

High-Throughput Measurement of Protein Stability using the FLUOstar

Jean P. Aucamp, Ana M. Cosme, Gary J. Lye and Paul A. Dalby
Dept. of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK

Introduction

The measurement of protein stability is essential for elucidating protein function *in vivo*, engineering the improved stability of therapeutic proteins or biocatalytic enzymes, and formulating proteins for therapeutic delivery. For example, therapeutic proteins require optimal formulation to improve shelf life and a high-throughput stability measurement would enable many combinations of excipients to be rapidly tested for their effect on protein stability.^{1,2}

High-throughput measurements of protein stability are often obtained indirectly by monitoring protein aggregation or residual activity after incubation at elevated temperatures.^{3,4} Both screens rely on irreversible inactivation of the protein upon unfolding, and also the assumption that positive variants result from resistance to denaturation under the test conditions. Although these indirect screens have been applied successfully, they may not easily distinguish differences in stability for proteins that spontaneously refold when returned to the activity-assay conditions.

The unfolding transition of proteins can be observed by measuring their tryptophan fluorescence upon perturbation with a chemical denaturant or a temperature shift. The average fluorescence reflects the change in local environment around the tryptophan residues and provides a direct means of assessing protein unfolding.⁵

Here we describe a high-throughput unfolding procedure using BMG LABTECH's FLUOstar OPTIMA microplate reader with titrating syringe pump. Protein unfolding transitions were monitored by tryptophan fluorescence at 340 nm, and assessed using bovine and equine cytochrome c (cyt c), as well as bovine serum albumin (BSA) stabilised with various amounts of palmitic acid.

Unfolding curves generated by the serial addition of denaturant into single wells, allowed high-throughput stability screens capable of identifying protein variants with unfolding midpoint differences of 0.15 M denaturant concentration or larger. Such a method would be suitable for screening large numbers of proteins or formulation conditions to rank the order of protein stability.

Materials & Methods

Stability measurement

All protein unfolding transitions were measured in F-type, polystyrene, 96-well microplates (Greiner Bio-One Ltd, UK) using a FLUOstar OPTIMA (BMG LABTECH Ltd, UK) microplate reader fitted with a syringe pump for automation of denaturant injection.

Protein unfolding was monitored by intrinsic tryptophan fluorescence using a 340 - 10 nm emission filter with excitation at 280 nm. Protein-unfolding curves were obtained by the serial addition of buffered denaturant stock to 50 μ L protein solution per well, containing 20 μ g cyt c or 40 μ g BSA. Each addition was followed by 30 seconds of mixing by orbital shaking at 350 rpm, and 15 minutes of equilibration prior to taking measurements. Stability calculations based on data fitting and analysis were performed as stated in reference.⁶

Results and Discussion

The incubation times for each addition of denaturant were optimised to ensure that cyt c and BSA unfolding both reached equilibrium. It was determined empirically that 15 minutes between injections were sufficient for both cyt c and BSA to fully equilibrate, resulting in serial addition experiments that took up to 10 hours for 25 denaturant concentrations.

Conformational transition curves for cyt c from bovine and equine heart, are plotted as nine replicates performed in parallel (Fig. 1).

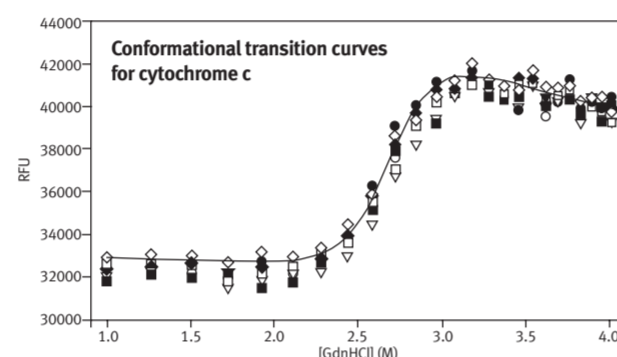


Fig. 1: Conformational transition curves for oxidized cytochrome c (cyt c) with GdnHCl. Serial addition method: multiple replicates of conformational transition curves for horse heart cyt c. Each curve represents data obtained from a single well in which 20 μ g cyt c was loaded. Wells were filled in small increments with the appropriate volume of 100 mM Tris, pH 7.0 containing 6.5 M GdnHCl to give the correct final denaturant concentration. Plates were agitated for 30 s followed by an equilibration period of 15 min before each measurement.

Transition mid-points, $C_{1/2}$ and m_G -values (estimated with non-linear fitting) are summarised in Table 1.

The reported stability values are averages of estimates for each of the nine parallel stability analyses. The inter-run variability was calculated from three separate experiments conducted on separate days, using different stock and protein solutions (Table 1). Mid-points of unfolding for proteins can be estimated to an accuracy of ± 0.15 M, but values of m_G are significantly overestimated.

Table 1: Summary of stability data for oxidized cytochrome c (cyt c) from bovine and equine heart.

	$C_{1/2}$ (M)	m_G ($\text{kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$)	ΔG ($\text{kcal} \cdot \text{mol}^{-1}$)	%CV		
				$C_{1/2}$	m_G	ΔG
(A) Serial addition in microplates						
Bovine	2.50 (0.08) ^a	6.19 (1.83) ^a	14.26 (4.60) ^a	2.25 ^b	22.66 ^b	49.09 ^b
Equine	2.67 (0.14) ^a	4.72 (1.19) ^a	11.83 (1.35) ^a	4.73 ^b	4.01 ^b	6.56 ^b
(B) Reported literature values						
Bovine	2.63 ^c	3.19 ^c	8.38 ^c	–	–	–
Equine	2.42 ^c – 2.60 ^d	3.01 ^c	7.27 ^c – 7.30 ^d	–	–	–

^a Standard deviations of the population calculated from nine datasets.

^b Percentage coefficient of variation calculated from twenty-seven datasets.

^c Ref. 7

^d Ref. 8

The ability to rank protein stabilities in terms of $C_{1/2}$ is sufficient for directed evolution screens, where the key requirement is to find enzymes that resist unfolding under the conditions required in a bioreactor.⁹

It is also useful for therapeutic formulation in which a combination of excipients is desired that improve the resistance of the protein to unfolding.² The large error on m_G -value calculation ($6.2 \pm 1.8 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$ for bovine cyt c) is a result of the inaccurate determination of the pre- and post-unfolding baselines.

Unfolding curves of various BSA:palmitate preparations using GdnHCl as denaturant are shown in Figure 2.

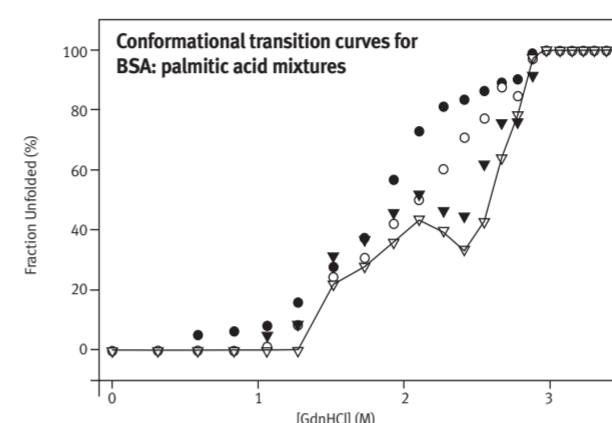


Fig. 2: Conformational transition curves for BSA. 50 μ L of a 0.8 mg/mL BSA solution stabilised with palmitic acid at molar ratios of (●) 1:0, (○) 1:3, (▼) 1:5, (▽) 1:6 and buffered with 60 mM sodium phosphate, pH 7.0, were loaded per well. Unfolding was induced by titrating small volumes of a 5.5 M GdnHCl stock solution buffered with 60 mM sodium phosphate, pH 7.0 into each well. Plates were agitated for 30 s followed by an equilibration period of 15 min before each measurement. Each plot represents data for an unfolding curve obtained from a single well. Data points are interpolated for the highest BSA:palmitate molar ratio (▽), to demonstrate the deviation from a two-state transition.

BSA unfolding curves at high-palmitate concentrations display at least one intermediate in the unfolding pathway, as seen from the deviation from a simple two-state unfolding curve. This intermediate is attributed to the early unfolding of the less stable C-terminal part of the protein. Fatty acid molecules partially stabilise the intermediate, increasing the mid-point of urea induced unfolding.¹⁰

Not all proteins will follow the ideal two-state model of unfolding and simple yet reliable high-throughput data processing protocols will be required for indexing the relative stability of related proteins. Three simple numerical methods were used to quantify the stability of the different BSA preparations: i) the unfolding datasets were fitted to a two-state transition model, calculating an approximate estimate for a global $C_{1/2}$ -value; ii) the areas below the unfolding curves were calculated using the trapezoidal rule; iii) the denaturant concentration at 60% or 70% unfolding was calculated using a linear equation defined by the two data points either side of the arbitrary set value. Figure 3 depicts the stability estimates using these techniques.

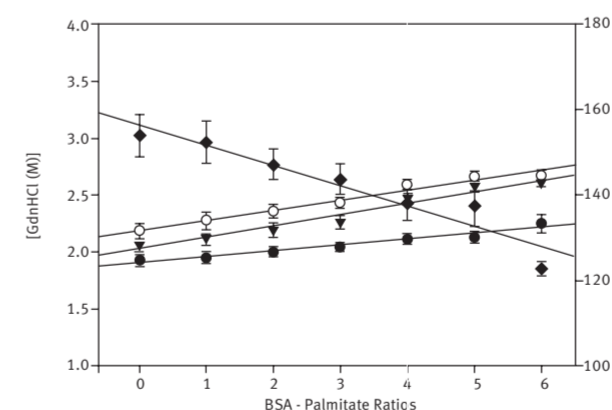


Fig. 3: Stability indexing for palmitate stabilised BSA using different numerical methods. Each dataset in Figure 2 was fit to two-state unfolding, even for those cases that clearly deviate from two-state models. Linear increases in stability are represented by values calculated from: (●) denaturant concentration at apparent midpoint of unfolding; (○) denaturant concentration at 60% unfolding; (◆) total area under unfolding curve.

Although these are crude estimates of stability, they are still functional and effective at identifying fatty-acid induced stabilisation. All three numerical methods can confidently determine the order of BSA stabilities present at four palmitate concentrations. Interestingly, the fluorescence unfolding curves for BSA in GdnHCl in microplates (Figure 2) can also detect the presence of intermediate states that occur as three palmitate molecules bind to each BSA molecule, i.e. at high palmitate concentrations.

Conclusion

We have developed and characterised an affordable high-throughput screening method for the direct measurement of the stability of proteins in a commercially available microplate reader. The method generates unfolding curves in microwells by autotitration of denaturant and measuring the resulting changes in tryptophan fluorescence. The method is useful when screening for changes in $C_{1/2}$ values of greater than 0.15 M. Combined with a suitable numerical data analysis technique it can be a powerful tool for screening mutant biocatalyst libraries for improved stability. The high-throughput determination of stability in microplates could be applied to the fields of directed evolution, proteomics and therapeutic protein formulation, to produce enzymes stable under process conditions, to characterise functions related to stability changes upon ligand binding, or to ascertain optimal combinations of excipients that prolong the shelf-life of biopharmaceuticals.

Acknowledgements

The authors would like to acknowledge the UK Biotechnology and Biological Sciences Research Council and the Association of Commonwealth Universities for financial support. The authors would also like to thank the UK Joint Infrastructure Fund, the Science Research Investment Fund and the Gatsby Charitable Foundation for funds to establish the UCL Centre for Micro Biochemical Engineering.

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Germany: BMG LABTECH GmbH Tel: +49 781 96968-0
Australia: BMG LABTECH Pty. Ltd. Tel: +61 3 59734744
China: BMG LABTECH Co. Ltd. Tel: +86 10 84110632
France: BMG LABTECH SARL Tel: +33 1 48862020
Japan: BMG LABTECH JAPAN Ltd. Tel: +81 48 647 7217
UK: BMG LABTECH Ltd. Tel: +44 1296 336650
USA: BMG LABTECH Inc. Tel: +1 919 806 1735

Internet: www.bmglabtech.com info@bmglabtech.com