Supporting Information

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SUPPLEMENTAL RESULTS

Protein Purification

We expressed variants from a T7 promoter with an N-terminal hexahistidine tag and an intervening TEV protease recognition sequence (MAHHHHHGGGENLYFQ). Sufficient protein is obtained by growth in 2 mL of media, using a combination of detergents and shaking with glass beads to lyse the cells. IMAC purification is accomplished with NiNTA magnetic beads in 96-well plates. For all variants tested, the fusion with the His\textsubscript{6} tag resulted in apparent dramatic destabilization compared to tagless counterparts, and the same apparent $T_M$s are observed if tag is cleaved from the protein but still in solution. This necessitates that the tag be purified away from the protein sample. To do so, we used an on-bead TEV cleavage method demonstrated to achieve near-quantitative cleavage from NiNTA magnetic beads used for HT purification.

Supplemental Figure 1. Effect of His\textsubscript{6}-TEV site tag on HTTS. Note the difference for the AV-Rop with the His\textsubscript{6} tag fused or cleaved and in solution, versus removed from the protein solution.
Comparison of HTTS with CD thermal and chemical denaturation

HTTS reports the relative stabilities of Rop variants as compared to CD thermal denaturation. The CD urea denaturation data differ more substantially from either thermal method. For example, the LMLL variant denatures cooperatively in increasing urea.

Supplemental Figure 2. Comparison of CD thermal melts (left) and HTTS (right) for 13 native-like Rop variants. The variants (Supplemental Table 1) are the same color in each plot.

Supplemental Figure 3. Comparison of stability measurements for molten globular Rop variants. In all cases examined so far, large initial fluorescence with small or no increases upon heating in HTTS (right) has corresponded to molten globules as characterized by CD thermal melt (left).
**Supplemental Figure 4.** Urea denaturation of six Rop variants. Due to lack of a pre-transition baseline, only two of the data sets could be fit to the model (the Clarke & Fersht equation 11).
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<th>Rop variant</th>
<th>HTTS $T_M$ (°C)</th>
<th>CD $T_M$ (°C)</th>
<th>$\Delta G_{D-N}$ kcal mol$^{-1}$</th>
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*These are variants of the Cys-free AV-Rop; --, not able to be fit; n.d., not determined

**Supplemental Table 1. Comparison of stability measurements for Rop variants.** Values are from least-squares fits to the data (see Methods). The melting temperatures for LMLL, LMLI, LIVL, MIVL and ITIL could not be determined from fits because they do not undergo a single, cooperative unfolding transition. The urea denaturation values for several variants could not be fit because there was no folded baseline (i.e., the proteins were already in the unfolding transition in 0 M urea). CD and HTTS measurements were carried out in 50 mM sodium phosphate (pH 6.3), 300 mM NaCl.
**Reversibility of Rop thermal denaturation**

The thermal unfolding of the native-like Rop variants above is fully reversible under standard CD conditions. The melting and re-annealing curves monitored by CD at 222 nm for AV-Rop is shown below.

*Supplemental Figure 5.* Full CD trace of AV-Rop at 222 nm heating to 90 °C and cooling back to room temperature (left) with wavelength scans before and after the melt (right). The data at the left are normalized to the range of the melting curve.
**Generality: Variants of a TIM Barrel Protein**

We subjected six variants of yeast triosephosphate isomerase to HTTS. The TIM variants examined here (with CD $T_M$) are: multi (L13M, K134R, Q82M, W90Y, F11W, A211V, 55.1°C); F11W (56.8 °C); W90Y (56.8 °C); Q82M (59.2 °C); wild-type (59.3°C); K134R (60.2 °C); L13M (60.4 °C).

![Graph](attachment:image.png)

**Supplemental Figure 6. Comparison of thermal stability of seven TIM variants.** Seven variants of *S. cerevisiae* triosephosphate isomerase (TIM) were subjected to both CD thermal denaturation and HTTS. The variants were expressed in *E. coli*, purified by NiNTA affinity, and cleaved from the His$_6$ tag by TEV protease. CD measurements at 222 nm were at 14 μM, and HTTS measurements were at 25 μM. The buffer in both cases was 50 mM potassium phosphate (pH 8), 300 mM NaCl.
Reproducibility

Several variants in our libraries appeared more than once (usually with a different DNA sequence but the same protein sequence). Despite slight changes in the fluorescence signal from sample to sample, it is evident that the point of inflection in the melting curve is very consistent.

Supplemental Figure 7. Reproducibility of HTTS. Each of the three panels are repeat protein sequences (noted in the top right of each panel) from the library that represent individual wells from the 96-well plate that differ in the amount of signal but exhibit a reproducible $T_M$. One of the IVLS repeats appears to be slightly different in $T_M$, which may be due to the very low signal (expression) or contamination of that particular well.
MATERIALS & METHODS

Rop Libraries

Two distinct libraries of Rop variants were used for this study. The NNK$_4$-2 library consists of variants in which residues 15, 19, 41, and 45 were randomized to all 20 amino acids. This library was synthesized using the wild-type Rop sequence and has been thoroughly characterized via standard biophysical methods (T.J.M. & Lynne Regan, manuscript in preparation). The DYV$_4$-2 library focuses on the same residues, but randomizes the positions to the hydrophobic and alcohol amino acids only using the DYV codon. This library was synthesized using an engineered cysteine-free Rop sequence, AV-Rop (S.B.H., Chang Byeon, J.J.L. & T.J.M., manuscript in preparation). Briefly, the library was created by PCR reassembly of synthetic oligonucleotides and cloned into the pACT7lac$^2$ screening vector, a low-copy plasmid that expresses Rop from a synthetic lac promoter. Screening for in vivo activity is accomplished in DH10B(pUCBADGFpuv). Active variants were amplified with PCR and subcloned into a variant of the pMR101$^3$ vector, fusing an N-terminal hexahistidine tag and TEV cleavage site, for T7 overexpression in BL21(DE3). Sequencing of clones directly from colonies was carried out by Genewiz (South Plainfield, NJ).

Protein Purification (large-scale)

Rop variants were overexpressed in BL21(DE3) in 500 mL 2YT media grown to an OD$_{600}$ = 0.7-0.9, induced with 0.1 mM IPTG, and incubated at 30 °C for 18 h. Cells were harvested by centrifugation and the cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, 2 mM βME [for Cys-containing variants], pH 8). Cells were lysed by adding 0.25 mg mL$^{-1}$ lysozyme, 2 μg mL$^{-1}$ DNase I, 200 ng mL$^{-1}$ RNase A, 5 mM MgCl$_2$, 0.5 mM CaCl$_2$, and 0.1% Triton X-100. The cell suspension was sonicated and centrifuged at 30,000 g. The soluble fraction was mixed with 750 μL NiNTA agarose slurry (Qiagen) and incubated for 1 h at 4 °C. The bound resin was washed (lysis buffer with 20 mM imidazole), and the protein was eluted with 1.5 mL elution buffer (lysis buffer with 250 mM imidazole). The His$_6$ fusion tag was cleaved twice with 0.5 mg rTEV protease (in 25 μL) with addition of 5 mM DTT, followed by overnight incubation at room temperature or 30 °C for 3 h. After diluting to 2.5 mL, the protein was exchanged into lysis buffer using one PD10 column (GE Healthcare) and mixed with 750 μL NiNTA agarose slurry at 4 °C. After incubation for 1 h at 4°C, the filtrate containing the free protein was brought to 3 mM TCEP and 1 mM DTT for Cys-containing variants. The protein was concentrated by centrifugation through a YM3 filter (Millipore) and exchanged into the appropriate buffer using a PD10 column for subsequent characterization by CD spectroscopy or HTTS.

High-throughput protein purification

The DH10B strain of E. coli was lysogenized with the DE3 lamboid phage using a kit from Novagen. Individual Rop variant seeds in DH10B(DE3) were grown in 2 mL 2YT media in each well of a 2 mL, 96 square deep-well TiterBlock plate (USA Scientific) covered with a porous membrane at 37 °C for 18 h. The seeds were diluted to 2 mL 2YT and OD$_{600}$ = 0.75-1.0, induced with 10 μM IPTG, and overexpressed at 30 °C for 18 h. Cell pellets were resuspended in 200 μL lysis buffer and lysed by adding 100 μg mL$^{-1}$ lysozyme, 0.5 μg DNase I, 40 ng RNase A, 5 mM MgCl$_2$, 0.5 mM CaCl$_2$, and 20 μL PopCulture reagent (Novagen), followed by
incubation at room temperature for 30 min. Glass beads (50 mg, Biospec) were added to each well, and the plate, covered with an Axymat (Oxygen), was processed with a Mini BeadBeater-96 (Biospec). The glass bead step is unnecessary if longer incubation with PopCulture (>1 h) is used instead. Soluble fractions were mixed with 50 μL NiNTA magnetic beads (Qiagen) and incubated at room temperature for 1 h. The bound resin was washed (lysis buffer with 20 mM imidazole) and resuspended in 25 μL lysis buffer. The proteins were cleaved off the resin by 10 μg rTEV protease (in 0.5 μL) and 11 mM βME with incubation at 30 °C for 3 h.

**CD Spectroscopy**

Spectra were obtained on Aviv 202 (Ohio State University Department of Chemistry) Circular Dichroism Spectrometer. Experiments were conducted at 50 μM protein monomer, determined by UV absorption (1,490 M⁻¹ cm⁻¹) at 280 nm, in CD buffer (50 mM sodium phosphate, 300 mM NaCl, 5 mM DTT [for Cys-containing variants], pH 6.3). Thermal denaturations were acquired at 1 °C min⁻¹, 25 to 90 °C, at 222 nm. Urea denaturations were performed in the same conditions but with 0, 1, 2, 3, 4, 5, 6, or 7 M urea, with spectra acquired after equilibrating 24-48 h at RT, at 222 nm. Denaturation profiles were fit to the model of Clarke & Fersht¹ (equation 11) to determine the $T_M$ or $D_{50}$ and $m$-value. $\Delta GD_N$ values are the product of $D_{50}$ and $m$ at the defined standard state of 50 μM protein monomer.

**High-Throughput Thermal Scanning (HTTS)**

SYPRO® Orange is provided at 5000× of the concentration needed for PAGE staining, but the absolute concentration is not disclosed. We give our concentrations relative to this same standard.

Spectra were obtained on a Bio-Rad iCycler iQ Real-Time Detection System (Ohio State University Plant-Microbe Genomics Facility). Samples of 20 μL per well were prepared by mixing 1 μL of 300× SYPRO® Orange dye (Invitrogen, final concentration 15×) with protein (100-200 μM), typically in lysis buffer, and loaded into iCycler 96-well 0.2 mL thin-wall PCR plates, sealed with iCycler optical quality sealing tape (BioRad). Thermal denaturations (0.2 °C per 12 s using the iCycler Melt Curve script) were acquired by measuring fluorescence intensities using a 490 ± 10 nm excitation filter (from the SYBR Green set) and a 575 ± 10 nm emission filter (from the HEX filter set). Background correction was provided by an external well-factor plate containing 15× dye in appropriate buffer with no protein, dwelling at 25 °C.

For HT data processing, we eliminated profiles from molten globules and variants that did not express or did not bind dye strongly at any temperature. To eliminate very low signals throughout the melt and very high signals at room temperature, we rejected profiles that did not increase at least 25% from room temperature to the fluorescence maximum and decrease at least two-fold from the maximum fluorescence to the final temperature. We also rejected profiles where the maximum raw fluorescence signals were less than 400. Although the correspondence between molten globular variants and those rejected by the test for increasing fluorescence has been 1:1 so far, we should caution that this is empirical and may result in the misassignment of some variants that bind dye tightly as a folded protein.

We then fit a variation on equation 11 of Clarke & Fersht,¹ which accounts for non-flat pre-transition baselines, to the data from room temperature to the temperature at the fluorescence maximum. Here, $\alpha_F$ and $\beta_F$ are the intercept and slope of baseline for the folded state, and $m$ is
an exponential factor related to the slope of the transition at the apparent melting temperature, $T_M$.

$$Signal = \frac{(\alpha_F + \beta_T T) + e^{m(T - T_M)}}{1 + e^{m(T - T_M)}}$$

The values of $\alpha_F$, $\beta_F$, $m$ and $T_M$ were fit from the normalized fluorescence signals at temperatures $T$ by least-squares using the Solver plug-in of Microsoft Excel. Note that the $T_M$ values for melting profiles that do not have an initial folded baseline, which are presumably partially unfolded at room temperature, cannot be estimated accurately.
REFERENCES (SUPPORTING INFORMATION)


FULL-AUTHOR REFERENCES (MANUSCRIPT)