

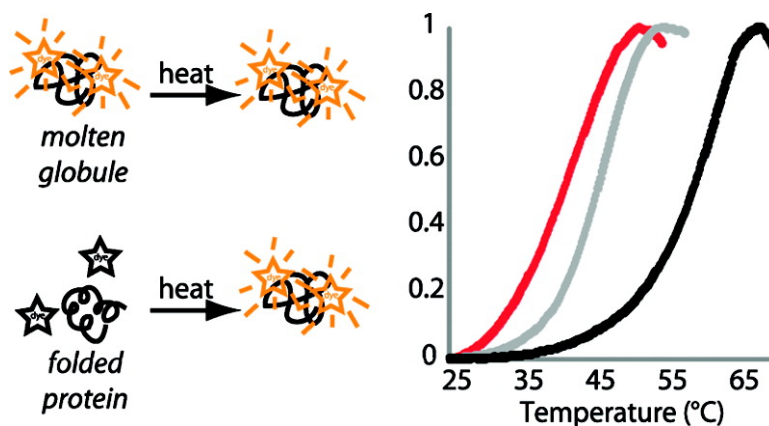
Communication

## High-Throughput Thermal Scanning: A General, Rapid Dye-Binding Thermal Shift Screen for Protein Engineering

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# High-Throughput Thermal Scanning: A General, Rapid Dye-Binding Thermal Shift Screen for Protein Engineering

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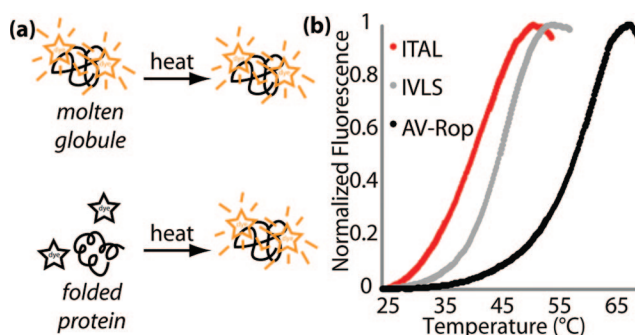
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Natural proteins are often too unstable for therapeutic or industrial applications or even for crystallography or directed-evolution experiments.<sup>1</sup> There is still no reliable way to predict stabilizing mutations, and biophysical characterization of proteins is generally large-scale and low-throughput.<sup>2</sup> Except for enzymes, where enzymatic activity can be screened at elevated temperatures, high-throughput methods of screening for stability are lacking. Notably, the dominant classes of protein drugs (hormones, antibodies, cytokines, etc.) are binding proteins or ligands, not enzymes. Here we demonstrate that a dye-binding thermal shift screen, an extension of the ThermoFluor method of screening for protein–ligand interactions,<sup>3</sup> reports the relative thermal stabilities of libraries of protein variants. We call the method high-throughput thermal scanning (HTTS).

In ThermoFluor, samples of a receptor protein are mixed with an analyte ligand and a fluorescent hydrophobic dye akin to 1-anilinonaphthalene-8-sulfonic acid (ANS). Folded proteins exclude these types of dyes, but molten globules and thermal denaturation intermediates bind them, resulting in a sharp increase in fluorescence. Binding of a ligand to the folded state of the receptor shifts the apparent melting temperature to a higher value, which can be observed by heating the sample in a fluorimeter. In addition to its use in drug discovery, this method has been applied to optimization of ligand and buffer conditions for crystallography.<sup>4</sup>

We wished to invert the format of the screen, instead using a library of protein variants under the same conditions of dye and buffer, to probe the approximate relative thermal stabilities of the mutants. Since dye binding is so physically different from circular dichroism (CD) spectroscopy or intrinsic fluorescence, and because many interesting mutants may have very different amino acid composition, it was not clear at the outset how the associated melting temperatures would correspond to standard large-scale approaches. Moreover, since a different protein is required in each well of the 96-well plate, compatible inexpensive, small-scale growth and purification had to be achieved.

We first validated the method using a small set of variants of the simple, well-studied four-helix bundle protein Rop.<sup>5</sup> The Rop variants are from two combinatorial libraries in which the two central layers (residues 15, 19, 41, and 45) of the hydrophobic core were repacked. We named the library variants by the amino acids at the four varied positions; hence, wild-type Rop is ITLA. AV-Rop is an engineered cysteine-free variant (C38A C52V) with wild-type-like thermal stability as measured by CD spectroscopy.<sup>13</sup> When AV-Rop is mixed with dye, there is little fluorescence at room temperature but a sharp increase in fluorescence between 52 and 63 °C (Figure 1b). As expected from the use of an ANS-like hydrophobic dye, HTTS is readily able to distinguish molten globular proteins from native-like proteins. When a molten globular variant of Rop, LMLL, is mixed with dye, there is a large



**Figure 1.** (a) Schematic of HTTS. Molten globular variants bind hydrophobic dyes such as ANS at room temperature. Native variants exclude the dye at low temperature but bind it strongly as the protein thermally denatures, resulting in a sharp increase in the fluorescence signal. (b) HTTS of three native-like variants of Rop whose thermal stabilities vary substantially.

fluorescence signal at room temperature and no increase in fluorescence upon heating (Figure 3a).

A real-time PCR machine was used for these experiments because it is compatible with very small volumes and because most multiwell plate readers cannot heat samples to 95 °C. Because the excitation and emission maxima of ANS are not accessible by commercial filter sets for real-time PCR machines, we used SYPRO Orange, which has been used successfully for ThermoFluor.<sup>6</sup> By application of a variation on the Clarke–Fersht equation<sup>7</sup> using data from 25 °C to the fluorescence maximum, we calculated an apparent  $T_M$  value.

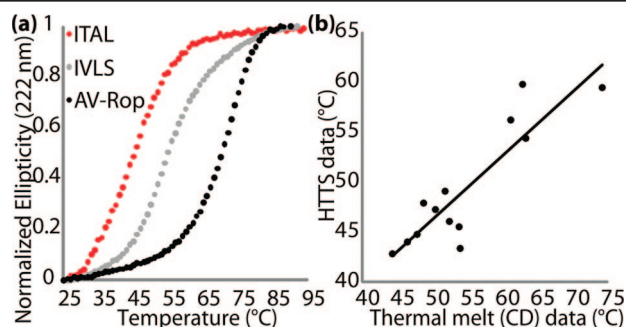
In general, the amount of protein required to obtain good signal-to-noise was  $\sim 100 \mu\text{M}$  with  $5\times$  dye (the absolute concentration of SYPRO Orange is not disclosed by the manufacturer). For Rop and other proteins tested in the laboratory, sufficient protein could be obtained from 2 mL of culture grown in deep 96-well plates with T7 overexpression. Saturated overnight cultures were diluted and induced with IPTG, and cells were lysed with glass beads or detergent. Hexahistidine-tagged protein was captured on magnetic NiNTA beads, washed, and released by proteolysis using TEV protease. Curiously, the His<sub>6</sub>-TEV site tag had to be removed from the sample because it resulted in a dye-mediated fluorescence signal at  $\sim 45$  °C. The NiNTA magnetic beads, the most expensive component of the method, could be regenerated for use many times.

To assess the validity of HTTS for measuring the approximate stabilities of a series of protein variants, 13 Rop variants were also analyzed using CD spectroscopy (Figure 2). The HTTS apparent  $T_M$  values are roughly related linearly to the CD  $T_M$  values, where the average offset between them depends upon buffer and dye conditions. Five variants bind large amounts of hydrophobic dye at room temperature, consistent with their noncooperative CD thermal melts. CD urea denaturation data differ more substantially from either thermal method.

We also subjected seven variants of yeast triosephosphate isomerase (TIM) to both CD thermal melts and HTTS, with

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**Figure 2.** (a) Thermal denaturation of the same three variants as in Figure 1b, monitored by CD at 222 nm. (b) Plot of CD  $T_M$  vs HTTS apparent  $T_M$  for 13 native-like Rop variants.

excellent correspondence (see the Supporting Information). TIM is a 240 amino acid ( $\beta/\alpha$ )<sub>8</sub> barrel protein that is radically different in size and overall fold, supporting the generality of this method.

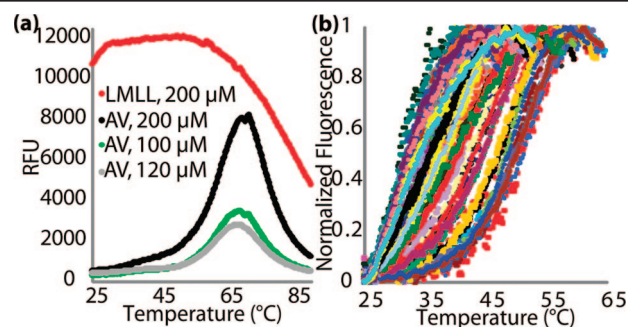
Randomly selected active variants from an AV-Rop library were purified and analyzed in 96-well format. This library was constructed so that residues 15, 19, 41, and 45 were subrandomized to the hydrophobic residues (AVILMF) and alcohols (ST). The variants in this library ranged from slightly stabilized to molten globular, as might be expected from the radical repacking of the core of a small protein. Native-like variants (Figure 3b) were separated from molten globules and variants that failed to express using empirical analysis of the fluorescence signals (i.e., identifying those with very high or very low fluorescence signals throughout the melt). A full sequence–stability analysis of this library will be presented separately.

Several of the variants from this library were identical in amino acid sequence. These repeats differ only in the amount of signal and not in the midpoint of denaturation or overall shape of the curve. The differences in signal can be attributed to differences in protein expression and purification and random differences from the normalization procedure employed by the real-time PCR software used here. One normally expects a concentration-dependent variation in  $T_M$  values for multimeric proteins. However, the Rop concentrations are quite uniform here, and Rop thermal stability is remarkably insensitive to concentration above 1  $\mu$ M.

The CD thermal melts of all Cys-free Rop variants tested are reversible. In HTTS, after the fluorescence passes through its maximum, decreasing fluorescence is always observed upon continued heating. This may be caused by aggregation or temperature-dependent dye fluorescence or binding (Figure 3a). Although we have called the midpoint values for the melting curves apparent  $T_M$ 's, the conditions are not fully reversible, and the system may not be at equilibrium, particularly after passing through the apparent melting points. Consequently, variants with very different unfolding rates may behave anomalously in HTTS.

Protein stability is typically characterized by calorimetry or by thermal or chemical denaturation observed using intrinsic fluorescence or CD spectroscopy. Automation increases the throughput; however, not all proteins have an intrinsic fluorophore, and specialized equipment is required.<sup>8</sup> Indirect screens (binding or proteolysis resistance of phage-displayed variants or protein solubility and expression) have also been applied.<sup>9</sup> In contrast, we present here one of the first high-throughput collections of thermodynamic data on a single protein scaffold, using a method that is simple and inexpensive enough to be carried out in any laboratory with access to a real-time PCR machine.

Thermal shift assays have been used to optimize the thermal stability of a single protein under different conditions, with either a battery of ligands or a battery of buffer conditions.<sup>3,4,6</sup> Inverting



**Figure 3.** (a) Full, non-normalized HTTS traces for a molten globular variant (LMLL) and AV-Rop at three concentrations in three independent experiments. (b) HTTS traces of native-like variants from a random selection of 96 core-repacked mutants of AV-Rop.

the format of the screen to examine a library of protein variants, as we demonstrate here, opens the door to new kinds of discovery. Protein-based therapeutics can have remarkable specificity, but the marginal stability of natural proteins is problematic for storage and pharmacological properties.<sup>10</sup> Protein engineering to increase solubility and expression,<sup>9</sup> remove or introduce surface interactions,<sup>11</sup> or generate stable subfragments<sup>12</sup> has become a critical tool in structural biology in recent years. The HTTS method goes beyond these, enabling a search for conservative mutants of a protein with enhanced stability.

HTTS is a simple, low-cost method that uses commonly available instrumentation. It requires only microgram quantities of protein that can be purified using commercially available reagents designed for high-throughput analysis. HTTS is able to provide an abundance of thermodynamic data on a large library of protein variants in a single hourlong experiment. The ability to acquire large sample sizes of thermodynamic and sequence data presents an obvious avenue for analyzing their relationship in a statistical manner.

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**Supporting Information Available:** Detailed procedures, additional results, and complete refs 1, 3–6, 8, and 11. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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