis is capable of quantitatively determining protein stability with minimal instrumentation in crude lysates without purification. We also demonstrate the method's ability to monitor the change in stability of a protein upon ligand binding, allowing for a simple indirect screen for ligand binding. Pulse proteolysis is a simple method for determining protein stability in various high-throughput applications, including mutation analysis, protein design and drug discovery.

RESULTS

We used the well-characterized, cysteine-free, recombinant *Escherichia coli* ribonuclease HI (herein referred to as RNase H*)[13] and its variants, I53A and I53D, as our test proteins. We used thermolysin as our proteolytic enzyme because it is robust and has broad specificity and robustness. Thermolysin hydrolyzes peptide bonds at hydrophobic residues[14,15] and retains its activity even in urea[12]. The excess amount of thermolysin we used (0.20 mg/ml) was comparable to, or even higher than, the concentration of substrate proteins.

Choice of the pulse length

To determine f_{fold} reliably, the length of pulse needs to be chosen properly; it must be long enough to digest unfolded protein completely but short enough that it will not digest folded proteins.

The lower limit of the pulse can be estimated by the intrinsic proteolysis kinetics of thermolysin using an unstructured substrate. The rate constant for unfolded proteins, k_p, was approximated by the product of k_{cat}/K_M and enzyme concentration, which is a pseudo-first-order rate constant for catalysis when substrate concentration is much lower than K_M. We determined k_{cat}/K_M values for the cleavage of the peptide *o*-aminobenzoyl-Ala-Gly-Leu-Ala-*p*-nitrobenzylamide (ABZ-Ala-Gly-Leu-Ala-NBA) by thermolysin in varying concentrations of urea. The urea dependence of k_{cat}/K_M was analyzed as reported previously[12]. The value of k_p was calculated by multiplying k_{cat}/K_M by the concentration of thermolysin used in this study, 5.8 μM (or 0.20 mg/ml). **Figure 2** shows the plot of τ_p (or $1/k_p$) for thermolysin as a function of urea concentration. At 0–8 M urea, τ_p varied from 0.26 to 27 s. Pulse durations of 1 min would therefore ensure complete cleavage of unfolded proteins at most concentrations of urea.

The upper limit of the pulse depends on the protein target. For proteins where proteolysis of the folded state is quite slow in the transition zone, a wide window of pulse lengths is available. If a protein unfolds quite quickly in the transition zone, or has an unstructured region in its native conformation, pulse proteolysis might not report f_{fold} reliably because of substantial proteolysis of folded protein within the pulse. Because the shortest possible pulse length is desirable, a 1-min pulse is a reasonable choice for most proteins. Monitoring proteolysis in the transition zone for longer than 1 min provides a simple test to verify that pulse proteolysis is suitable for a particular protein. If the protein is not digested much more by the extended proteolysis, then the determination of f_{fold} by a 1-min pulse will be reliable.

Denaturation of RNase H* in urea

The amount of intact RNase H* remaining after a 1-min proteolysis pulse with 0.20 mg/ml thermolysin was measured by SDS-PAGE (**Fig. 3a**). At concentrations of urea below 4 M, RNase H* seemed resistant to the proteolysis. The fraction of remaining protein decreased rapidly in 5.2–6.0 M urea, consistent with the cooperative transition of protein unfolding. In 7 M urea, RNase H* was completely digested by the pulse. In 8 M urea, some of the protein remained after proteolysis, which we attributed to the reduced activity of thermolysin in this high concentration of urea (τ_p was

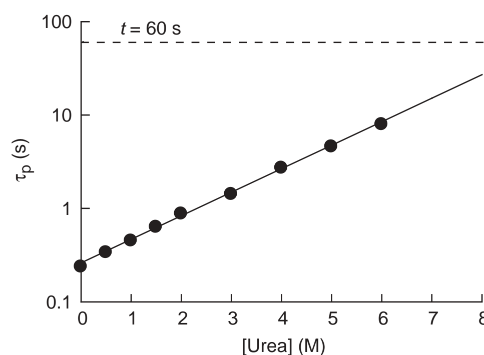

Figure 2 | Effect of urea concentration on intrinsic proteolysis rate. τ_p (or $1/k_p$) was calculated for 0.20 mg/mL of thermolysin from the experimentally determined k_{cat}/K_M for cleavage of ABZ-Ala-Gly-Leu-Ala-NBA. The data were fitted with an empirical equation reported previously[12]. The dashed line indicates $t = 60$ s, the pulse length used in this study.

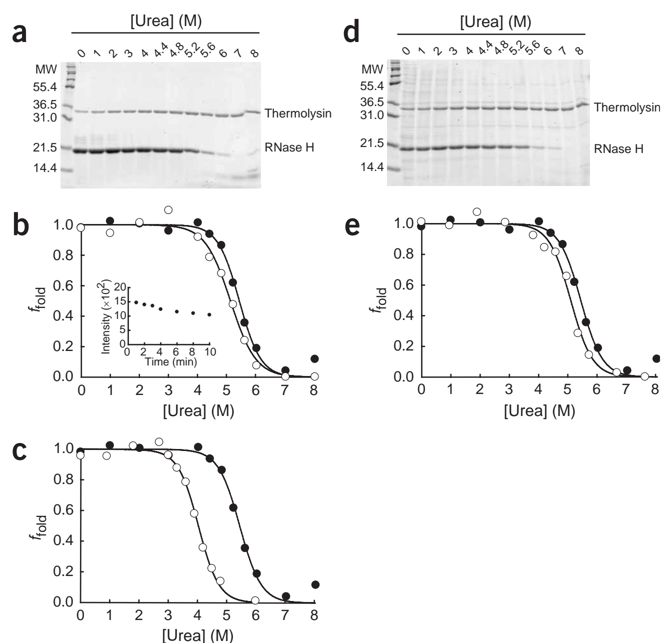

Figure 3 | Determination of C_m of RNase H* by pulse proteolysis. We used 0.20 mg/ml thermolysin to digest 0.50 mg/ml wild-type or I53A RNase H* equilibrated in 0.10 M sodium acetate buffer (pH 5.5) containing 0.010 M $CaCl_2$ and urea (0–8 M). (**a**) SDS-PAGE gel of wild-type RNase H* after 1-min pulse proteolysis. (**b**) f_{fold} of wild-type RNase H* determined by pulse proteolysis for 1 min (●) and 10 min (○). Inset: proteolysis of RNase H* in 5.0 M urea was monitored up to 10 min. The band intensity of the intact protein on a SDS-PAGE gel was plotted against incubation time. (**c**) f_{fold} of wild-type (●) and I53A (○) RNase H* were determined by 1-min pulse proteolysis. (**d**) SDS-PAGE gel after 1-min pulse proteolysis of crude extract of *E. coli* cells overexpressing wild-type RNase H*. Extract was diluted in 0.10 M sodium acetate buffer (pH 5.5) containing 0.010 M $CaCl_2$ and urea (0–8 M), and digested with 0.20 mg/ml thermolysin after overnight equilibration. (**e**) f_{fold} of purified RNase H* (●) and RNase H* in crude extract (○) determined by 1-min pulse proteolysis. Curves were drawn by nonlinear curve-fitting of f_{fold} to equation (2).

~27 s). The f_{fold} values determined from the band intensities fit well with a two-state equilibrium unfolding model (**Fig. 3b**). The denaturant concentration at which half of the protein is unfolded (C_m) was determined to be 5.44 ± 0.05 M. To confirm that the proteolysis of folded proteins was insignificant in the 1-min pulse, RNase H* was digested with 0.20 mg/ml thermolysin in 5.0 M urea for longer times (**Fig. 3b**, inset). This extended proteolysis showed that the amount of folded protein cleaved during the 1-min pulse was negligible. Increasing the pulse length tenfold did not substantially change the C_m (5.14 ± 0.06 M), indicating that our determination of C_m by pulse proteolysis was quite robust and insensitive to variations in pulse length (**Fig. 3b**). The C_m of the RNase H* variant I53A was determined to be 4.03 ± 0.03 M (**Fig. 3c**), which was 1.4 M lower than the C_m of wild-type RNase H*. The error in C_m determined from repeated experiments was typically less than 0.1 M.

We carried out similar experiments on crude lysates of *E. coli* cells overexpressing RNase H*, which can be easily quantitated by SDS-PAGE. Pulse proteolysis yielded a C_m of 5.10 ± 0.05 M (**Fig. 3d**), which was consistent with the C_m determined for purified RNase H* (**Fig. 3e**).

Quantitative determination of global stability

The global stability of a protein, ΔG_{unf}°, in water can be calculated directly by multiplying the C_m by the *m*-value (the denaturant dependence of ΔG_{unf}°). The *m*-values determined from our proteolysis experiment, however, were not reliable enough for this purpose, probably because of insufficient data in the transition zone. Instead, we used a technique based on statistical data[16] that estimates *m*-values from the size of a protein. For urea denaturation, the *m*-value of a protein is approximated by multiplying the number of residues of the protein by –0.013 (ref. 17). The *m*-value of RNase H* was estimated to be −2.0 kcal/(mol · M), which was in agreement with the *m*-value determined by circular dichroism under the same conditions (−2.2 ± 0.1 kcal/(mol · M); data not shown).

Table 1 shows the ΔG_{unf}° values determined by pulse proteolysis for RNase H* and its variants, I53A and I53D. The values were consistent with those determined by circular dichroism. Pulse proteolysis also reliably reported the effect of mutations and pH on global stability (**Table 1**). The stability of I53D RNase H* was strongly dependent on pH, probably because of the titration of Asp53 in its core. Pulse proteolysis revealed that the global stability of I53D RNase H* decreased from 7.1 to 3.9 kcal/mol when pH was increased from 5.5 to 8.0. The ΔG_{unf}° values determined by circular dichroism were 7.6 ± 0.4 kcal/mol at pH 5.5 and 3.7 ± 0.1 kcal/mol at pH 8.0, corroborating the findings from pulse proteolysis. Together, these data indicate that pulse proteolysis is a simple, reliable and quantitative method for determining protein stability.

Monitoring ligand binding

Association with a ligand stabilizes a protein when the ligand binds preferentially to the protein in its native conformation[18,19]. When a ligand (L) binds selectively to the folded state of a protein, ligand

Table 1 | Determination of ΔG_{unf}° by pulse proteolysis

Proteins	pH	C_m	ΔG_{unf}° (proteolysis)	ΔG_{unf}° (CD)
Wild-type RNase H*	5.5	5.20[a]	10.4	10.6 ± 0.5
	8.0	5.14[b]	10.3	11.0 ± 0.3
I53A RNase H*	5.5	4.05[a]	8.1	9.0 ± 0.2
	8.0	3.89[b]	7.8	8.9 ± 0.3
I53D Rnase H*	5.5	3.53[a]	7.1	7.6 ± 0.4
	8.0	1.97[b]	3.9	3.7 ± 0.1
MBP	8.0	2.78[c]	13.3	14.6 ± 0.7
MBP + 100 μM maltose	8.0	3.59[c]	17.2	–

The C_m values were determined by fitting band intensities in varying concentrations of urea to equation (2). The experimental error for C_m from repeated experiments was typically less than 0.1 M. ΔG_{unf}°(proteolysis) was calculated by multiplying the C_m from pulse proteolysis by estimated *m*-values. The *m*-values were estimated by multiplying −0.013 by the number of residues based on the reported statistical data[16].

Experimental conditions for pulse proteolysis: [a]0.10 mg/ml protein in 0.10 M sodium acetate buffer (pH 5.5) containing 0.050 M NaCl and 0.010 M $CaCl_2$; [b]0.030 mg/ml protein in 0.020 M Tris-HCl buffer (pH 8.0) containing 0.050 M NaCl and 0.010 M $CaCl_2$; [c]0.50 mg/ml protein in 0.020 M Tris-HCl buffer (pH 8.0) containing 0.050 M NaCl and 0.010 M $CaCl_2$. ΔG_{unf}°(CD) was determined by CD experiments with 0.030 mg/ml protein in the same buffer as that used for proteolysis.

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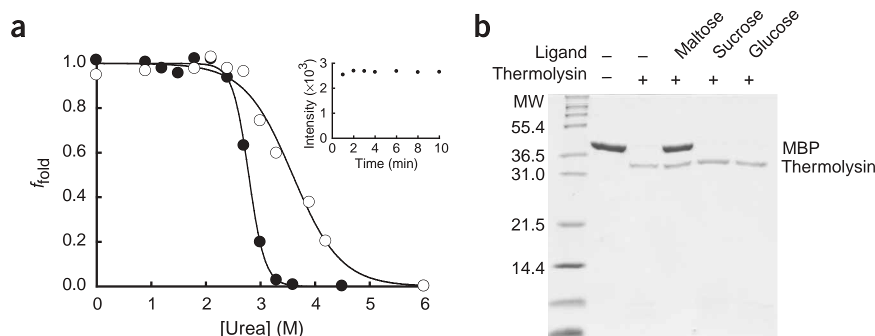

Figure 4 | Monitoring ligand association by pulse proteolysis. (**a**) Determination of C_m of maltose binding protein (MBP) with (○) and without (●) 100 μM maltose. Inset, proteolysis of MBP in 2.5 M urea was monitored up to 10 min. Band intensity of intact protein on SDS-PAGE gel was plotted against incubation time. (**b**) Detection of unfolding of MBP by pulse proteolysis in 4.5 M urea, with and without ligands.

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binding increases the apparent stability of the protein ($\Delta G_{unf}^{\circ}{}_{,app}$; ref. 19) according to the equation

$$\Delta G_{unf,app}^{\circ} = \Delta G_{unf}^{\circ} + RT\ln\left(1+\frac{[L]}{K_d}\right) \quad (1)$$

This thermodynamic linkage allows one to measure ligand binding to a protein by monitoring changes in protein stability[20].

To demonstrate that pulse proteolysis can be used to monitor ligand binding, we determined the C_m of maltose-binding protein (MBP) with and without maltose (**Fig. 4a**). Without maltose, the C_m of MBP was 2.78 ± 0.01 M. We also digested MBP with 0.20 mg/ml thermolysin in 2.5 M urea for a longer period of time to show that the proteolysis of folded proteins was negligible and that the protein was amenable to pulse proteolysis (**Fig. 4a**, inset). Incubating the protein with 100 μM maltose for 2 d increased the C_m to 3.59 ± 0.07 M (**Fig. 4a** and **Table 1**).

Based on the method described above, we estimated the m-value of MBP to be −4.8 kcal/(mol · M), which was in agreement with the value determined by circular dichroism (−5.2 ± 0.3 kcal/(mol · M); data not shown). The ΔG_{unf}° values for MBP were 13.3 and 17.2 kcal/mol without and with 100 μM maltose, respectively. Binding of maltose therefore contributed 3.9 kcal/mol to the global stability of the protein at that concentration. From this difference in ΔG_{unf}° values, the dissociation constant (K_d) for the MBP-maltose complex was determined to be 0.15 μM by equation (1). When the propagation of errors from ΔG_{unf}° determination was considered, this equilibrium constant was in agreement with the reported K_d values for MBP-maltose (0.8 μM (ref. 21) and 1.2 μM (ref. 22) by fluorescence titration and 3 μM by the rate-of-dialysis method (ref. 23)).

The apparent m-value of the MBP-maltose complex determined by pulse proteolysis was substantially smaller than that of MBP alone (**Fig. 4a**), which suggests that the 2-d incubation period was not long enough for the MBP-maltose complex to establish equilibrium in urea. This slow relaxation of the complex imposes some uncertainty in the C_m determination. Circular dichroism studies confirmed that even 6 d were not enough to ensure complete relaxation of the MBP-maltose complex in urea (data not shown), as the binding of maltose to MBP slows its unfolding significantly.

This slow unfolding of the MBP-maltose complex allowed us to screen ligand binding using proteolysis. MBP was incubated in 4.5 M urea for 1 h with or without ligand and then digested with 0.20 mg/ml thermolysin for 1 min. Without ligand, MBP was fully digested under this experimental condition (**Fig. 4b**). In contrast, 100 μM maltose successfully protected MBP against proteolysis by slowing its unfolding, whereas 100 μM sucrose or glucose did not protect MBP at all, in spite of their structural similarity. These results indicate that pulse proteolysis can be used to screen and detect ligand binding.

DISCUSSION

We have developed a simple, quantitative measure of protein stability using pulse proteolysis. Although the proteolytic susceptibility of some proteins is associated with their conformational stability[24], proteolytic susceptibility under native conditions does not necessarily reflect global stability and is not an accurate measure of protein stability. Proteolysis can occur through local fluctuations; that is, localized unfolding not coupled with global unfolding[12]. The rate of proteolysis can also reflect the unfolding kinetics of a protein rather than its thermodynamic stability[10,25]. Pulse proteolysis, however, reports directly on f_{fold}. The substantial differences in proteolysis kinetics between folded and unfolded proteins allow selective digestion of unfolded proteins by pulse proteolysis. The pulse length is chosen to ensure complete digestion of unfolded protein and minimal digestion of folded protein. Once this condition is satisfied, the stability determination by pulse proteolysis is robust to variation in pulse lengths (**Figure 3b**).

Protein stability is traditionally determined using biophysical methods such as circular dichroism, fluorospectrometry and spectrophotometry[5]. Recently, a mass spectrometry technique called SUPREX was also successfully used to determine protein stability *in vivo*[26] by monitoring the mass change from hydrogen exchange[17]. The most notable advantage of pulse proteolysis, however, is that it does not require any biophysical instrumentation other than electrophoresis and a scanner. In its simplest form, quantitation can be performed with Coomassie blue staining and a generic scanner. Another merit of pulse proteolysis is that it requires minimal amounts of protein. Typically, a sample 30–500 μg was enough for one set of pulse proteolysis experiments. In the 1980s, urea-gradient gels were used to monitor protein unfolding by urea, harnessing the difference in electrophoretic mobilities of folded and unfolded proteins[27]. However, preparing and running urea-gradient gels is even more technically challenging than using the conventional biophysical instruments. Unlike urea-gradient gels, pulse proteolysis uses regular SDS-PAGE gels and does not need to maintain equilibrium during electrophoresis. Pulse proteolysis is therefore unique in its simplicity as a quantitative method to determine ΔG_{unf}° from urea denaturation.

The ΔG_{unf}° values determined by pulse proteolysis for RNase H*, its variants and MBP agreed with those determined by more traditional probes (**Table 1**). We also found that pulse proteolysis can be used to determine stability over a wide range of ΔG_{unf}°

values (3.9–17.2 kcal/mol). In both pulse proteolysis and the more traditional methods, protein stability is directly measured under conditions where a protein has only marginal stabilities near the C_m (See **Figs. 3** and **4**), and the data are extrapolated to determine stabilities under native conditions. Unlike the traditional probes, however, pulse proteolysis determines f_{fold} directly from the amount of total protein and can be used even if a protein is not fully folded under native conditions. As long as the native protein resists proteolysis, pulse proteolysis can therefore be applied to quite unstable proteins with ΔG_{unf}° values of ~ 0 kcal/mol.

We found that pulse proteolysis was effective in analyzing the effect of mutations on protein stability. Because this method circumvents the need for extensive protein purification, it should prove useful in high-throughput screening for stabilizing or destabilizing mutations. In combination with two-dimensional electrophoresis, a proteomic approach to evaluating protein stability is also feasible. Other more sensitive measures of protein detection, such as a specific antibody, should allow determination of protein stability without the need for cloning and overexpression. Pulse proteolysis should be a simple diagnostic tool for the detection of specific phenotypes related to changes in protein stability.

For pulse proteolysis to be successful, digestion of the folded protein must be minimal during the pulse. The unfolding kinetics and proteolytic susceptibility of most proteins are not known. However, pulse proteolysis of a crude cell lysate revealed that about a third of the total intensity of soluble *E. coli* proteins on SDS-PAGE gels remains after 1 min of pulse proteolysis with 0.20 mg/ml thermolysin (C.P. and S.M., unpublished data), indicating that a substantial population of the proteome is resistant to pulse proteolysis.

Our results with MBP show that pulse proteolysis can be used to monitor ligand binding (**Fig. 4a**). Ligand binding affects both the stability and kinetics of unfolding, both of which were easily detected for MBP. Depending on the rate of relaxation, one can use pulse proteolysis to monitor ligand binding by either the increase in C_m or the decrease in unfolding rate. The C_m determination is more quantitative and can give information on the K_d. Monitoring the decrease in unfolding rate, while qualitative, provides a more marked difference in an all-or-none fashion. Both methods could easily be adapted for high-throughput ligand screening. The method relies on energetic properties and thus obviates the need for a new assay system for each protein.

METHODS

Materials. Wild-type RNase H* and its I53A variant were prepared as described elsewhere[28,29]. I53D RNase H* was a generous gift from E. Miller (University of California, Berkeley). MBP was cloned by amplifying the coding region in the *E. coli* genome (*malE*) without the leader sequence. The protein was expressed in *E. coli* BL21(DE3)pLysS under the control of the T7 promoter, and purified with DEAE and gel-filtration columns. Thermolysin (EC 3.4.24.27) from *Bacillus thermoproteolyticus rokko* (Sigma Chemical) was used without further purification. A stock solution of thermolysin was prepared in 2.5 M NaCl containing 10 mM $CaCl_2$ (ref. 30). The concentration was determined spectrophotometrically in 20 mM sodium phosphate buffer (pH 6.5) containing 6.0 M guanidinium chloride using an ε of 2.3 $cm^{-1}(mg/ml)^{-1}$ at 280 nm (ref. 31). Urea was from Shelton Scientific; $CaCl_2$ and sodium acetate were from Fisher Scientific.

Determination of k_p. To determine the effect of urea on the catalytic activity of thermolysin, the proteolysis rate of ABZ-Ala-Gly-Leu-Ala-NBA (a generic fluorogenic substrate for thermolysin; MD Biosciences) was measured in varying concentrations of urea[12]. The assays were performed at 25 °C in 0.10 M sodium acetate (pH 5.5) containing 50 mM NaCl, 10 mM $CaCl_2$, 1.5 μM ABZ-Ala-Gly-Leu-Ala-NBA and 0–6 M urea. Reactions were initiated by adding thermolysin to a final concentration of 0.29 μM or 1.4 μM. Fluorescence was measured with a SPEX FluoroMax-3 spectrofluorometer (Jobin Yvon) using 323 and 420 nm as the excitation and emission wavelengths, respectively.

Pulse proteolysis of proteins in urea. To establish equilibrium, proteins were incubated in urea at least overnight before proteolysis. Proteolysis was initiated by adding a 50-fold-concentrated stock solution of thermolysin in 2.5 M NaCl and 10 mM $CaCl_2$ to a final concentration of 0.20 mg/ml. After either 1 or 10 min incubation, 15 μl of reaction solution were removed, quenched with 5 μl of 50 mM EDTA (pH 8.0) and loaded onto 15% (w/v) SDS-PAGE gels. Gels were run with a Laemmli buffer system[32], stained with SYPRO Red fluorescent dye (Molecular Probes) and scanned with a Typhoon imaging system (Amersham Biosciences). Band intensities were quantified with the image analysis software ImageJ (http://rsbweb.nih.gov/ij/).

To determine C_m and m-values, we used the linear extrapolation method to fit the band intensity to the following equation[4]:

$$I = I_0\left(\frac{1}{1+\exp(-\Delta G_{unf}^{\circ}/RT)}\right) \quad (2)$$

where I is the band intensity, I_0 is the band intensity of the total protein. ΔG_{unf}° was calculated as $-m(C_m - [\text{urea}])$, where m is the urea dependence of ΔG_{unf}° and C_m is the urea concentration at which f_{fold} is 0.5. Because the m-values determined by equation (2) were not reliable, ΔG_{unf}° was calculated by multiplying the C_m and an m-value estimated by multiplying –0.013 by the number of residues, based on reported statistical data[16].

Proteolysis kinetics in the transition zone. Proteolysis kinetics were analyzed quantitatively using SDS-PAGE gels as reported elsewhere[12,25]. Briefly, 0.50 mg/ml RNase H* was prepared in 100 mM sodium acetate (pH 5.5) containing 10 mM $CaCl_2$ and 5.0 M urea and incubated at 25 °C overnight to establish equilibrium. Proteolysis was initiated by adding 50-fold-concentrated stock solution of thermolysin to a final concentration of 0.20 mg/ml. At designated time intervals, 15 μl of reaction were removed, quenched with 5 μl of 50 mM EDTA (pH 8.0) and analyzed by SDS-PAGE as described.

Detection of ligand binding by proteolysis. We incubated 0.5 mg/ml MBP with 100 μM ligand (maltose, sucrose or glucose) in 20 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl, 10 mM $CaCl_2$ and 4.5 M urea. All the components of the reaction were premixed and incubated for 10 min before adding urea and incubating further for 1 h at 25 °C. Proteolysis was initiated by adding 0.20 mg/ml thermolysin; the reaction was quenched with EDTA after 1 min and the remaining proteins were analyzed by SDS-PAGE as described above.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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