The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability

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Differential scanning fluorimetry (DSF) is a rapid and inexpensive screening method to identify low-molecular-weight ligands that bind and stabilize purified proteins. The temperature at which a protein unfolds is measured by an increase in the fluorescence of a dye with affinity for hydrophobic parts of the protein, which are exposed as the protein unfolds. A simple fitting procedure allows quick calculation of the transition midpoint; the difference in the temperature of this midpoint in the presence and absence of ligand is related to the binding affinity of the small molecule, which can be a low-molecular-weight compound, a peptide or a nucleic acid. DSF is best performed using a conventional real-time PCR instrument. Ligand solutions from a storage plate are added to a solution of protein and dye, distributed into the wells of the PCR plate and fluorescence intensity measured as the temperature is raised gradually. Results can be obtained in a single day.

INTRODUCTION

Most applications that determine the activities, interactions or structures of proteins require that the protein samples be stable over long periods of time. Conditions under which a protein is most stable are also the best to prevent denaturation while proteins are stored and during the process of freezing and thawing. In addition, often the tendency to aggregate would decrease under stabilizing conditions. Therefore, identifying these conditions would help in experiments involving analytical and biophysical techniques that require high protein concentrations and are sensitive to protein aggregation.

Among the factors that may influence protein stability are generic ingredients like buffers, salts and detergents whose interactions with the protein are nonspecific, but also ligands that bind to the protein at a specific site. In addition to their effect as protein stabilizers, protein–ligand interactions can be of high value for functional studies such as substrate specificity studies and for identifying allosteric effectors that would help in providing better protein annotations. Furthermore, identifying ligands is a valuable starting point for developing drug candidates.

The stability of a protein is related to its Gibbs free energy of unfolding, ΔG_u, which is temperature-dependent[1,2]. The stability of most proteins decreases with temperature; as the temperature increases, the ΔG_u decreases and becomes zero at equilibrium where the concentrations of folded and unfolded protein are equal. At this point, the temperature is considered as melting temperature (T_m). If the protein unfolds in a reversible two-state manner, the equilibrium thermodynamics models will apply[3]. If a compound binds to a protein, the free energy contribution of ligand binding in most cases results in an increase in ΔG_u, which may cause an increase in the T_m. It has been shown that the stabilizing effect of compounds upon binding is proportional to the concentration and affinity of the ligands[4–6]. Although for many proteins unfolding is not a reversible (equilibrium) monomolecular two-state reaction as described, assuming equilibrium conditions allows one to approach protein stability reasonably well and compare it under different conditions. However, interpretations regarding thermodynamics need to be done with caution and confirmed by other methods. Also one should be careful with ranking compounds that have been identified as ligands for a protein based on their ΔT_m values, as these values are not always a reflection of their relative affinities. The magnitude of the T_m shift observed for different compounds with the same affinity at the relevant temperature is dependent on the contributions of enthalpy and entropy of binding. Larger T_m shifts are observed for more entropically driven (e.g., hydrophobic) binding. Similarly, a given T_m shift is not unique to a given binding affinity, because a range of different affinities, with different entropic and enthalpic components, might give rise to the same change in T_m. Competing effects could also mask ligand binding. For example, a compound binding tightly and enthalpically to the native state could be masked by weaker, entropically driven binding to the denatured state. However, one can rank compounds with similar physicochemical properties based on their relative ΔT_m (reviewed by Holdgate and Ward[7]).

Description of the method

DSF monitors thermal unfolding of proteins in the presence of a fluorescent dye[8,9] and is typically performed by using a real-time PCR instrument[10] (e.g., Stratagene Mx3005p, Bio-Rad iCycler5, DNA Engine Opticon 2). DSF can be applied to a wide range of proteins[6,11]. The fluorescent dyes that can be used for DSF are highly fluorescent in non-polar environment, such as the hydrophobic sites on unfolded proteins, compared to aqueous solution where the fluorescence is quenched. The various dyes that have been used differ with respect to their optical properties[12], particularly in the fluorescence quantum yield caused by binding to denatured protein (**Fig. 1**).

DSF is an excellent platform to screen for conditions that stabilize proteins, owing to the small amounts and low concentrations of protein required. As a consequence, the method can be applied to samples for which aggregation or low stability hinders purification efforts[6]. In DSF, the fluorescence intensity is plotted as

a function of temperature; this generates a sigmoidal curve that can be described by a two-state transition (**Fig. 2**). The inflection point of the transition curve (T_m) is calculated using simple equations such as the Boltzmann equation,

$$y = \mathrm{LL} + \frac{(\mathrm{UL} - \mathrm{LL})}{1 + \exp\left(\frac{Tm - x}{a}\right)} \quad (1)$$

where LL and UL are the values of minimum and maximum intensities, respectively, and a denotes the slope of the curve within T_m. The simplest way to calculate T_m values is to determine the maximum of the first derivative; this feature is offered by most PCR software packages (e.g., in the Mx3005p, scans using the type 'SYBR green' allow display of derivatives after the run is finished).

To date, the dye with the most favorable properties for DSF is SYPRO orange, owing to its high signal-to-noise ratio. For example, with hen egg lysozyme, the increase in the fluorescence intensity of the dye bound to denatured protein compared to aqueous solution is almost 500% (**Fig. 1**). The relatively high wavelength for excitation for SYPRO orange, near 500 nm (**Fig. 3**), also decreases the likelihood that any small molecule would interfere with the optical properties of the dye and cause, for example, quenching of the fluorescence intensity. For comparison, many compounds interfere with the spectral properties of the commonly used 1-Anilino-8-naphthalene sulfonate (1,8-ANS), whose excitation maximum is ~350 nm. In addition, the signal intensities with 1,8-ANS, for example, are relatively small (~80%; see **Fig. 1**)[12]. However, not all proteins can be analyzed using SYPRO orange. Other dyes should be tested for cases where no unfolding transition is observed using SYPRO orange.

Other thermal scanning methods for small-molecule screening

The stabilization of proteins against thermal denaturation, and the influence of small molecules, can also be measured by differential scanning calorimetry (DSC) or differential scanning light scattering (DSLS). DSC in an automated version (capDSC, MicroCal, LLC[13]) allows a series of up to 192 protein/compound combinations. Although generally less favorable because of the need for a reporter dye, DSF is superior to DSC with respect to throughput (time per sample in DSC: ~90 min). DSLS[5], another technique available for assessing protein stability, monitors the aggregation that occurs for most proteins as they denature[6]. It is performed in a 384-well format (StarGazer, Harbinger Biotech). Although most proteins are amenable to DSLS and DSF, a small percentage of proteins might not work with DSLS but could be analyzed by DSF and vice versa[6]. DSF performed on RT-PCR instruments also eliminates the need to add oil on top of the samples to prevent evaporation and provides a wider range of temperature for scanning (>80 °C).

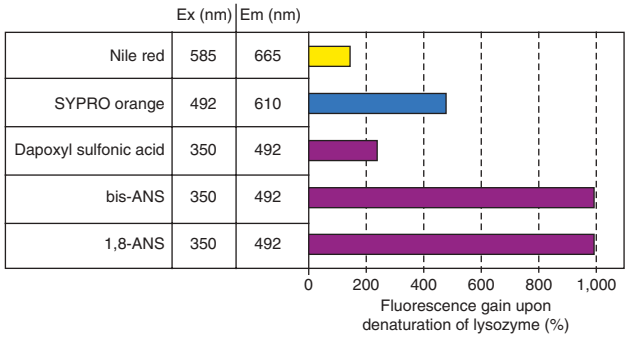

Figure 1 | Optical properties and fluorescence signal in the presence of lysozyme (native versus denatured) for selected dyes that can be used for DSF. All dyes (at 43 µM except SYPRO orange, which was diluted 1:250) were in a solution of 75 µg ml^{-1} hen egg lysozyme in buffer (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid pH 9.0, 150 mM NaCl). The graph shows the difference in fluorescence intensity before and immediately after incubation for 5 min at 100 °C, respectively. The excitation and emission wavelengths given in the table refer to the custom filters for the Stratagene Mx3005p instrument.

Setting up the plate

The small molecules to be screened may comprise salts, buffers or compounds, such as substrates, inhibitors or co-factors that are thought to selectively interact with the protein of interest. If a protein or closely related proteins are known to bind to specific ligands, the screening collection may include those specific ligands and their analogues (for a more detailed discussion on library construction, see Vedadi *et al.*[6]). We typically select 10–15 compounds per lead scaffold to add to the screening library. These synthetic compounds are customarily distributed in 2 mg amounts, allowing hundreds of screens and sufficient initial follow-up studies, such as co-crystallization or inhibition assays. Most suppliers deliver the ordered amount in a vial, and for accuracy and practicality reasons it is best to dissolve the entirety of the compound in the required volume of the inorganic solvent dimethyl sulfoxide (DMSO). The majority of compounds that are hydrophobic are dissolved in DMSO at relatively high concentrations. We found a solubility limit of less than 10 mM for only 1% of over 2,000 compounds dissolved, whereas for 85% the solubility was 50 mM or higher. For long-term storage of light-sensitive compounds, brown glass vials with DMSO-resistant lids and seals

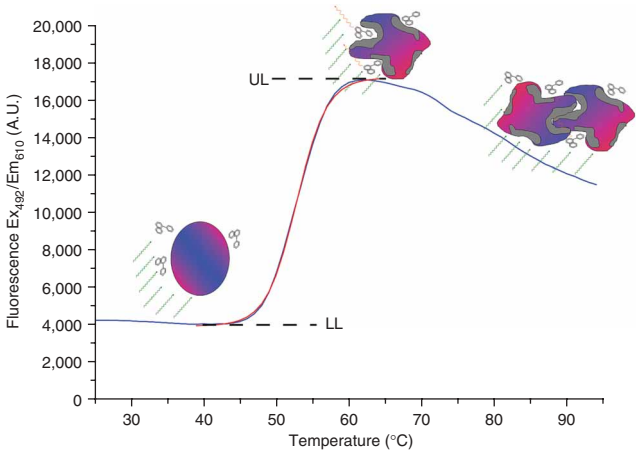

Figure 2 | Typical recording of fluorescence intensity versus temperature for the unfolding of protein (citrate synthase) in the presence of SYPRO orange. The dye, the molecular structure of which is undisclosed, is symbolized as a three-ring aromatic molecule. In the presence of a globular protein (spherical shape at the baseline of the curve), a basic fluorescence intensity is excited by light of 492 nm (depicted schematically by green curved arrows). Through unfolding of the protein, hydrophobic patches (in gray) become exposed, and strong fluorescent light of 610 nm (depicted by orange curved arrows) is emitted by the dye molecules bound to them. Following the peak in the intensity, a gradual decrease is observed, which is mainly explained by protein being removed from solution owing to precipitation and aggregation. The lower and upper level in the fluorescence intensity, LL and UL, respectively, defined by equation (1), are depicted in the figure.

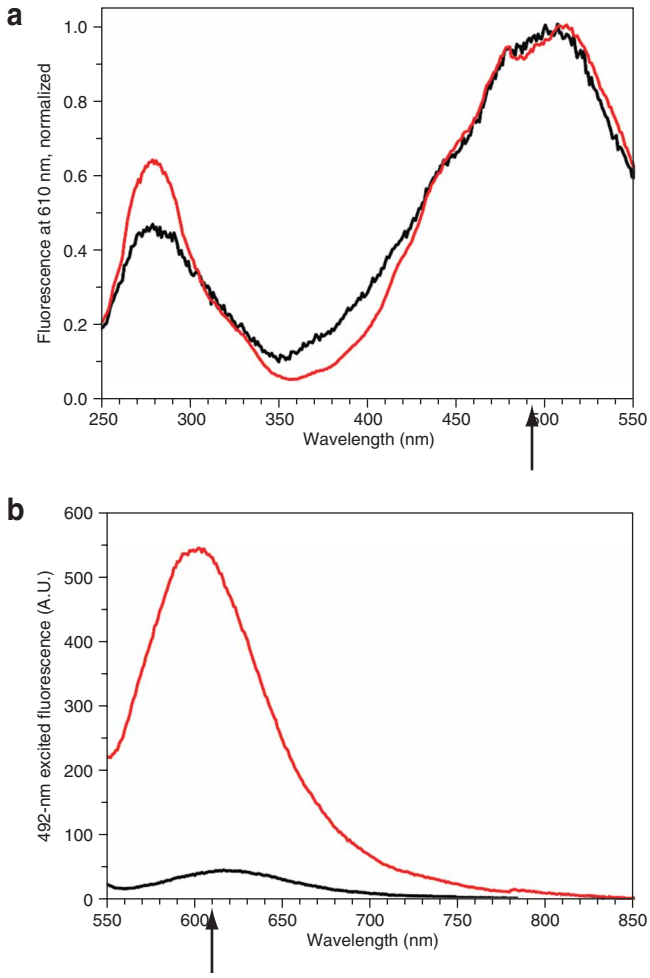

Figure 3 | Optimum excitation of SYPRO orange in protein buffer and gain in fluorescence upon protein unfolding. Excitation/emission spectra of dye diluted 1:1,000 in a solution of 75 μg ml^{-1} hen egg lysozyme in buffer (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid pH 9.0, 150 mM NaCl), before (black traces) and immediately after incubation for 5 min at 100 °C (red traces), are shown. Arrows depict position of the custom filters used in the Stratagene Mx3005p PCR instrument. (**a**) Normalized excitation spectra, recorded at a wavelength of 610 nm. (**b**) Non-normalized emission spectra upon excitation at 492 nm.

should be employed. For simplicity, it is easiest to use these vials for all compounds.

We recommend screening against natural compounds, such as buffers, salts, etc. (e.g., when low solubility or low stability of the protein of interest is detected, usually during purification), in aqueous solutions that have been adequately pH adjusted. It is most practical to prepare 4× concentrations of all conditions (see **Table 1** for a suggested set of conditions for buffers/pH values, NaCl concentrations and glycerol). For small molecules that require freezing for storage, it may be most practical to prepare aliquoted PCR plates and freeze them at less than −20 °C until use. The plates should be covered with DMSO-resistant lids such as aluminum foil. During freezing, it is likely that moisture will build up on the lid; so it is critical to briefly centrifuge the plate (1 min at room temperature (20±5 °C), 200*g*) before removing the foil, to avoid cross-contamination.

The compounds are stored in DMSO in mother plates, which serve as source plates in setting up the experiments in the PCR plate. Controls should be included to ensure that DMSO does not significantly influence the stability of the protein. The final concentration of DMSO in the screen should not exceed 2% (vol/vol). We divide the plate layout into three parts (**Fig. 4**), which is useful

TABLE 1 | Suggested conditions for small-set buffer/additive screening.

No.	Buffer component (all 100 mM)	pH	$\Delta pH/\Delta t$[a]	NaCl concentration (mM)	Additive	Additive concentration (%)
1	Sodium acetate-AcOH	5.0	0.000	150		
2	Pyridine-HCl	5.5		150		
3	Sodium cacodylate-HCl	6.0		150		
4	MES-NaOH	6.0	−0.011	150		
5	Imidazole-HCl	7.0		150		
6	PIPES-NaOH	7.0	−0.009	150		
7	MOPS-NaOH	7.2	−0.011	150		
8	HEPES-NaOH (reference)	7.5	−0.015	150		
9	Tricine-NaOH	8.0	−0.021	150		
10	Tris-HCl	8.1	−0.028	150		
11	Bicine-NaOH	8.3	−0.018	150		
12	Glycine-NaOH	9.0	−0.026	150		
13	BORAX-Boric acid	9.0	−0.008	150		
14	CHES-NaOH	9.5	−0.011	150		
15	Ethanolamine-NaOH	9.5	−0.030	150		
16	CAPS-NaOH	10.0		150		
17	HEPES-NaOH	7.5	−0.015			
18	HEPES-NaOH	7.5	−0.015	50		
19	HEPES-NaOH (reference)	7.5	−0.015	150		
20	HEPES-NaOH (reference)	7.5	−0.015	150		
21	HEPES-NaOH	7.5	−0.015	500		
22	HEPES-NaOH	7.5	−0.015	150	Glycerol	5
23	HEPES-NaOH	7.5	−0.015	150	Glycerol	10
24	HEPES-NaOH (standard GF buffer)	7.5	−0.015	500	Glycerol	5

[a]Data taken from Dawson *et al.*[16].

	1	2	3	4	5	6	7	8	9	10	11	12
A	Compound A-1 at 0.4 mM	Compound A-2 at 0.4 mM	Compound A-3 at 0.4 mM	Compound A-4 at 0.4 mM	Compound B-1 at 0.4 mM	Compound B-2 at 0.4 mM	Compound B-3 at 0.4 mM	Compound B-4 at 0.4 mM	Compound C-1 at 0.4 mM	Compound C-2 at 0.4 mM	Compound C-3 at 0.4 mM	Compound C-4 at 0.4 mM
B	Compound A-5 at 0.4 mM	Compound A-6 at 0.4 mM	H_2O control	Reference	Compound B-5 at 0.4 mM	Compound B-6 at 0.4 mM	H_2O control	Reference	Compound C-5 at 0.4 mM	Compound C-6 at 0.4 mM	H_2O control	Reference
C	Compound A-7 at 0.4 mM	Compound A-8 at 0.4 mM	Compound A-9 at 0.4 mM	Compound A-10 at 0.4 mM	Compound B-7 at 0.4 mM	Compound B-8 at 0.4 mM	Compound B-9 at 0.4 mM	Compound B-10 at 0.4 mM	Compound C-7 at 0.4 mM	Compound C-8 at 0.4 mM	Compound C-9 at 0.4 mM	Compound C-10 at 0.4 mM
D	Compound A-11 at 0.4 mM	Compound A-12 at 0.4 mM	Compound A-13 at 0.4 mM	Compound A-14 at 0.4 mM	Compound B-11 at 0.4 mM	Compound B-12 at 0.4 mM	Compound B-13 at 0.4 mM	Compound B-14 at 0.4 mM	Compound C-11 at 0.4 mM	Compound C-12 at 0.4 mM	Compound C-13 at 0.4 mM	Compound C-14 at 0.4 mM
E	Compound A-15 at 0.4 mM	Compound A-16 at 0.4 mM	Compound A-17 at 0.4 mM	Compound A-18 at 0.4 mM	Compound B-15 at 0.4 mM	Compound B-16 at 0.4 mM	Compound B-17 at 0.4 mM	Compound B-18 at 0.4 mM	Compound C-15 at 0.4 mM	Compound C-16 at 0.4 mM	Compound C-17 at 0.4 mM	Compound C-18 at 0.4 mM
F	Compound A-19 at 0.4 mM	Compound A-20 at 0.4 mM	Compound A-21 at 0.4 mM	Compound A-22 at 0.4 mM	Compound B-19 at 0.4 mM	Compound B-20 at 0.4 mM	Compound B-21 at 0.4 mM	Compound B-22 at 0.4 mM	Compound C-19 at 0.4 mM	Compound C-20 at 0.4 mM	Compound C-21 at 0.4 mM	Compound C-22 at 0.4 mM
G	Reference	Compound A-23 at 0.4 mM	Compound A-24 at 0.4 mM	Compound A-25 at 0.4 mM	Reference	Compound B-23 at 0.4 mM	Compound B-24 at 0.4 mM	Compound B-25 at 0.4 mM	Reference	Compound C-23 at 0.4 mM	Compound C-24 at 0.4 mM	Compound C-25 at 0.4 mM
H	Compound A-26 at 0.4 mM	Compound A-27 at 0.4 mM	Compound A-28 at 0.4 mM	Compound A-29 at 0.4 mM	Compound B-26 at 0.4 mM	Compound B-27 at 0.4 mM	Compound B-28 at 0.4 mM	Compound B-29 at 0.4 mM	Compound C-26 at 0.4 mM	Compound C-27 at 0.4 mM	Compound C-28 at 0.4 mM	Compound C-29 at 0.4 mM

Figure 4 | Layout for a 96-well plate, enabling screening of sets of 29 compounds under three different conditions in parallel. The layout is divided vertically into three parts (colored in light green, white and orange, respectively), each of which contains two reference (DMSO, purple boxes) conditions and one control (water, blue boxes). The figure depicts a screen shot of a layout created with the setup & analysis file available online (ftp://ftp.sgc.ox.ac.uk/pub/biophysics).

to screen against a set of compounds under different conditions in parallel (e.g., in the presence of different cofactors).

Experimental setup

When setting up a compound screen, we distribute the buffered protein solution including the dye into the wells of the plate and then transfer the compound solution (in a small volume, to limit the DMSO concentration) from each well of the source plate into the PCR plate. The SYPRO orange dye (Invitrogen) is delivered as a 5,000× solution in 100% (vol/vol) DMSO. We dilute the dye first into the buffer solution (1:1,000) before adding the protein, to prevent damage to the protein by contact with high concentrations of DMSO. Compounds in 100% (vol/vol) DMSO solutions need to be diluted in final screening conditions at least 50× to avoid DMSO concentrations of more than 2% (vol/vol). As a result of these limitations, the smallest volume that can be dispensed determines the final volume per well in the PCR plate. Most manual 10 µl multichannel pipettes allow accurate pipetting of 1 µl. So, in most laboratories, the total reaction volume will be 40 µl.

A protein concentration of 75 µg ml^{-1} (i.e., 2 µM, for a molecular mass of 35 kDa) is normally sufficient to accurately measure the T_m. We maintain a ratio between protein and compound of at least 1:10. For compounds that have an expected affinity (K_d) between 1 nM and 1 µM, it is most practical to screen the compounds at 10 µM concentrations because most compounds are soluble in aqueous solution at this concentration. For most proteins, we find that lower concentrations are applicable; so for most of our screens, we employ protein and compound concentrations of 1 and 10 µM, respectively. We find, however, that varying protein and compound concentrations are frequently necessary, for example for proteins, where a higher concentration is necessary to obtain an acceptable signal-to-noise ratio, or compounds, such as nucleotides, that bind with relatively low affinity.

Analysis to identify best conditions

To identify a ligand or a buffer condition that stabilizes a protein, the T_m value of the protein under each condition of the screen needs to be compared with the reference T_m. The first step of the analysis, therefore, is the calculation of the position of the midpoint of the curves. The easiest way is to use the instrument software to display the first derivatives (e.g., choose the third program, type 'SYBR green' from the Mx3005p menu for the run). More accurate fitting can be achieved by using software packages such as XLfit (http://www.idbs.com/Decision/xlfit), Kaleidagraph (http://www.synergy.com), GraFit (http://www.erithacus.com/grafit) or Origin (http://www.originlab.com). Visualization of the data and comparison of the stabilization under various conditions is accomplished through custom calculation software based on Microsoft Excel (ftp://ftp.sgc.ox.ac.uk/pub/biophysics).

Compound titration

We have observed that the stability of proteins usually increases as the concentration of the compound is increased, which indicates that the thermal stability of the protein is proportional to the concentration of the ligand[4,6]. To confirm that a compound that has been identified from primary screening as a stabilizer binds and stabilizes the protein in a concentration-dependent manner, we recommend re-screening the protein in the presence of different concentrations of the compound. An example of such an experiment is presented in **Figure 5**, where the effect of different

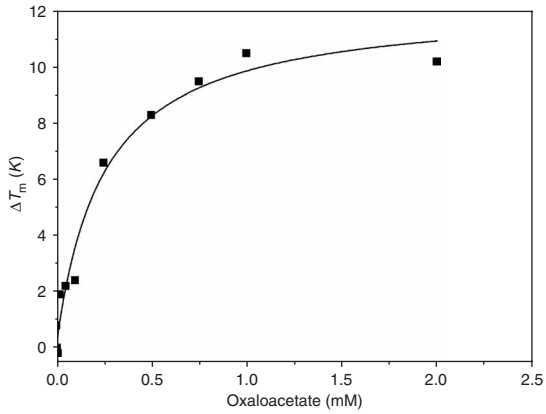

Figure 5 | Concentration-dependent stabilization of citrate synthase by oxaloacetate. The T_m for 1 µM of protein in 10 mM HEPES-NaOH pH 7.5, containing 100 mM NaCl, was measured by DSF at oxaloacetate concentrations between 10 and 2,000 µM. The curve represents an exponential fit to the ΔT_m values plotted against the compound concentration.

concentrations of oxaloacetate on the stability of porcine citrate synthase has been investigated. Compound titration is critical to investigate if small stabilizing effects would be valid and reproducible. This type of experiment would also help to determine the concentration at which a maximum stabilizing effect of a compound can be obtained. This information is especially valuable for protein crystallization, using a concentration of compound that helps reaching maximum occupancy.

MATERIALS

REAGENTS

- DMSO (Sigma Aldrich, cat. no. D8418)
- 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; Sigma Aldrich, cat. no. H3375) ▲ CRITICAL Other buffer components may be used, dependent on the requirements of the protein under investigation. It is, however, not advisable to use buffers whose properties are strongly temperature-dependent, such as buffers that comprise a high coefficient of $\Delta pH/\Delta t$ (e.g., Tris; see **Table 1**).
- Sodium chloride (NaCl; Sigma Aldrich, cat. no. S7653)
- SYPRO orange (Invitrogen, cat. no. S6650)
- Citrate synthase (from porcine heart; Roche, cat. no. 103381)

EQUIPMENT

- Compound storage vial combinations comprising brown glass vial, PTFE/silicone seal and cap (Chromacol). Specifically, for a 300 µl volume: vial; 03-FISV(A), seal (9 mm); AGE A1005, cap (9 mm); cat. no. 9-SCS-8RTI. For a 2 ml volume: vial; 2-SV(A), seal (8 mm); 8-ST15, cap (8 mm); cat. no. 8-SCS. For a 4 ml volume: vial; 4-SV(A), seal (12 mm); 12-ST2, cap (12 mm); cat. no. 12-SCS
- DMSO-resistant 96-well plates (e.g., ABgene, cat. no. AB-0796)
- Adhesive aluminum seals (e.g., ABgene, cat. no. AB-0626)
- U-bottom 96-well microplates, total volume 360 µl (e.g., Corning, cat. no. 3355)
- 10 and 50 µl multichannel pipettes
- White quantitative PCR low-profile plates (e.g., ABgene, cat. no. AB-0700/w) ▲ CRITICAL Compared to frosted plates, white plates have more consistent optical properties and decrease refraction, thus enhancing the signal. ▲ CRITICAL For pipetting, stack the flexible PCR plates into standard microplates.
- Optical PCR seals (Bio-Rad, cat. no. 223-9444)
- Smooth rubber roller for aluminum plate foil (from any DIY store)
- Hand applicator for plate foil (3M 'PA1', cat. no. 75-3454-4264-6)
- Desalting columns (GE Healthcare PD-10)
- Real-time PCR instrument (e.g., Stratagene Mx3005p; Bio-Rad iCycler5 or DNA Engine Opticon 2)
- Worksheet 'DSF analysis.xls' (ftp://ftp.sgc.ox.ac.uk/pub/biophysics). In addition, software for accurate analysis such as XLfit, Kaleidagraph, GraFit, Origin (see text for company websites)

EQUIPMENT SETUP

RT-PCR instrument Because of its broad range of excitation and emission (**Fig. 3**), SYPRO orange can be monitored in RT-PCR instruments using the filters commonly provided with the machines: FAM (492 nm) and ROX (610 nm), for excitation and emission, respectively (for wavelengths, refer to Stratagene filters). Alternatively, other dyes may be used (**Fig. 1**).

PROCEDURE

Preparation of compound solutions ● TIMING Typically 0.1 h per compound

1| Calculate the volume of DMSO required to yield a 50 mM compound solution for the amount of compound in the vial (typically 2 mg). Alternatively, use the calculator 'Helpfile Weigh&Dissolve Compounds.xls' (ftp://ftp.sgc.ox.ac.uk/pub/biophysics).

2| Add DMSO to the vial and dissolve using a pipette and then vortex.

? TROUBLESHOOTING

3| Place the vial in the tube (e.g., 50 ml) and centrifuge (200*g*, room temperature, 1 min) to collect all of the solution at the bottom.

4| Label the compound storage vial according to the final volume and concentration of compound solution.

5| Transfer compound solution into compound storage vial and store at −20 °C.

▲ CRITICAL STEP Prepare and store all compound stock solutions that are needed for one plate before setting up the storage plate. DMSO is very hygroscopic and should be protected from exposure to moisture. Such exposure can cause precipitation of the compound during storage at high concentrations in DMSO if the absorption is high. Additionally, avoid exposing the compounds to warm temperatures whenever possible.

■ PAUSE POINT Compounds can be stored at −20 °C. Note, however, that the stability of different compounds may vary at this temperature.

Preparation of buffer/additive screen plates ● TIMING 2 h (excluding setup of buffer/additive solutions)

6| This can be done following option A or B. Option A is recommended for quick setup of experiments or if additives unstable at temperatures above 0 °C are used, whereas option B can be followed with additives that can be stored for a short time at 4 °C.

(A) Standard method

(i) Prepare PCR plates by aliquoting one-fourth of the final volume (e.g., 5 µl) of the 4× concentrated test solutions directly into the wells.

(ii) Seal the plates with aluminum foil.

▲ CRITICAL STEP Ensure that the plates are sealed well (use rubber roller) to prevent evaporation and cross-contamination.

■ PAUSE POINT Store plates at −80 °C until use.

(B) Alternative method

(i) Set up solutions with desired buffers and salts at 4× the desired experimental concentration (e.g., in volumes of 2 ml).

▲ CRITICAL STEP Ensure that the plates are sealed well (use rubber roller) to prevent evaporation and cross-contamination.

■ PAUSE POINT Store solutions at 4 °C in 2 ml deep-well microplates and use within 2 months.

Preparation of compound storage plates (example for manual pipetting: 0.4 mM compound concentrations) ● TIMING 3–4 h per plate

7| Thaw all compound stock solutions for the plate layout (e.g., place vials for ~5 min into a trough filled to ~1 cm depth with room temperature water).

8| Fill the reference and the control wells of the 96-well storage plate with 100% (vol/vol) DMSO and distilled water, respectively.

9| Pipette a sufficient volume of each compound stock solution (in DMSO) into the wells of the compound plate (DMSO-resistant standard microplate) to yield a final concentration of 0.4 mM of each compound in 200 µl volumes. It may be necessary to vortex the compound stock solution to ensure that it is completely dissolved after thawing. Use a pipette to mix the well solutions.

10| Prepare copies of the master plate (20–50 µl).

11| Attach aluminum foil seals to plates and store at −20 °C or below.
▲ **CRITICAL STEP** Ensure that the plates are sealed well (use rubber roller) to prevent evaporation and cross-contamination.
■ **PAUSE POINT** Plates can be stored at −20 °C.

Equipment preparation ● TIMING Steps 12 and 13: 0.25 h

12| Warm up the xenon lamp in the PCR instrument for the time recommended by the manufacturer (20 min for the Mx3005p). This process may start upon launching the software (if the 'Turn on lamp for warm up' option has been selected previously). Alternatively, start warming up the lamp selecting 'Instrument\Lamp On' from the menu. For further details on equipment preparation, refer to the instructions provided by the manufacturer of your RT-PCR machine.
▲ **CRITICAL STEP** To establish proper communication between computer and instrument, it is necessary to turn on the instrument before launching the software.

13| Thaw the compound or buffer/additive plate during the lamp warm-up time by placing it in a shallow trough of room temperature water.

Sample preparation ● TIMING 0.25 h

14| Measure the protein concentration, preferably by its absorbance at 280 nm and using an extinction coefficient calculated by the method described by Pace *et al.*[14]. For proteins containing neither tryptophan nor tyrosine residues, alternative procedures may be employed (refer, e.g., to ref. 15).

15| Follow one of the following three options for plate setup, depending on whether compound (option A) or buffer/additive screening (option B) will be performed or if compounds are to be tested under different (up to three) conditions (option C).

(A) Full-plate compound screen

(i) Calculate the volume of protein stock solution necessary to prepare 4.2 ml of a 1 µM solution (in some cases, higher concentrations may be necessary; see text).

(ii) Prepare 4.2 ml protein solution in 10 mM HEPES-NaOH pH 7.5, 150 mM NaCl and 5× SYPRO orange (1:1,000 dilution of the stock solution) in a 10 ml tube.
▲ **CRITICAL STEP** Add the protein to a solution of the other ingredients and mix carefully using a 1 ml pipette.

(iii) Aliquot (340 µl each) protein solution into wells of one row of the U-bottom-shaped microplate.

(iv) Using a 50 µl multichannel pipette, aliquot 39 µl of the protein solution into each of the wells of the PCR plate.
▲ **CRITICAL STEP** To avoid creating bubbles, mix gently and empty the pipette on the side of the well.

(v) Spin the compound plate briefly (200*g*, room temperature, 1 min) to collect the solution at the bottom of the wells and then remove the aluminum sealing foil.

(vi) Using a 10 µl pipette, transfer 1 µl of each compound into the PCR plate containing the protein solution. Mix carefully using the pipette's first stop and dispense the remaining solution on the upper wall of the well to avoid creating bubbles.

(vii) Reseal the compound plate with a new aluminum sealing foil.
▲ **CRITICAL STEP** Ensure that the plate is well sealed by using a smooth rubber roller.

(B) One plate third/three conditions compound screen

(i) Calculate the volume of protein stock solution necessary to prepare 1.5 ml of a 1 µM solution (in some cases, higher concentrations may be necessary; see text).

(ii) Prepare three 1.5 ml protein solutions in 10 mM HEPES-NaOH pH 7.5, 150 mM NaCl and 5× SYPRO orange (1:1,000 dilution of the stock solution) each in a 2 ml vial. Add the desired cofactors, metals or salts at an appropriate concentration to each of the individual solutions.
▲ **CRITICAL STEP** Add the protein to a solution of the other ingredients and mix carefully using a 1 ml pipette.

(iii) For each solution, aliquot (185 µl each) protein solution into wells of one column of the standard U-bottom-shaped microplate.

(iv) Using a 50 µl multichannel pipette, aliquot 39 µl of the protein solution into each of the wells of the PCR plate. A standard microplate may be used as support for the flexible PCR plate.
▲ CRITICAL STEP To avoid creating bubbles, mix gently and empty the pipette on the side of the well.
(v) Spin the compound plate briefly (200*g*, room temperature, 1 min) to collect solution in the bottom of the wells and then remove the aluminum sealing foil.
(vi) Using a 10 µl pipette, transfer 1 µl from each well of the selected part (i.e., third) of the source plate into the left part of the PCR plate. Mix carefully using the pipette's first stop and dispense the remaining solution on the upper wall of the well to avoid creating bubbles. Then proceed in the same manner, adding the selected compounds into the central and right part of the PCR plate.
(vii) Reseal the compound plate with a new aluminum sealing foil.
▲ CRITICAL STEP Ensure that the plate is well sealed by using a smooth rubber roller.

(C) Buffer/additive screen

(i) Calculate the volume of protein stock solution needed to prepare 1.8 ml of a 0.1 mg ml^{-1} solution (in some cases, higher concentrations may be necessary; see text). Should the dilution be less than 20×, the protein should be transferred into a low-strength buffer without salt, to ensure that the desired change in conditions is achieved within the screen. To do this, equilibrate a fresh PD-10 column (Amersham) with 25 ml of 10 mM HEPES-NaOH pH 7.5. Apply 2.5 ml of 0.2 mg ml^{-1} protein solution to the column and discard the flow-through. Apply 3.5 ml of buffer and collect the flow-through. Dilute protein solution to 0.1 mg ml^{-1}.
(ii) Add SYPRO orange to a final concentration of 5× to the 1.8 ml protein solution (1:1,000 dilution) and mix using a 1 ml pipette.
(iii) Aliquot (145 µl each) protein solution into wells of one row of the standard U-bottom-shaped microplate.
(iv) If buffer/additive solutions are stored in a deep-well plate, aliquot 5 µl of each into the wells of the PCR plate. When using prepared frozen PCR plates containing the screen conditions, spin at 200*g* (1 min, room temperature) to collect solution in the bottom of the wells before removing the aluminum seal.
(v) Add 15 µl of the protein solution into each of the wells of the PCR plate, using a 50 µl multichannel pipette. Mix carefully using the pipette's first stop and dispense the remaining solution on the upper wall of the well to avoid creating bubbles.

Performing the scan ● TIMING 1.5 h

16| Seal the PCR plate with the optical foil seal.
▲ CRITICAL STEP Use the applicator and press down hard, starting from the middle to avoid bulges. Then, turn the plate by 180° and repeat the procedure for the other side.

17| Spin the PCR plate (200*g*, room temperature, 1 min).
▲ CRITICAL STEP This step may remove bubbles from the solution.

18| Place the PCR plate into the PCR instrument and run the temperature scan from 25 to 95 °C, at 1 °C min^{-1} (on the Mx3005p, choose program type 'SYBR green', to enable display of selected curves as first derivatives after the run).

Data analysis (details refer to the Mx3005p) ● TIMING 0.75 h

19| Export the data to Excel. In the software, choose the 'Analysis' tab, then click on the 'Analysis Selection/Setup' tab and select all wells by mouse-clicking the upper left corner of the displayed plate layout. Click on the tab 'Results', tick 'Amplification plots' from the options given on the upper right corner and select 'R (Multicomponent view)'. From the main menu, select 'File\Export Chart Data\Export Chart Data to Excel\Format 2—Horizontally Grouped by Plot'. Excel will open and the file can then be processed further (see below).

20| If the experiment performed was of the type 'SYBR Green (With Dissociation curve)', derivative curves can be exported to Excel. In the software, select the tab 'Analysis', click on 'Analysis Selection/Setup' and select all wells by mouse-clicking the upper left corner of the displayed plate layout. Select the tab 'Results', tick 'Dissociation curve' from the options given on the upper right corner and select '-R'(T)'. From the main menu, select 'File\Export Chart Data\Export Chart Data to Excel\Format 2—Horizontally Grouped by Plot'. Excel will open in a new window and the file can be processed further (see below).

21| Open the 'DSF analysis' Excel file that is available for download from ftp://ftp.sgc.ox.ac.uk/pub/biophysics. Open the Excel file that contains the thermal denaturation plots (called 'amplification plots' in the Mx3005p software). Mark the entire data table, by clicking the upper left corner. Drag (Ctrl + C) and drop (Ctrl + V) data into the worksheet 'Import chart data horizontal'.

22| To calculate T_m values, the 'DSF analysis' worksheet uses the first derivative curves. Open the Excel file with the first derivatives. Mark the entire data table, by clicking the upper left corner. Drag (Ctrl + C) and drop (Ctrl + V) data into the worksheet 'Import 1st deriv. data horiz.' of the analysis file 'DSF analysis'. T_m values will be calculated (accurately to zero digits). As an

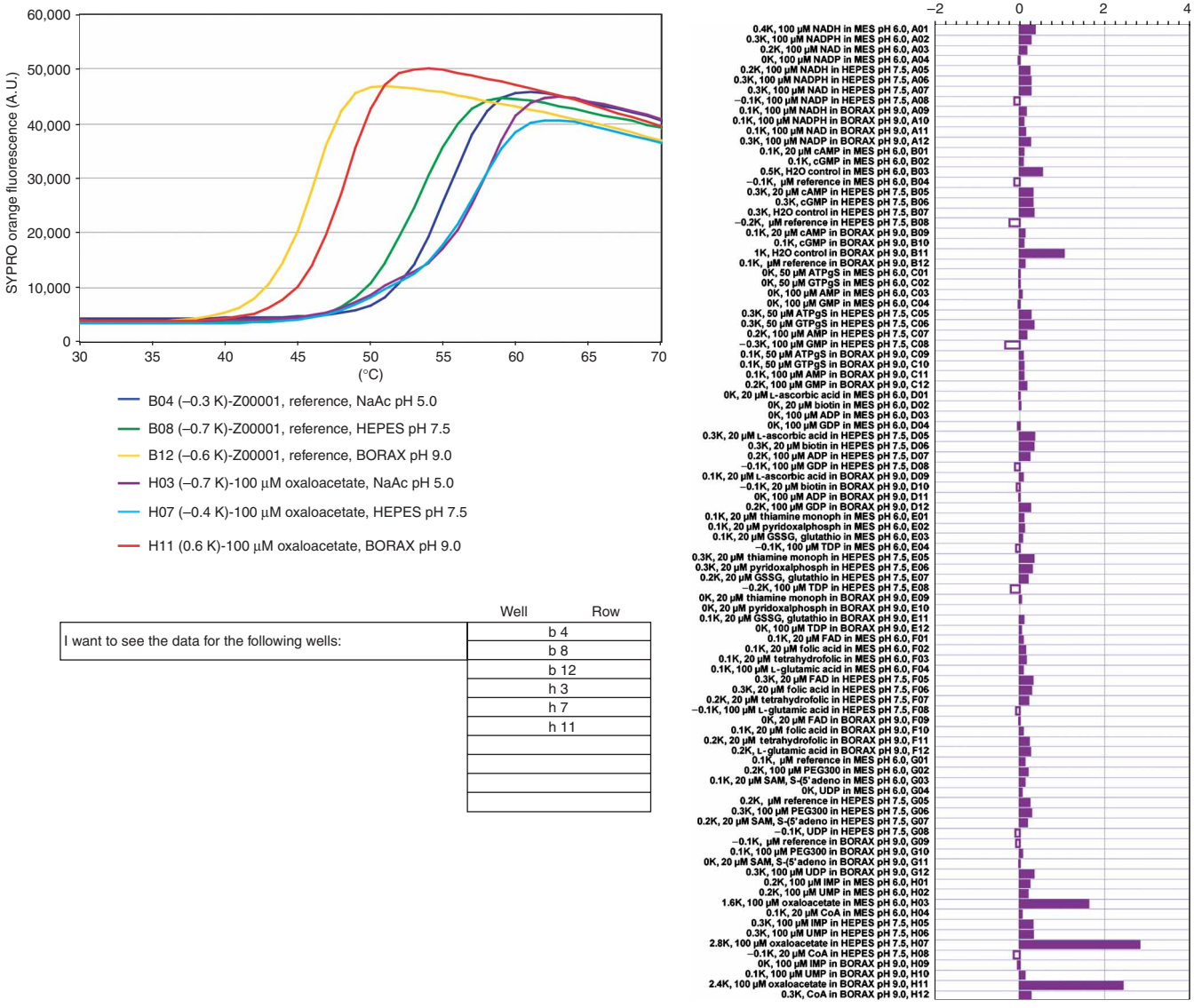

Figure 6 | Visualization of the screen results for citrate synthase against physiologically relevant compounds. Porcine citrate synthase (1 µM) was screened against a selection of 29 compounds (**Table 3**), under three different buffer conditions (entry numbers 4, 8 and 13 in **Table 1**; pH 6.0, 7.5 and 9.0, respectively). The figure depicts a screen shot of two features provided with the DSF analysis tool (ftp://ftp.sgc.ox.ac.uk/pub/biophysics), showing the selection of up to ten curves (left panel) and an overview of tested conditions regarding the ΔT_m values.

alternative, more accurate fitting to the Boltzmann equation can be performed using proprietary software, such as XLfit, Kaleidagraph, GraFit or Origin. Transfer the table of T_m values into the worksheet 'T_m table' of the 'DSF analysis' file, to replace the data with more accurate values.

? TROUBLESHOOTING

23| The 'DSF analysis' Excel file may be used for further analysis and visualization of data, such as display of selected curves in one graph after normalization or a column-type comparison between the ΔT_m values for conditions of an entire plate.

● TIMING

Preparation of compound solutions, Steps 1–5: 0.1 h per compound (typically)
Preparation of buffer/additive screen plates, Step 6: 2 h (excluding setup of buffer/additive solutions)
Preparation of compound storage plates, Steps 7–11: 3–4 h per plate

Equipment preparation, Steps 12 and 13: 0.25 h
Sample preparation, Steps 14 and 15: 0.25 h
Performing the scan, Steps 16–18: 1.5 h
Data analysis, Steps19–23: 0.75 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Poorly soluble compounds	Cloudy suspensions are a sign of low solubility and indicate that further dilution is necessary	Try sonicating or moderate heating of poorly soluble compounds
22	High initial fluorescence intensity, small transitional increase	Binding of dye to hydrophobic domains exposed in native state Dye binding to compound or solvent ingredient	Option 1: Perform titration of protein concentration: Increase dye concentration to 10× or higher when using >4 μM of protein Option 2: Use a different dye Option 3: Use an alternative method (see INTRODUCTION)
		Protein not folded	Option 1: Re-purify the protein Option 2: Perform buffer/additive screen
	No transition	Protein partially unfolded or aggregated	Option 1: Re-purify the protein Option 2: Perform buffer/additive screen
		Protein stability higher than temperature range	Option 1: Extend temperature range Option 2: Perform buffer/additive screen, identify de-stabilizing conditions to be used for compound screens
	Transition curve not monophasic	Protein comprises more than one domain or forms oligomers	Option 1: Perform buffer/additive screen, to identify condition under which protein unfolding is two-state Option 2: If protein is known to comprise >1 domain, try separate expression of domain comprising active site
		Compound concentration suboptimal, indicated by the intensity peak being at higher temperature than observed with the reference	Titrate the compound concentration Check if compound is soluble in aqueous solution. Replace insoluble compound with analogue

ANTICIPATED RESULTS

Citrate synthase is a commercially available enzyme (Roche; see the REAGENTS section for further details) with well-defined properties that can serve as a standard for DSF. We used this protein, for example, to compare different thermal scanning methods[6]. We included its natural substrate, oxaloacetate, into a screen containing 29 physiologically relevant compounds (cofactors, nucleotides, etc.; see **Table 3**; note the relatively high concentrations that reflect the relatively low affinities usually

TABLE 3 | Set of 29 conditions for screening of citrate synthase.

No.	Compound	Storage plate concentration (mM)	Final assay concentration (μM)
1	NADH	4	100
2	NADPH	4	100
3	NAD	4	100
4	NADP	4	100
5	cAMP	0.8	20
6	cGMP	0.8	20
7	ATPγS	2	50
8	GTPγS	2	50

TABLE 3 | Set of 29 conditions for screening of citrate synthase (continued).

No.	Compound	Storage plate concentration (mM)	Final assay concentration (μM)
9	AMP	4	100
10	GMP	4	100
11	L-Ascorbic acid	0.8	20
12	Biotin	0.8	20
13	ADP	4	100
14	GDP	4	100
15	Thiamine monophosphate	0.8	20
16	Pyridoxalphosphate	0.8	20
17	GSSG, glutathione oxidized	0.8	20
18	TDP	4	100
19	FAD	0.8	20
20	Folic acid	0.8	20
21	Tetrahydrofolic acid	0.8	20
22	L-Glutamic acid	4	100
23	Polyethylene glycol 300	4	100
24	SAM (*S*-(5′adenosyl)-L-methionine)	0.8	20
25	UDP	4	100
26	IMP	4	100
27	UMP	4	100
28	Oxaloacetate	4	100
29	Coenzyme A	0.8	20

encountered for such compounds). Under three different buffer conditions (numbers 4, 8 and 13 in **Table 1**; pH 6.0, 7.5 and 9.0, respectively), 10 μM citrate synthase was screened against this set of compounds in a 'one plate third/three conditions' scenario as described (see Step 15B).

Among the tested compounds, only oxaloacetate induced a stabilization >2 K (at pH 7.5 and 9.0). The titration of the oxaloacetate concentration confirmed the effect and showed that citrate synthase would be maximally stabilized by approximately 11 K, and that for saturation of this effect at least 2 mM oxaloacetate is needed (**Fig. 5**). **Figure 6** shows a visualization of the screen results for citrate synthase against physiologically relevant compounds.

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