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ReBaTSA: A simplified CeTSA protocol for studying recombinant mutant proteins in bacterial extracts

Maria Monticelli [a,b], Demi Marie Wright [a,c], Maria Vittoria Cubellis [a,b,d,*], Giuseppina Andreotti [a,*]

[a] Institute of Biomolecular Chemistry ICB, CNR, Via Campi Flegrei 34, 80078 Pozzuoli, Italy
[b] Department of Biology, University of Napoli "Federico II", Complesso Universitario Monte Sant'Angelo, Via Cinthia, 80126 Napoli, Italy
[c] Institute of Chemistry and Biochemistry, Freie Universität Berlin, D-14195 Berlin, Germany
[d] Stazione Zoologica "Anton Dohrn", Villa Comunale, Naples, Italy

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ABSTRACT

Introduction: The study of protein stability is crucial to biochemistry and relies on different methodologies. Recently, the Cellular Thermal Shift Assay has been introduced to study protein stability in whole cells.
Methods: We report a novel application of CeTSA named ReBaTSA. This Recombinant Bacterial TSA was performed using clear extracts from bacteria expressing a recombinant protein, incubated at different temperatures, centrifuged and analyzed via SDS-PAGE.
Results and conclusions: We demonstrated the feasibility and reliability of this simplified approach. We validated the method using the protein phosphomannomutase-2 and its common mutants, which were compared in the presence or the absence of a known ligand.

The melting temperature of a protein (T_M) is the temperature at which 50% of the molecules are in the native state. Its value depends on the difference in free energy between the native and denatured forms. The process must be reversible to correctly determine the melting temperature, the enthalpy of fusion, and other thermodynamic quantities. This condition is challenging because chemical reactions that lead to the irreversible denaturation of proteins accelerate as the temperature increases. Nevertheless, estimating T_M, even under non-reversible conditions can provide comparisons of numerous variants of the same protein.

Comparing the T_M of wild-type and variant proteins is advantageous for many reasons, ranging from biotechnological to medical applications. Most missense mutations associated with disease do not occur in the active site of proteins or other functionally important sites. Some mutations prevent folding; others cause a decrease in stability, manifesting in a decreased melting temperature [1]. Evaluating the impact of a mutation on a protein is a complex but essential task in exomic analyses. Several in silico methods exist to assess whether the mutation is deleterious, and experimental datasets are used to validate these methods by measuring the melting temperature. Moreover, analyzing the effect of a mutation on a protein also allows for patient stratification and personalised therapies.

In some cases, unstable proteins exhibit a decreased intracellular concentration compared to their wt counterpart. Protein stability is dependent on several factors: environment, pH, ionic strength, and physical-chemical environment, as well as the presence of ligands. It has been demonstrated that in the presence of specific small molecules, corresponding mutated and unstable proteins exhibit an increased melting temperature. This apparent stabilisation leads to increase concentration – and consequently the total activity - of mutated proteins.

Cellular Thermal Shift Assay (CeTSA) is a versatile technique introduced in 2013 [2,3] as an alternative to Thermal Shift Assay (TSA [4]). It measures the residual amount of protein after heating at a specific temperature. This value is sensitive to the environment, pH, ionic strength, protein-protein and protein-ligand interactions. CeTSA, as it is for TSA, can be used to plot melting profiles and estimate T_M, carrying out experiments at different temperatures and recording the ratio between the amount of protein after and before heating.

Abbreviations: CeTSA, Cellular Thermal Shift Assay; G16P, Glucose-1,6-Bisphosphate; G1P, Glucose-1-Phosphate; ITDRF-CeTSA, Isothermal dose response fingerprint-CeTSA; M1P, Mannose-1-Phosphate; M6P, Mannose-6-Phosphate; ReBaTSA, Recombinant Bacterial TSA; T_M, Melting temperature; TSA, Fluorescence-based Thermal Shift Assay.

* Corresponding author at: Institute of Biomolecular Chemistry ICB, CNR, Via Campi Flegrei 34, 80078 Pozzuoli, Italy.
E-mail addresses: cubellis@unina.it (M.V. Cubellis), gandreotti@icb.cnr.it (G. Andreotti).

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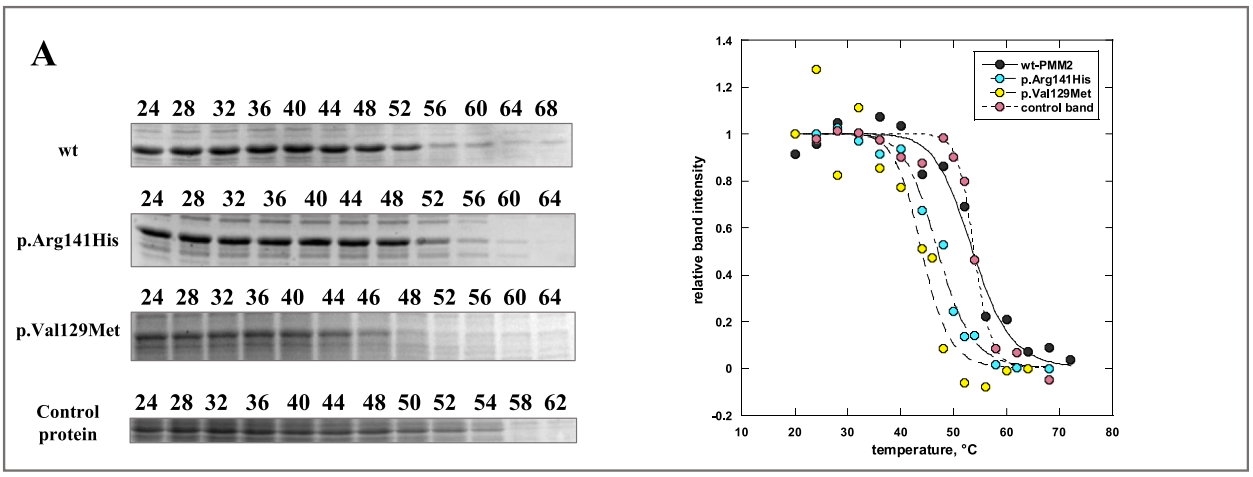

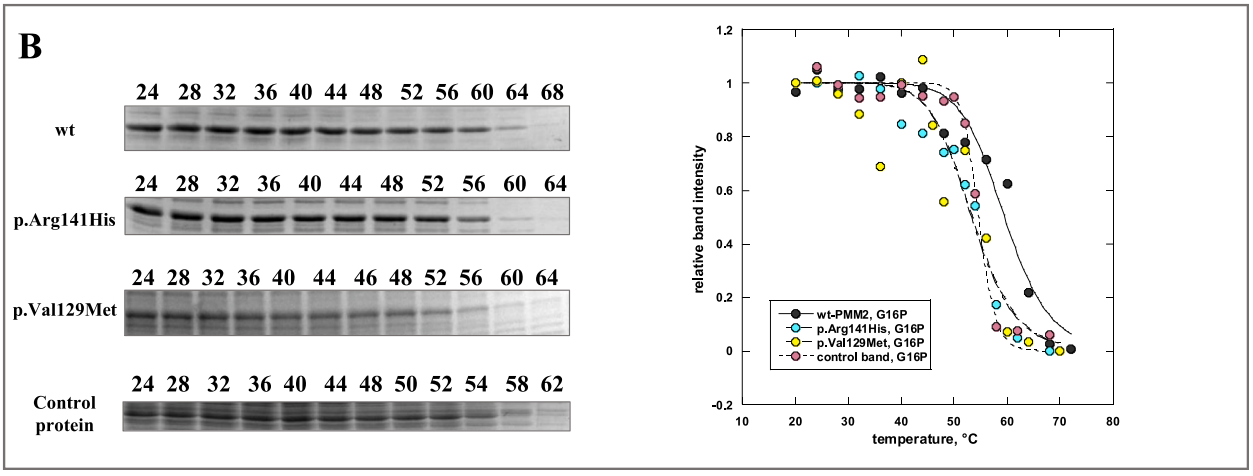

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PROTEIN	TSA* T_M,°C		ReBaTSA T_M,°C	
	-	+ G16P 0.5mM	-	+ G16P 0.5mM
wt-PMM2	53.3±0.8	59.9±3.8	51.8±1.7	57.9±1.5
p.Arg141His, PMM2	49.5±0.5	53.0±0.8	48.3±1.8	53.7±0.6
p.Val129Met, PMM2	47.7±0.6	58.1±1.3	45.2±1.6	52.7±1.0
Control protein	-	-	54.2±0.4	53.2±1.9

* Mean from data available in literature. Details in the caption.

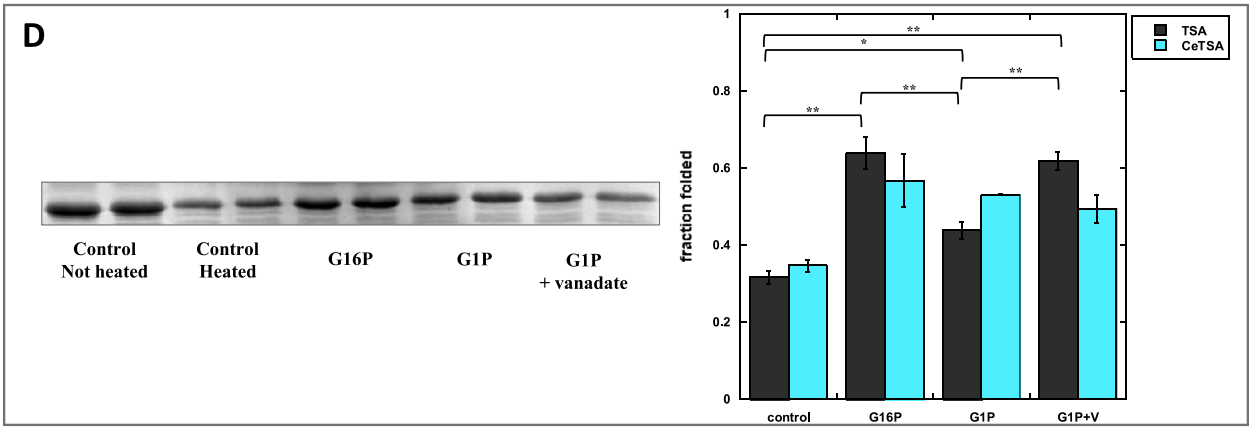

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Fig. 1. (A) Heat-induced melting profiles of *E. coli* extract expressing wt or mutant PMM2. The clear extracts in 50 mM Tris HCl pH 7.5, 1 mM $MgCl_2$, 0.1 mM DTT, were diluted up to 0.3 mg/mL and aliquots were incubated at different temperatures in the 20–74 °C range for 4 min each, then immediately cooled on ice. Centrifuge at 18400 xg for 15 min at 4 °C allowed pelletizing denatured proteins, and supernatants containing the folded fraction were collected and analyzed via SDS-PAGE. The Coomassie staining is showed on the left, quantitative analysis performed with the software ImageLab™ is shown on the right. (B) Heat-induced melting profiles of *E. coli* extract expressing wt or mutant PMM2 in the presence of 0.5 mM G16P. (C) The table summarizes the results ($n = 2$). The mean of published results by fluorescence-based Thermal Shift Assay (TSA) - obtained with the purified wt or mutant PMM2 in the same experimental conditions - are shown for comparison[7,8,11,12,14,15]. (D) Isothermal ReBaTSA. *E. coli* extract expressing wt-PMM2, was used and the effects of different ligands (published in [2], G16P, G1P, G1P + vanadate 0.5 mM each) were compared. The incubation was conducted at 54 °C. Quantitative analysis of bands highlighted the different binding of the ligands considered (Tukey's HSD, * $p < 0.05$, ** p. < 0.01, $n = 2$). For a comparison, data extracted from Fig. 11 in Andreotti et al. [15] - obtained by TSA using purified wt-PMM2 under the same experimental conditions - are shown.

Isothermal CeTSA can also be used to assess the stabilizing effect of ligands. In this case, the temperature is kept constant and close to the T_M of the protein, and the unfolded fraction is measured upon incubation with or without small molecules. Isothermal dose response fingerprint-CeTSA can be carried out by varying a small molecule's concentration (ITDRF-CeTSA) [5,6].

The novelty of CeTSA is related to its application to proteins in complex mixtures, such as cellular lysates or tissue homogenates. The original method required an immunoblot for the detection, and the latest versions used mass-spectrometry (MS-CeTSA).

In this brief application note, we describe a simplified version of CeTSA on raw extracts from transformed bacteria that hyper-express the protein of interest, named ReBaTSA (Recombinant Bacterial TSA). It does not require protein purification, immunoblotting, or mass-spectrometry detection and can be adapted to isothermal analysis. The simplified method described below allows for comparison of wild-type protein with its unstable variants with or without ligands. The difficulty of purifying and preserving relatively unstable proteins should not be underestimated when evaluating the method's usefulness. Results obtained by ReBaTSA are comparable with those obtained by TSA. Our method can also be applied to isothermal CeTSA.

We tested ReBaTSA on the human enzyme phosphomannomutase-2 (PMM2). PMM2 converts mannose-6-phosphate (M6P) to mannose-1-phosphate (M1P) in the early glycosylation steps [7]. It requires glucose-1,6-bisphosphate (G16P) for its activity [8]. > 115 mutations in phosphomannomutase-2 (PMM2) that cause congenital disorder of glycosylation (PMM2-CDG) have been described [9]. Here, we focus our attention on two mutants of interest, namely p.Arg141His and p.Val129Met. p.Arg141His is the first most common pathogenic mutant worldwide [10]. Arg141 occurs in the active site; hence, the variant is not active and has never been observed in homozygosis. It is often found in patients associated with hypomorphic mutants with which it forms heterodimers [11]. p.Val129Met is common in Italian patients and is often heterozygous with p.Arg141His [12].

Wild-type and mutant PMM2 were expressed in *E. coli* as previously described [12]. Upon lysis, aliquots of raw protein extract were incubated at different temperatures in the 20–74 °C range for 4 min each [13], then immediately cooled on ice. Samples were centrifuged (18,400 x*g*) for 15 min at 4°, allowing the separation of pellets containing insoluble proteins and supernatants containing the soluble fraction. Supernatants were collected and analyzed via SDS-PAGE followed by Coomassie staining (Fig. 1A). A quantitative analysis of the SDS-PAGE bands performed using Image Lab ™ (Bio-Rad) allowed the determination of melting curves, where the band intensities of the target proteins were plotted versus the incubation temperatures. The T_M were then mathematically evaluated, applying the sigmoidal fit with KaleidaGraph™.

Figure 1A shows the residual soluble target protein fraction compared to the untreated sample plotted versus the incubation temperature. T_M of wt-PMM2, p.Arg141His and p.Val129Met are 51.8 ± 1.7 °C, 48.3 ± 1.8 °C and 45.2 ± 1.6 °C respectively. In our experiments, the T_M perfectly aligned with those evaluated using fluorescence-based Thermal Shift Assay (TSA) on purified proteins [7,8,11,12,14,15].

The coincidence between the obtained results with ReBaTSA and TSA is also observed when the experiment is carried out in the presence of a ligand. Shift induced by G16P binding increased the T_M by 6 °C for wt-PMM2, 5 °C for p.Arg141His, and >7 °C for p.Val129Met (Fig. 1 B).

The experiments were performed in analogous experimental conditions respect to published results, and G16P was used at a concentration 0.5 mM, as previously described. Fig. 1C summarizes the results and shows the comparison with published data [7,8,11,12,14,15]. Data from the papers above were extracted for comparison, and the mean was calculated.

ReBaTSA consents to use a non-specific abundant protein band as an internal control. The T_M of this protein did not change in the presence of G16P, thereby excluding a non-specific effect of the ligand (Fig. 1C).

Determining the melting temperature (T_M) of a protein is a preliminary step to carry out isothermal CeTSA, a simplified method to test the effect of ligands incubating the protein with or without the molecule of interest at a temperature close to the melting point.

Wt-PMM2 was incubated at a fixed temperature to compare various ligands. G16P, glucose-1-phosphate (G1P) and G1P plus vanadate were selected based on previous investigation [15]. The stabilizations were recorded at 54 °C. The ratio between the residual amount of protein and its total amount was calculated by the bands' intensity and compared with the values obtained by melting curves previously carried out with the same protein and the same ligands by TSA (Fig. 1D).

The IsoThermal Dose Response Fingerprint-CeTSA (ITDRF-CeTSA), dedicated to analyzing dose-response ligand binding, exhibits the development of this approach. We performed ITDRF-ReBaTSA on our target proteins using G16P. According to the melting profiles (Fig. 1A), we used a varying incubation temperature for each mutant: 53 °C for p.Arg141His and p.Val129Met, 57 °C for wt-PMM2. The results are shown in Supplementary Fig. 1. As already mentioned, p.Arg141His is an inactive variant of PMM2. For this reason, it was not possible to evaluate the affinity for G16P by enzymatic assays. Our experiments demonstrate that G16P binds to the inactive protein with an affinity comparable to that of wild-type protein, at least at high temperature.

In conclusion, this report demonstrated the feasibility and robustness of the ReBaTSA approach. The limitations associated with ReBaTSA protocol are mainly related to the recombinant protein expression. It is necessary that the target protein is expressed at quite high levels, to use Coomassie staining rather than immunoblot. Also, the effects mediated by relevant post-translational modifications would not be recorded, representing false negatives. Another restraint comes from the limited permeability of bacterial cells that hampers the possibility to test ligands using whole cells. Besides this, the study of protein complexes is not suitable with this approach. Despite its limitations, we believe it is a valuable biochemical tool for comparing wt and mutant variations of the target protein. This is an ever-expanding field since the study of mutated proteins covers a wide range of biotechnological applications, ranging from biomedical fields to bio-productive processes.

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CRediT authorship contribution statement

Maria Monticelli: Formal analysis, Investigation, Validation, Visualization, Writing – original draft. **Demi Marie Wright:** Formal analysis, Investigation, Validation, Visualization, Writing – review & editing. **Maria Vittoria Cubellis:** Conceptualization, Formal analysis, Resources, Supervision, Writing – review & editing. **Giuseppina Andreotti:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2023.130526.

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