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# Characterization of aminoacyl-tRNA synthetase stability and substrate interaction by differential scanning fluorimetry

Jamie A. Abbott [a,*], Nathan M. Livingston [a], Shawn B. Egri [a], Ethan Guth [b], Christopher S. Francklyn [a,*]

[a] Department of Biochemistry, University of Vermont, Burlington, VT 05405, USA
[b] Chemistry & Biochemistry Department, Norwich University, Northfield, VT 05663, USA

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ABSTRACT

Differential scanning fluorimetry (DSF) is a fluorescence-based assay to evaluate protein stability by determining protein melting temperatures. Here, we describe the application of DSF to investigate aminoacyl-tRNA synthetase (AARS) stability and interaction with ligands. Employing three bacterial AARS enzymes as model systems, methods are presented here for the use of DSF to measure the apparent temperatures at which AARSs undergo melting transitions, and the effect of AARS substrates and inhibitors. One important observation is that the extent of temperature stability realized by an AARS in response to a particular bound ligand cannot be predicted *a priori*. The DSF method thus serves as a rapid and highly quantitative approach to measure AARS stability, and the ability of ligands to influence the temperature at which unfolding transitions occur.

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* Corresponding authors.
*E-mail addresses:* Jamie.Abbott@uvm.edu (J.A. Abbott), Christopher.Francklyn@uvm.edu (C.S. Francklyn).

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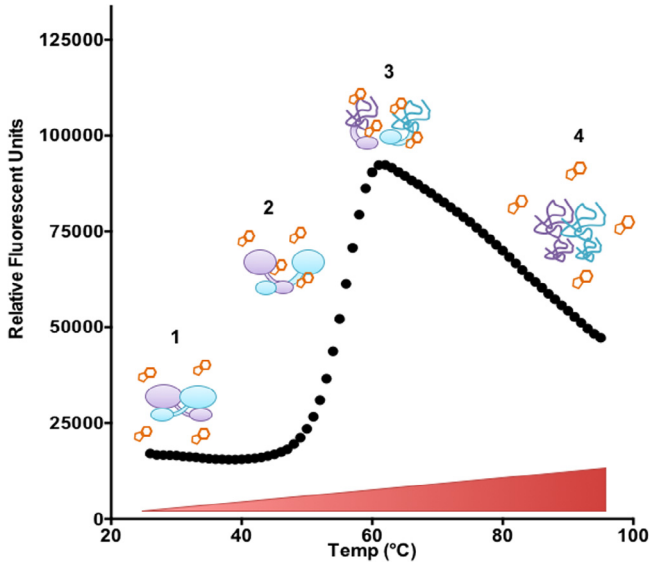


**Fig. 1.** Molecular basis of differential scanning fluorimetry. (1) Folded protein is incubated in solution with SYPRO Orange dye. (2) Temperature increases, causing protein to unfold and SYPRO Orange dye to bind exposed hydrophobic residues. This results in an increase in fluorescence signal. (3) Protein is denatured and maximum SYPRO Orange dye binding is achieved. (4) Denatured protein begins to aggregate and SYPRO Orange dye binding decreases.

## 1. Introduction: Development of differential scanning fluorimetry assay

Aminoacyl-tRNA synthetases (AARS) synthesize aminoacylated tRNA in one of the earliest steps of protein synthesis. The aminoacylation reaction involves binding of the three substrates amino acid, tRNA, and ATP to each AARS to produce the final aminoacylated tRNA product. Traditionally, biophysical techniques to measure AARS enzyme stability and unfolding have included spectroscopic methods such as intrinsic tryptophan fluorescence in the presence of a denaturant like urea or guanidine-HCl, monitoring fluorescence with 1-anilino-8-naphthalene-sulfonic (ANS) dye, and circular dichroism [1–4]. An example of a classic method for determining a protein melting point without the use of exterior dyes is differential scanning calorimetry (DSC) [5,6]. Differential scanning calorimetry calculates the amount of heat required to raise the temperature of a sample by a given number of degrees, computing the difference between the sample and a buffer reference cell. This approach has been employed to study protein stability in several AARS systems [7,8]. Isothermal titration calorimetry (ITC) has also proven useful for studying the interactions of small molecule ligands with AARSs is [9–11]. In addition to providing binding constants and stoichiometry, ITC is a direct process that does not involve chemical alteration of either the enzyme or the substrate, while providing detailed thermodynamic parameters for a given interaction. The chief disadvantage of ITC is that it requires expensive instrumentation and, depending on the specific heat of a particular interaction, may require relatively high concentrations of the target protein in order derive adequate signal. Accordingly, both calorimetric approaches can provide valuable information, but typically require large quantities of protein, extensive analysis, and are low throughput.

Differential scanning fluorimetry (DSF) is a method that enables researchers to monitor thermal denaturation of purified proteins with a fluorescent reporter. The DSF assay relies on the interactions of the fluorophore dye SYPRO Orange with the exposed regions of partially unfolded proteins rich in hydrophobic amino acids (Fig. 1). The folded structure of proteins sequesters these hydrophobic amino acid residues from water, burying them in the highly folded regions of the protein's core domains. As the solution temperature is increased incrementally, denaturation of the protein exposes increasing numbers of hydrophobic residues. This creates an opportunity for SYPRO Orange dye binding, which gives rise to an increase in fluorescence (excitation and emission of 490 and 575 nm, respectively).

DSF was first developed as a drug screen for small molecule studies, but is also applicable as a screen to optimize stabilizing buffer conditions for aaRS crystallization [12–14] and to obtain ligand binding parameters [15]. Many examples of DSF have been reported for proteins such as Carbonic Anhydrase-II [16], cytoplasmic sulfotransferase 1C1 [17], cAMP-dependent protein kinase (PKA) [18], MAPK13 [19], and pregnane xenobiotic receptor (PXR) [20] to name a few [21]. A particular advantage of the DSF method is that the experimental set up can be implemented in a high-throughput mode by use of a 96-well microtiter plate [22]. The measurements can be performed in a real-time PCR instrument that is standard equipment for most molecular biology core facilities [23]. Fluorescence output is plotted against temperature to determine the melting temperature ($T_m$), by fitting the Boltzmann equation to fluorescent data [24].

Here, we present a set of protocols for determining the apparent melting transitions of aminoacyl-tRNA synthetases by use of DSF. After a presentation of the specific methodology for a single experiment, additional examples are discussed in which the melting transitions of three bacterial Class II AARS enzymes are determined. In addition, we also show how DSF can be used to study the binding of cognate amino acids and inhibitors to AARSs, and how DSF can be used to evaluate tRNA binding to certain AARS enzymes.

## 2. Differential scanning fluorimetry method

### 2.1. Protein (aaRS) preparation and purification

- Purified *E. coli* enzymes Alanyl-tRNA synthetase (AlaRS), Histidyl-tRNA synthetase (HisRS) and Threonyl-tRNA synthetase (ThrRS) were isolated by Ni-column chromatography as previously described [25–27]. This represents the minimum degree of recommended purification; some enzymes may require additional steps for best results. We typically employ an additional step, such as ion exchange chromatography. The effect of affinity tags on protein unfolding has not been investigated systematically. Given that affinity tags have limited structure, there is little rationale to expect major deviations from the melting temperatures determined in the absence of the 6X-His tag. The results might also be affected by non-specifically bound residual RNA or DNA, which can sometimes co-purify with

**Table 1**
Reagents necessary for DSF experiment.

| Reagent or supply | Supplier | Catalogue number |
|---|---|---|
| Ultrol grade HEPES buffer | CalBioChem | 391338 |
| KCl | Fisher | 138149 |
| SYPRO Orange Dye 5000 X | Molecular probes | S6651 |
| MillexGV 0.22 uM filter | MerkMillipore | 309603 |
| Sterile syringe 5 mL | BD Biosciences | 309603 |
| Siliconized low retention micocentrifuge tubes | Fisherbrand | 02-681-311 |
| 96 well PCR Plate non-warping | Phenix Research | MPS-3580-NW |
| ThermalSeal RT2 Fi | Phenix Research | LMT-RT2-RR |

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