

A quantitative, high-throughput screen for protein stability

S. Ghaemmaghami*, M. C. Fitzgerald†, and T. G. Oas**

*Department of Biochemistry, Duke University Medical Center, Durham, NC 27710; and †Department of Chemistry, Duke University, Durham, NC 27710

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In proteomic research, it is often necessary to screen a large number of polypeptides for the presence of stable structure. Described here is a technique (referred to as SUPREX, stability of unpurified proteins from rates of H/D exchange) for measuring the stability of proteins in a rapid, high-throughput fashion. The method uses hydrogen exchange to estimate the stability of microgram quantities of unpurified protein extracts by using matrix-assisted laser desorption/ionization MS. The stabilities of maltose binding protein and monomeric λ repressor variants determined by SUPREX agree well with stability data obtained from conventional CD denaturation of purified protein. The method also can detect the change in stability caused by the binding of maltose to maltose binding protein. The results demonstrate the precision of the method over a wide range of stabilities.

The function of a protein is contingent on the stability of its native conformation. Consequently, in the field of protein biochemistry, stability measurements frequently are performed to establish a polypeptide as a stably folded protein and to study the physical forces that lead to its folding (1). Stability measurements also provide important biological information; a decrease in stability can be a sign of misfolding, which in some proteins leads to disease (2), whereas an increase in stability can be indicative of ligand binding (3). Despite their utility, stability measurements currently necessitate time-consuming experiments with pure protein samples. In proteomic experiments (4), where a large number of polypeptides often need to be analyzed, stability measurements are not practical. We have developed the SUPREX (stability of unpurified proteins from rates of H/D exchange) technique to rapidly screen a large number of protein samples for the presence of stable structure. The method uses hydrogen exchange coupled with matrix-assisted laser desorption/ionization (MALDI) MS to obtain quantitative measurements of stability from crude extracts of recombinant *Escherichia coli* cultures grown in 96-well microtiter plates.

Within a polypeptide, certain labile hydrogen atoms can exchange freely with the surrounding solvent. Native structure protects a subset of these hydrogens from exchange (5), and some of these protected protons exchange only if the protein globally unfolds (6). The stability of a protein can be analyzed by monitoring the exchange rates of these “globally protected” hydrogens (7). Protein hydrogen exchange rates typically are measured by allowing labile protons to exchange with D₂O. The proton/deuteron exchange reaction can be monitored by NMR (6) or MS (8). MS is experimentally more convenient than NMR for several reasons: it requires less protein, is faster and simpler to use, does not require complicated spectral assignments, and does not require pure protein samples. These advantages come at the expense of the residue-specific information NMR affords, but that information is not necessary for assessing global stability.

Recent studies have demonstrated that hydrogen exchange coupled with electrospray ionization MS can qualitatively distinguish native-like proteins from unfolded polypeptides in partially purified samples (9) and can be used to study the kinetics and thermodynamics of folding (8, 10). In contrast, the experiments described here use MALDI MS to detect hydrogen exchange, an approach previously described by Komives and

coworkers (11). MALDI is ideally suited for fast, high-throughput screening because a large number of samples can be analyzed in a short period. More importantly, the MALDI technique is tolerant of impure samples that contain moderate levels of salts and other small molecule contaminants (12). This feature allows the measurement of hydrogen exchange as a function of denaturant concentration to give a quantitative measurement of the global protein stability. To demonstrate the accuracy and utility of the method, the analysis was performed on maltose binding protein (MBP) and eight variants of monomeric λ repressor (λ_{6-85}) expressed in bacterial cultures grown in microtiter plates. The change in stability resulting from the mutations in λ_{6-85} and maltose binding to MBP were measured. The stability measurements are verified by comparison with CD denaturation curves obtained with purified proteins.

Materials and Methods

Sample Preparation. The gene encoding the protein of interest was cloned into a T7 expression vector and expressed in *E. coli* strain BL21-(DE3). LB cultures (200 μ l) of the recombinant *E. coli* were grown in 96-well plates and induced by the addition of 0.4 mM isopropylthio- β -D-galactosidase. The cells subsequently were pelleted and lysed by suspension in 10 μ l Bug Buster solution (Novagen). The lysates were centrifuged, and the supernatant was used for hydrogen exchange experiments without any further manipulation. SDS/PAGE indicated that the crude samples consisted of \approx 10–50% expressed protein in a background of cellular impurities. From the gel, the concentrations of the proteins were estimated to range between 50 and 500 μ M.

Hydrogen Exchange. Hydrogen exchange was initiated by adding 10-fold excess deuterated exchange buffer (20 mM sodium phosphate/20 mM sodium acetate/100 mM NaCl, pH 6.3 for MBP or pH 6.7 for λ_{6-85}) to the lysed cultures. The exchange buffers contained different concentrations of guanidinium chloride (GdmCl). At a given exchange time, 0.5 μ l of the exchange reaction was added to 50 μ l of matrix solution. The matrix used for these experiments was sinapinic acid. It was prepared as a saturated aqueous solution containing 45% acetonitrile/0.1% trifluoroacetic acid (pH 3.0) and was kept on ice (2°C) before the addition of the protein. The choice of sinapinic acid as the MALDI matrix was critical because it was more tolerant than other MALDI matrices to the GdmCl used in our experiments. To obtain highly accurate mass measurements, a reference protein was added to the matrix solution. The reference proteins used were BSA for the MBP experiments and a 10-kDa biosyn-

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Abbreviations: MALDI, matrix-assisted laser desorption/ionization; SUPREX, stability of unpurified proteins from rates of H/D exchange; MBP, maltose binding protein; λ_{6-85} , monomeric λ repressor; GdmCl, guanidinium monochloride.

**To whom reprint requests should be addressed. E-mail: T.Oas@duke.edu.

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thetic non-natural polypeptide for the λ_{6-85} experiment. Once the sample protein was added to the matrix, 2 μl of the solution was immediately placed on a MALDI target and rapidly dried under an air stream. Typically, the samples were placed in the matrix and dried within 40 sec. For a given data point five MALDI spectra were analyzed and the results were averaged. No more than 10 min passed between matrix formation and data collection. It is estimated that the amount of protein in each spot was approximately 1 pmol, based on the estimated protein concentration in cell lysates.

Measurement of Back Exchange in the Protonated Matrix. Control experiments were performed to measure the rate of exchange of protons from the matrix back into deuterated protein sites, both in the matrix solution and in the solid matrix crystals. To examine the exchange in the matrix solution, fully deuterated MBP was placed in a prechilled (2°C) matrix solution. At given time intervals the sample was dried on a MALDI target and processed as described below. To examine the exchange in the solid matrix, fully deuterated MBP was placed in a prechilled (2°C) matrix solution and immediately dried on a MALDI target at room temperature. At given time intervals mass spectra were collected and processed as described below.

Data Collection. Mass spectra were collected on a Voyager Biospectrometry Workstation from PerSeptive Biosystems (Framingham, MA) by using the autosampler mode. All spectra were obtained in the positive ion mode and summed over 32 laser shots.

Analysis of SUPREX Data to Determine Protein Stability. According to the classical hydrogen exchange model (5):

$$k_{\text{ex}} = k_{\text{open}}k_{\text{int}}/(k_{\text{open}} + k_{\text{close}} + k_{\text{int}}), \quad [1]$$

where k_{ex} is the observed exchange rate constant for each hydrogen, k_{open} and k_{close} are the rate constants for the conformational changes leading to exchange competent and exchange incompetent states, respectively, and k_{int} is the exchange rate for the unprotected hydrogen. Under EX2 conditions where k_{close} (or k_{open}) are much greater than k_{int} :

$$k_{\text{ex}} = K_{\text{open}}k_{\text{int}}/(K_{\text{open}} + 1), \quad [2]$$

where K_{open} is the equilibrium constant between the exchange competent and exchange incompetent conformations of the protein ($k_{\text{open}}/k_{\text{close}}$). For the hydrogens that are exchanging through a global unfolding mechanism (see *Results and Discussion*),

$$K_{\text{open}} = 1/K_{\text{fold}}. \quad [3]$$

Substituting Eq. 3 into Eq. 2:

$$k_{\text{ex}} = k_{\text{int}}/(1 + K_{\text{fold}}). \quad [4]$$

Because k_{int} is similar among the majority of the backbone amide hydrogens (13), the total exchange of the hydrogens that exchange through global unfolding can be estimated by a single rate constant. The increase in mass due to the exchange of labile protons (ΔMass) as a function of time can thus be estimated by the following first-order rate equation:

$$\Delta\text{Mass} = \Delta M_{\infty} + (\Delta M_0 - \Delta M_{\infty})e^{-k_{\text{ex}}t}. \quad [5]$$

ΔM_0 is ΔMass before global exchange, ΔM_{∞} is ΔMass after complete exchange, t is the exchange time. Substitution of Eq. 4 into Eq. 5 gives an equation for ΔMass vs. $[\text{GdmCl}]$:

$$\Delta\text{Mass} = \Delta M_{\infty} + (\Delta M_0 - \Delta M_{\infty})e^{-((k_{\text{int}})/(1+K_{\text{fold}}))t}, \quad [6]$$

where (14):

$$K_{\text{fold}} = e^{-(\Delta G_f + m[\text{GdmCl}])/RT}. \quad [7]$$

ΔG_f is the free energy of folding in the absence of GdmCl, $[\text{GdmCl}]$ is the GdmCl concentration, m is $\delta\Delta G_f/\delta[\text{GdmCl}]$, R is the gas constant, and T is the temperature in kelvin. The m value determines the sharpness of the transition in the ΔMass vs. $[\text{GdmCl}]$ plot. Myers *et al.* (15) have shown that m values can be estimated from the size of the protein. The average GdmCl m value per residue for the 34 proteins in table 1 of ref. 15 is 26 ($\sigma = 7.2$) $\text{cal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}$ per residue. According to this analysis, MBP and λ_{6-85} are predicted to have m values of 9.7 ± 2.6 and 2.1 ± 0.6 $\text{kcal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}$. These measurements are close to the previously reported experimental m values (12 ± 1 and 2.1 ± 0.1 $\text{kcal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}$, respectively) measured from CD denaturation curves (16, 17). To assess the error involved in using estimated m values, as would be the case in a high-throughput screen of proteins, the calculated m values were used in this analysis. In Eq. 6, $\langle k_{\text{int}} \rangle$ is the average exchange rate of unprotected amide hydrogens and is a function of pH and temperature. In principle, $\langle k_{\text{int}} \rangle$ can be estimated by averaging the values for all of the backbone amide hydrogens using the measurements of Englander and coworkers (13). However, for these studies, the simple relationship $\langle k_{\text{int}} \rangle = 10^{\text{pH}-5} \text{min}^{-1}$ was used to estimate the rate at room temperature and $\text{pH} > 4$.

Results and Discussion

In the SUPREX method, 200 μl recombinant *E. coli* cultures overexpressing the protein of interest are pelleted in a 96-well plate. The cells are lysed by the addition of a nonionic detergent, and hydrogen exchange is initiated by the addition of deuterated exchange buffer containing GdmCl at concentrations ranging from 0 to 8 M. After exchange occurs for a fixed period the protein extract is diluted into a MALDI matrix solution that has been prechilled to 2°C. This addition unfolds the protein and causes a fixed number of deuterons to re-exchange immediately with protons. Control experiments show that the remaining deuterons are relatively stable and re-exchange at a slower rate of 0.001s^{-1} (Fig. 1A). Once the matrix forms a solid crystal, the exchange slows to a rate of 0.001min^{-1} (Fig. 1B). By rapidly drying the sample on a MALDI target, the slowly exchanging deuterons are trapped in the time frame of the experiment and their number can be determined from a mass spectrum (Fig. 2A). To obtain highly accurate mass measurements, a reference protein is added to the sample/matrix solution. The mass of the sample protein is determined by using the reference protein as an internal standard.

As deuterium atoms replace protons during the hydrogen exchange period, the mass of the protein increases. The extent of exchange is determined by monitoring the change in mass relative to a fully protonated sample (ΔMass). The observed ΔMass is caused by deuterated hydrogens that do not re-exchange in the protonated matrix. This set is restricted to the backbone amide hydrogens because they have the slowest intrinsic exchange rates among the exchangeable hydrogens.

Under native conditions, most of these observable amide hydrogens do not provide global stability information because they exchange by local (partial) unfolding processes. However, the addition of denaturant enhances the relative contribution of global unfolding to the exchange rates because local unfolding mechanisms are less denaturant dependent (18, 19). For this reason, many amide hydrogens exchange through a global unfolding mechanism at moderate denaturant concentrations below that required to significantly denature the protein. Fig. 2B shows the exchange of MBP as a function of time in the presence of different GdmCl concentrations. The amplitude of the 6 M GdmCl curve shown in Fig. 2B represents the total number of exchangeable hydrogens observable by SUPREX for MBP. Of

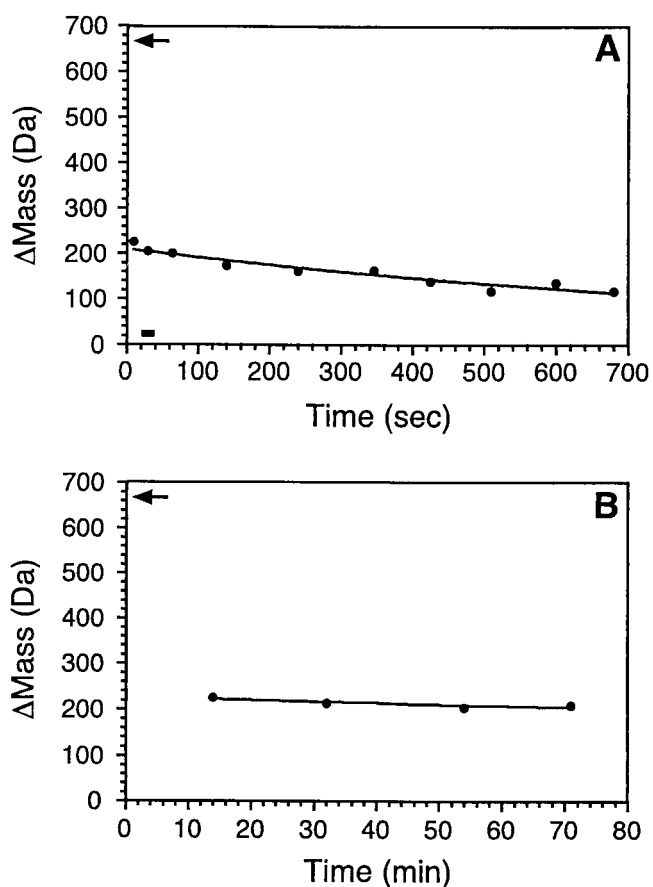


Fig. 1. Exchange of a fully deuterated MBP with the MALDI matrix as a function of time. Δ Mass is the increase in mass relative to the protonated sample. The arrow is the Δ Mass of the fully deuterated protein. (A) The exchange in the matrix solution at 2°C. The bar represents the range of time required for the sample to form a solid matrix and trap the deuterons. The plot is fitted to a single exponential equation with a rate of 0.001 s^{-1} . (B) The exchange in the crystallized solid matrix at room temperature. The plot is fitted to a single exponential equation with a rate of 0.001 min^{-1} .

these ≈ 230 hydrogens, ≈ 100 exchange within 1 h in the absence of GdmCl. These ≈ 100 protons are not globally protected under these conditions and exchange with deuterons relatively quickly. The remaining mass increase is caused by backbone amide hydrogens that are more protected and exchange at a slower rate. The addition of GdmCl dramatically increases the exchange rate of these hydrogens through global unfolding (18, 19). Fig. 3A shows the mass spectra and Fig. 3B depicts the Δ Mass of MBP after 60 min of exchange as a function of GdmCl concentration in the presence and absence of $100 \mu\text{M}$ maltose. Both curves in Fig. 3B indicate the cooperative unfolding of the protein induced by the addition of GdmCl. The presence of maltose increases the stability by binding to the native state and the protein unfolds at higher concentrations of GdmCl. These data were fit to Eq. 6 to determine the stability under native conditions (Table 1). This analysis involves the extrapolation of the linear free energy versus denaturant concentration curves to 0 M. The presence of $100 \mu\text{M}$ maltose stabilizes the protein by 3.0 kcal/mol, a value that is consistent with the stabilization expected from the previously published binding constant (20).

To demonstrate that the method is capable of accurately measuring stability perturbations caused by mutations, SUPREX assays were performed on a series of λ_{6-85} variants known to have

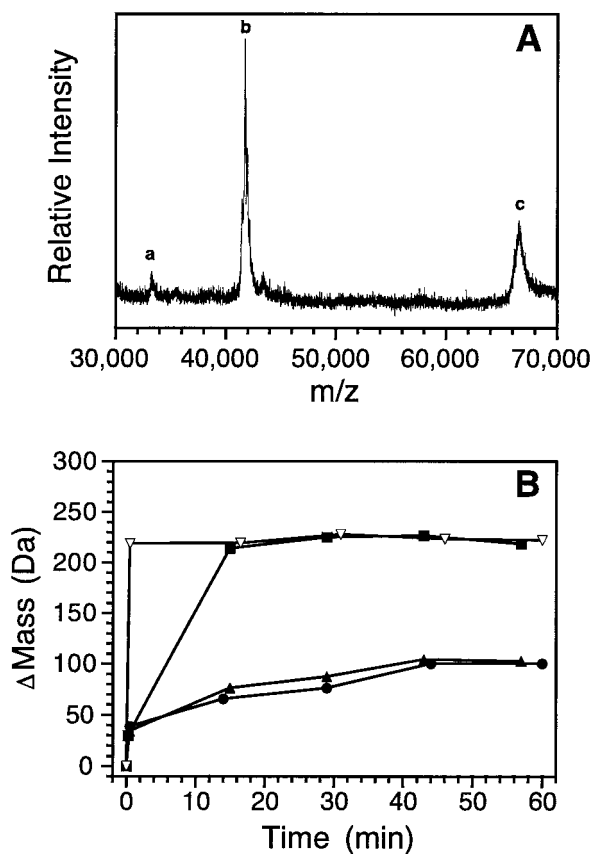


Fig. 2. Hydrogen exchange of MBP detected by MALDI. (A) The MALDI-TOF mass spectrum of a nonexchanged (all protonated) sample. Peak b is the signal from the singly charged molecular ion of MBP, peaks c and a are the signals from the singly and doubly charged molecular ions of BSA, which is added as an internal mass reference. (B) The change in mass (Δ Mass) of MBP as a function of exchange time in the presence of different GdmCl concentrations: \bullet , 0 M; \blacktriangle , 1 M; \blacksquare , 2 M; and ∇ , 6 M.

different stabilities (21–23). The exchange was performed at room temperature and pH 6.7 for 60 min. Fig. 4 depicts the stability curves for this set of proteins. Stabilizing mutations shift the titration curves to higher GdmCl concentrations. Table 1 compares the calculated ΔG_f values obtained by the SUPREX analysis of the crude samples of wild-type λ_{6-85} , the G46A/G48 variant, and MBP with published values obtained by conventional CD denaturation curves of the purified proteins under similar conditions. Table 1 also lists the calculated change in ΔG_f values relative to wild-type proteins ($\Delta\Delta G_f$) for a series of λ_{6-85} variants whose stabilities were determined at either 25°C or 37°C by conventional methods (23). Protein-dependent variations in $\langle k_{\text{int}} \rangle$ values (24) and the uncertainty involved in estimating m values introduces systematic errors in the SUPREX ΔG_f estimates. However, because variants of a protein have nearly identical $\langle k_{\text{int}} \rangle$ and m values, this error does not affect the $\Delta\Delta G_f$ measurements. Thus, even in the absence of exact $\langle k_{\text{int}} \rangle$ and m values, SUPREX can accurately determine the change in ΔG_f . The results in Table 1 show a good correlation between the stability changes measured by CD denaturation and SUPREX.

It should be noted that the stability curves obtained from SUPREX analysis (Figs. 3B and 4) are not identical to conventional denaturation curves. The midpoint of transition in conventional denaturation curves is a function of the stability and the m value of the protein (see *Materials and Methods* for definition of m). In the SUPREX curves the midpoint is not only

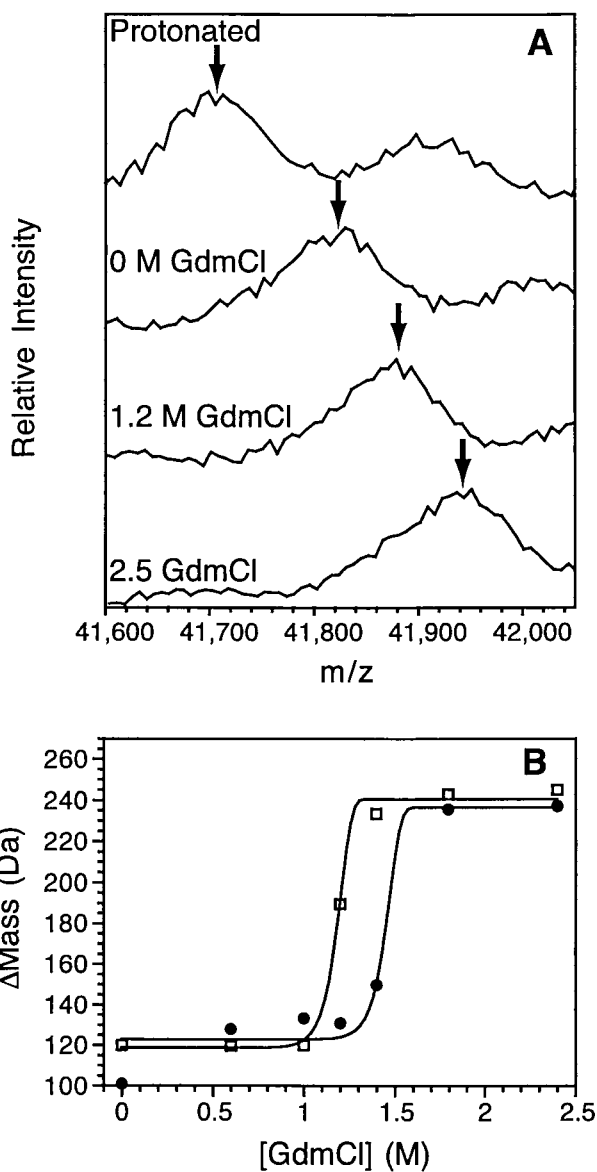


Fig. 3. Stability of MBP obtained by SUPREX. (A) MALDI-TOF mass spectra after 60 min of exchange in the presence of different [GdmCl] with no maltose present. The spectra have been calibrated by using an internal reference (BSA). The arrow indicates the population averaged mass of the singly charged state of MBP. The small peak at m/z MBP + 224 is due to a sinapinic acid matrix adduct of MBP. (B) Δ Mass as a function of [GdmCl]. \square , No maltose present; \bullet , in the presence of 100 μ M maltose. The lines represent fits to Eq. 6. Hydrogen exchange was conducted at room temperature at pH 6.3.

a function of these parameters but also depends on the time of exchange (t) and $\langle k_{\text{int}} \rangle$.

$$C_{1/2}^{\text{SUPREX}} = C_{1/2}^{\text{den}} - (RT/m) \ln(\langle k_{\text{int}} \rangle t / 0.693 - 1), \quad [8]$$

where $C_{1/2}^{\text{SUPREX}}$ and $C_{1/2}^{\text{den}}$ are the midpoints of transition in the SUPREX analysis and conventional denaturation curves, respectively and $\langle k_{\text{int}} \rangle$ is itself a function of pH and temperature (13). As Eq. 8 demonstrates, a SUPREX curve is shifted to the left relative to a conventional denaturation curve, because $\langle k_{\text{int}} \rangle t$ is always much greater than 0.693 ($\ln 2$). Under the conditions used for the experiments described here, this effect

Table 1. Stability of proteins by SUPREX and CD denaturation

Protein	ΔG_f , kcal/mol		$\Delta \Delta G_f$, kcal/mol	
	SUPREX ^a	CD	SUPREX ^a	CD
λ_{6-85}	-5.0 ± 0.4	-4.4 ± 0.2^b	—	—
λ_{6-85} (A66G)	> -4.0		> 1.0	1.5^c
λ_{6-85} (A63G)	-4.8 ± 0.4		0.2	0.4^c
λ_{6-85} (G46A/G48A/A66G)	-5.2 ± 0.4		-0.2	-0.4^c
λ_{6-85} (G46A/G48A/A49G)	-5.6 ± 0.4		-0.6	-1.0^c
λ_{6-85} (Q33Y)	-6.5 ± 0.4		-1.5	-1.5^d
λ_{6-85} (G46A/G48A)	-6.7 ± 0.4	-6.1 ± 0.2^b	-1.7	-1.7
λ_{6-85} (G46A/G48A/Q33Y)	-7.9 ± 0.7		-2.9	-3.4^d
MBP	-16 ± 3	-14.5 ± 0.4^e		
		-12.5 ± 0.2^f		
MBP + 100 μ M maltose	-19 ± 3	—	-3.0	-2.9^e

^aStabilities measured at 23°C.

^bStabilities measured at 25°C.

^c $\Delta \Delta G_f$ ($\Delta G_f^{\text{variant}} - \Delta G_f^{\text{wild type}}$) were measured at 37°C (23).

^d $\Delta \Delta G_f$ measured at 25°C by G. Kapp, personal communication.

^eStability measured at 25°C by urea titration (17).

^fStability measured at 25°C by GdmCl titration (17).

^gA dissociation constant of 1 μ M (20) and an estimated protein concentration of 5 μ M were used to calculate the expected change in stability (3).

shifts the SUPREX curves of MBP and λ_{6-85} by 0.5 and 2.4 M, respectively. It is important to note that the $C_{1/2}^{\text{SUPREX}}$ can purposely be altered by changing the pH, temperature, or exchange interval, t . High stabilities can be measured at higher pH and/or temperature, low stabilities at lower pH and/or temperature. Additionally, the exchange interval can conveniently range from a few minutes to hours. For example, according to Eq. 8, the midpoint of the SUPREX curve for λ_{6-85} can be shifted to the right by 1 M relative to the curves shown in Fig. 4 by changing the pH to 5.9 and the exchange time to 11 min. This sort of adjustment makes SUPREX very flexible for measurements over a wide range of stabilities.

As with all denaturation experiments, the major caveat of the quantitative analysis of SUPREX data is that the protein must unfold in a cooperative, two-state process (1). If one or more stable intermediates are present, the data will not fit Eq. 6. In such cases, a multiphasic titration curve is observed or the transition is broadened. Under these conditions, an extrapolated stability measurement is impossible. However, the midpoint of the curve still serves as a good qualitative gauge of stability. Multiphasic or broadened SUPREX curves would give poor fits with predicted m values; thus this method potentially can be used to identify proteins that have stable folding intermediates.

Another possible complication in the quantitative analysis of SUPREX data is the tendency of some proteins to enter the EX1 regime at high denaturant concentrations and/or pH (24–26). Under such conditions, the intrinsic exchange rate is greater than the folding rate and the exchange rate becomes a function of the unfolding rate rather than the folding equilibrium constant. This situation will cause distortions in the SUPREX curves and it would not be possible to properly analyze the data without knowledge of the denaturant dependence of the unfolding rate. In practice, as is the case with MBP and λ_{6-85} , SUPREX measurements most frequently will be obtained at lower denaturant concentrations and pH where EX1 conditions usually do not prevail.

Although the data described here were obtained by manual methods, the entire methodology of SUPREX can be easily automated and implemented for the analysis of a very large number of samples. For the above experiments, the *E. coli* cultures were grown and induced on 96-well plates. The exchange reaction and dilution into the matrix also were conducted in microtiter plates. The spotting onto a MALDI target from a

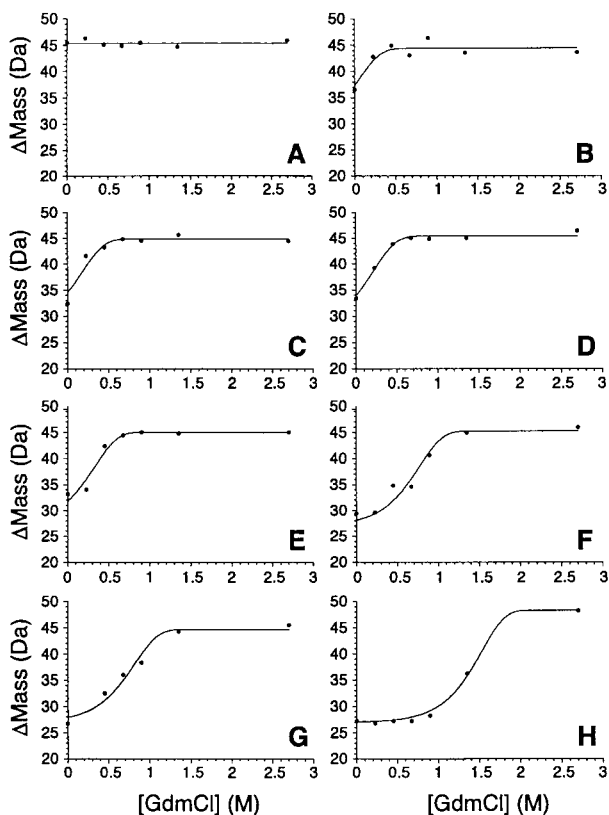


Fig. 4. Stability plots for eight mutants of λ_{6-85} obtained by SUPREX. The curves are arranged in order of the stability of the mutant, from the least to the most stable: (A) A66G, (B) A63G, (C) wild type, (D) G46A/G48A/A66G, (E) G46A/G48A/A49G, (F) Q33Y, (G) G46A/G48A, and (H) Q33Y/G46A/G48A. The lines represent fits to Eq. 6. Under these conditions (60 min of exchange, pH 6.7, room temperature), the midpoints of the SUPREX curves are shifted by 2.4 M [GdmCl] to the left relative to their corresponding CD denaturation curves (see Eq. 8).

microtiter well also can be done robotically, and commercially available MALDI instruments can record the mass spectra of all of the samples in quick succession. The analysis of the data also can be easily automated. We estimate that a single person, with access to a MALDI instrument, could use this technology to measure the stability of as many as 1,000 proteins per day. However, there are likely restrictions on the type of proteins amenable to this analysis. MBP and λ_{6-85} are both fairly soluble proteins with high expression levels in *E. coli* (≈ 50 mg protein per liter of culture). Poorly expressed proteins will be more difficult to visualize on a mass spectrum and might require an initial concentration procedure. Proteins that form aggregates in the lysate also will produce misleading results. Aggregation is likely to protect some hydrogens that are free to exchange in the monomeric soluble form, thus creating artificially high observed stability. Complications also will arise if the recombinant protein requires some manipulation such as reduction or renaturation for its folding. However, many of these complications have been minimized in the past by the addition of moderate concentrations of denaturant sufficient to disaggregate or encourage

proper disulfide bond formation but not high enough to unfold the protein. Fortunately, these are just the sort of conditions under which SUPREX measurements are made. For this reason, we are optimistic that SUPREX may be useful even for proteins that are difficult to study by conventional methods.

We foresee several immediate applications of the SUPREX method. Recent genomic sequencing efforts have provided the DNA sequences of thousands of previously unknown genes (TIGR Microbial Genome Database, www.tigr.org). Assigning a function to all of these unknown sequences will be one of the major scientific tasks of the coming decades. Whether this process involves biochemical analysis or structure determination, the magnitude of the task is so great that it will be important to intelligently select out the most promising candidates for study. Many cellular proteins are not stable under typical *in vitro* conditions. Because both structural and functional analyses require stability, SUPREX can act as a fast screen for selecting promising stable proteins for further study. Similarly, large-scale screening of cloned and expressed polymorphic genes could efficiently aid in the identification of alleles that code for unstable proteins that cause a diseased phenotype.

Combinatorial and directed-evolution methods have proven to be promising techniques for designing proteins of novel structure and function (27, 28). These methods systematically generate a large number of sequences and it is imperative to be able to detect successful *de novo* designs from a large background of unfolded polypeptides. The ability to rapidly screen a large number of sequences for stable folding is crucial to the success of these methods. With its ease of use and high-throughput capability, SUPREX can perform this function and provide a fast convenient way to select for stable designs.

Finally, the results with MBP show that SUPREX can detect binding through a change in protein stability. The thermodynamic linkage between stability and binding is well established (3, 29) and recently has been implemented as a method to detect ligand binding (30). A large number of potential ligands can be rapidly screened for this effect. These ligands could be small molecules, proteins, or nucleic acids. SUPREX provides a convenient alternative in experiments where a binding assay is not available or difficult to use. This approach could be applied to a method analogous to the yeast two-hybrid screen (31), in which two proteins of interest (e.g., a target and a library) are coexpressed and the target protein is screened for stability by SUPREX. The method also could be modified to allow screening of large ligand libraries directly on MALDI plates with predeposited protein. Additional studies should help determine the applicability of the method to expression cell types other than bacteria to allow binding studies under nearly *in vivo* conditions.

SUPREX provides a simple, rapid, and economical way to measure the stability of a large number of cloned proteins and is useful in any application where stability provides important functional and thermodynamic information about a series of proteins. The method should prove to be a very important component in the arsenal of modern proteomic technology.

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