

# Supporting Information

## Temperature-Jump Fluorescence Provides Evidence for Fully Reversible Microsecond Dynamics in a Thermophilic Alcohol Dehydrogenase

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### Methods

#### Site-Directed Mutagenesis and Protein Purification

The W87F mutant was generated from the wild-type *B. stearrowophilus* ht-ADH gene previously cloned into the *E. coli* pET-24b(+) expression vector<sup>1</sup>. The W87F:H43A mutant was subsequently made from the W87F plasmid. The following primers and their reverse complements (not shown) were used to make changes to the ht-ADH gene (underlined) using the Quikchange II site-directed mutagenesis kit (Stratagene):

W87F-5'-CCGCGTTGGAATTCCTTTTCTTTATATTCTGCATGC-3';

H43A-5'-GTTTGTCTACTGACTTGGCGGCCGCTCACGGCGATTGG-3'. DNA sequencing was verified by the UC Berkeley DNA sequencing facility. Cell harvest, lysis, and subsequent purification protocols were executed in a manner described previously<sup>1</sup>.

#### Enzyme Kinetics

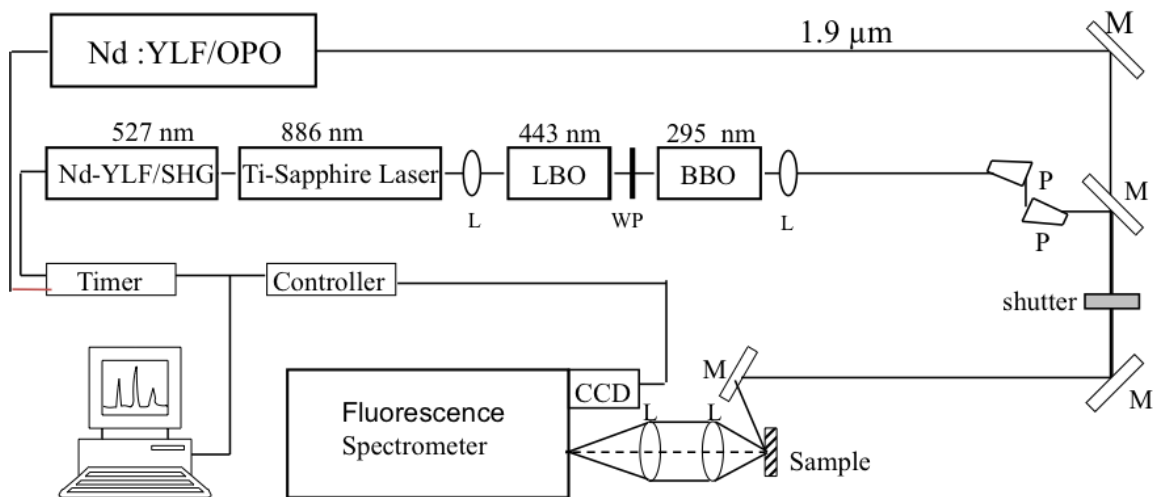
Kinetics for W87F and W87F:H43A were assessed and subsequently analyzed in a manner similar to that published elsewhere<sup>2</sup> with some modification. All assays were dissolved in 50 mM potassium phosphate buffer to a final volume of 495  $\mu$ L; reactions were then initiated using 5  $\mu$ L of appropriately diluted enzyme. Kinetic isotope effects at 30°C were still determined by comparing rates of protio-benzyl alcohol (h-BnOH) to that observed with d-BnOH (CDN Isotopes). The concentrations were simultaneously varied from 0.5–18 mM for NAD<sup>+</sup> and from 1–25 mM for substrate. Initial rates were determined by measuring the slopes of the linear traces acquired for at least 3 min. Such conditions for turnover were assayed over temperatures ranging from 10°C–45°C at 5°C intervals.

## T-jump/Fluorescence

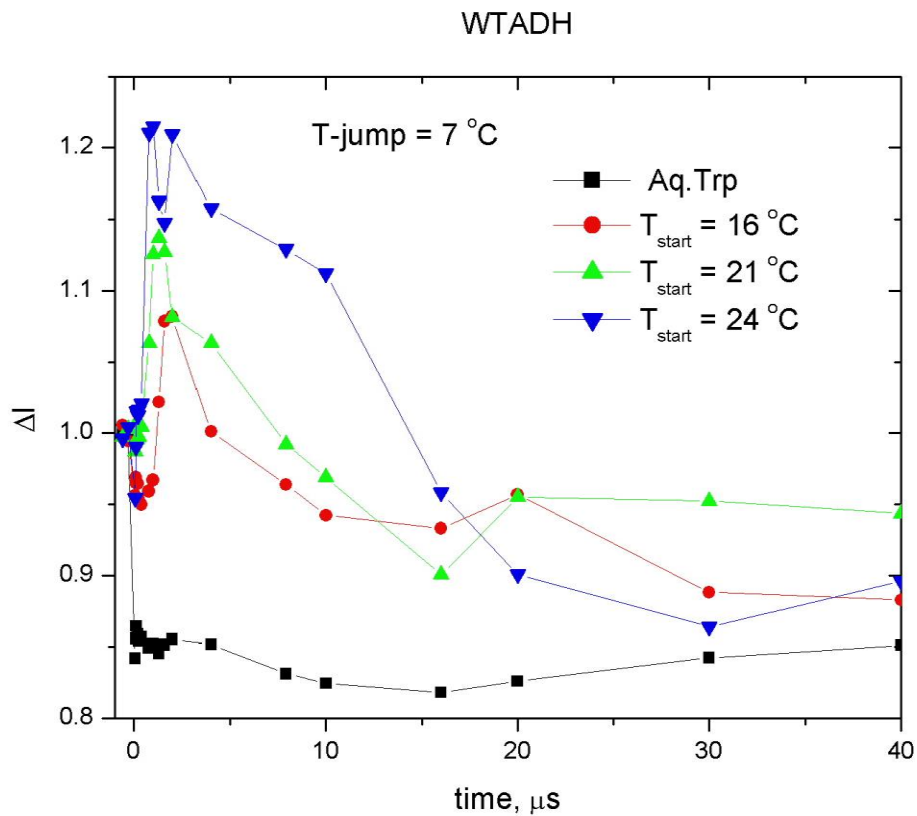
UV probe pulses for the fluorescence excitation at 295 nm ( $\sim 1 \mu\text{J}/\text{pulse}$ , 20 ns, 1 kHz) were obtained by frequency tripling the output of a Ti:sapphire laser, which was pumped (527 nm,  $\sim 10 \text{ mJ}/\text{pulse}$ , 70 ns, 1 kHz) by an intracavity frequency-doubled Nd:YLF laser (GM30, Photonics International, Inc.). T-jump pulses at 1.9  $\mu\text{m}$  ( $\sim 0.5 \text{ mJ}/\text{pulse}$ , 20 ns) were obtained from an intracavity diode pumped Nd:YLF laser optical parametric oscillator (OPO) operating at 1 kHz (Photonics International, Inc.). The timing between T-jump and probe pulses was adjusted with a computer-controlled pulse generator (DG 535, Stanford Research Systems). The T-jump amplitude,  $7 \pm 1 \text{ }^\circ\text{C}$ , was measured using a calibration curve generated from the static temperature dependent fluorescence intensity of the aqueous tryptophan solution. The final temperature of the sample within the laser interaction volume is achieved instantaneously after a T-jump pulse (complete thermalization is reached within 10-20 ps).

Tryptophan fluorescence was collected at  $135^\circ$  geometry and focused into a 0.25 m spectrograph (Acton SP2156), which was equipped with a holographic grating (300 groove/mm) and an liquid nitrogen cooled CCD detector (Spec-10, Roper Scientific). The sample solution was pumped through a wire-guided free-flowing cell to minimize the background. Temperature control of the sample was achieved with a water bath (RTE-100, Neslab), which regulates the temperature of the sample cell and reservoir. The final pump + probe (with T-jump) and probe-only (without T-jump) fluorescence spectra were averages of 1-2 minutes obtained through 30 cycles of 2-4 sec/point exposure, to minimize artifacts from spectrometer drift and photoproduct accumulation, which is critical for this study, since no internal standard was added. Wavelength axis of the spectra was calibrated using the Ne-Ar emission lines. Time dependent intensity at the tryptophan emission maximum near 355 nm was modeled with a biexponential function to obtain T-jump relaxation time constants.

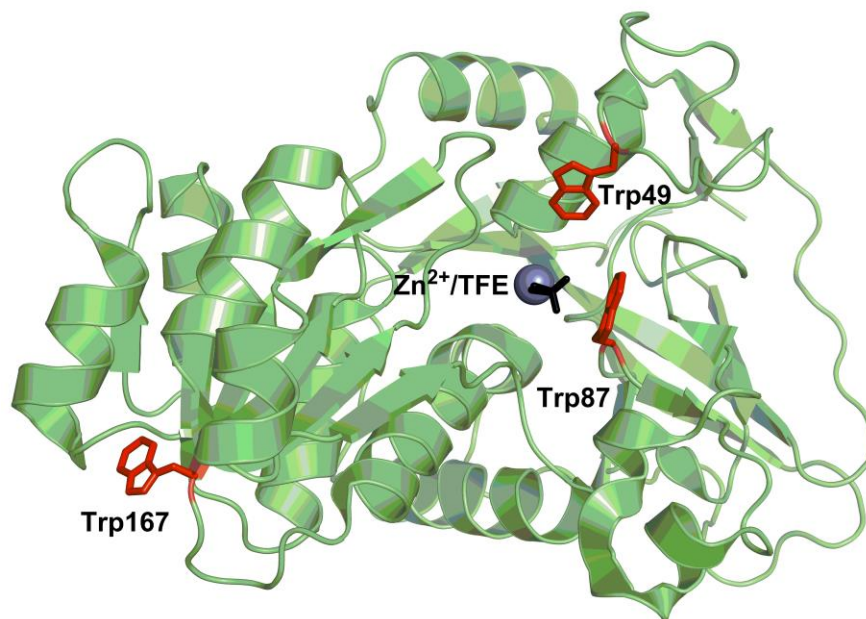
**Figure S1.** Setup of the temperature-jump fluorescence apparatus described in Methods. Abbreviations associated with the block diagram: LBO – lithium triborate crystal; BBO – BaB<sub>2</sub>O<sub>4</sub> crystal; M – UV reflective mirrors; P – Pellin-Broca prism; L – silica lenses; WP – waveplate; CCD – charged coupled device.



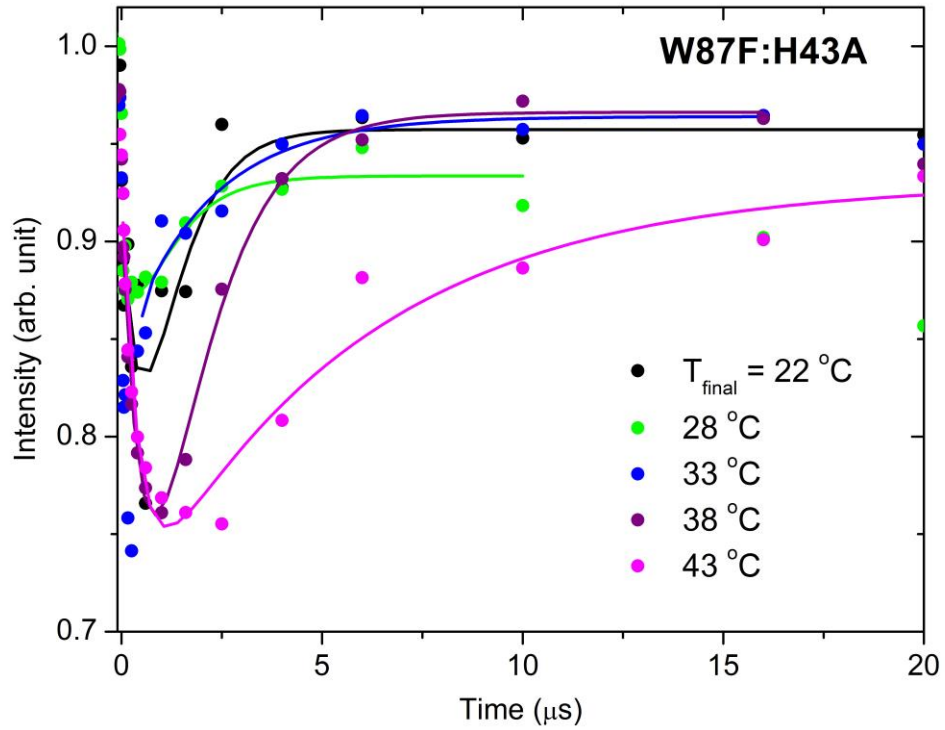
**Figure S2.** T-Jump fluorescence kinetic traces for wild type ht-ADH with initial temperatures at 16, 21, and 24°C. The final increase in temperature for each trace is 7°C, yielding final temperatures of 23, 28, and 31°C, respectively. Aqueous tryptophan's kinetic trace was collected at 16°C and is shown with black squares. While free tryptophan has a well-defined trace that decreases in intensity on the sub- $\mu$ s timescale, the wild-type traces fluctuate wildly with no consistent change in behavior at any temperature.



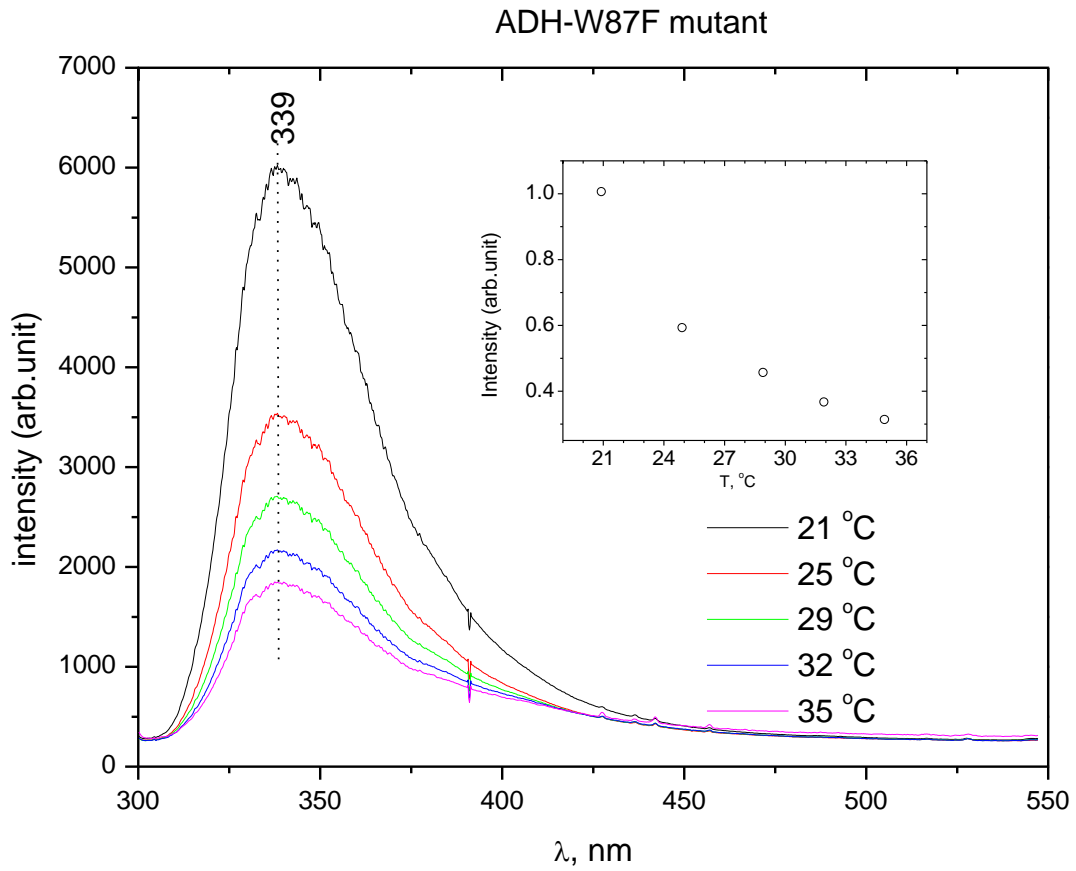
**Figure S3.** Monomeric view of ht-ADH(PDB ID: 1RJW) highlighting all three of its tryptophans (red). Also included is the catalytic  $Zn^{2+}$  cofactor (grey) and the trifluoroethanol (TFE) inhibitor (black). Images generated using PyMOL<sup>3</sup>.



**Figure S4.** T-jump ( $^{\circ}\text{C}$ ) induced fluorescence ( $\lambda_{\text{ex}} = 295 \text{ nm}$  and  $\lambda_{\text{em}} = 355 \text{ nm}$ ) time courses as a function of final temperature for the W87F:H43A form of ht-ADH. The plotted intensities were normalized to ‘probe-only’ emission spectra obtained at  $-0.6 \mu\text{s}$ , and the experimental data were fit to a double exponential with the exception of  $33^{\circ}\text{C}$  (Table 1).



**Figure S5.** Static fluorescence emission spectra of W87F shown as a function of temperature. The relative intensities in the inset are normalized to the 21°C intensity.



**Table S1.** Kinetic parameters of W87F and W87F:H43A.

	<b>W87F</b>	<b>W87F:H43A</b>
30°C $k_{\text{cat}}$ , s <sup>-1a</sup>	29 ± 2	1.2 ± 0.1
$K_{\text{M}}(\text{NAD}^+)$ , mM	2.5 ± 0.3	3.0 ± 0.2
$K_{\text{M}}(\text{H-BnOH})$ , mM	18.7 ± 0.9	20 ± 3
$\Delta H^\ddagger(\text{lo})$ , kcal/mol	17.0 ± 0.4	17.6 ± 1.1
$\Delta H^\ddagger(\text{hi})$ , kcal/mol	11.0 ± 0.6	12.0 ± 1.1
$\Delta\Delta H^\ddagger$ , kcal/mol <sup>b</sup>	6.0 ± 0.7	5.6 ± 1.6
KIE <sup>c</sup>	2.7 ± 0.2	3.2 ± 0.5

<sup>a</sup>Reported for H-BnOH; <sup>b</sup>Computed as  $\Delta H^\ddagger(\text{lo}) - \Delta H^\ddagger(\text{hi})$ ; <sup>c</sup>Computed as the ratio of  $k_{\text{cat}}(\text{H-BnOH})/k_{\text{cat}}(\text{D-BnOH})$ .



## References

- (1) Liang, Z.-X.; Lee, T.; Resing, K. A.; Ahn, N. G.; Klinman, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9556.
- (2) Meadows, C. W.; Ou, R.; Klinman, J. P. *Journal of Physical Chemistry B* **2014**, *118*, 6049.
- (3) Schrodinger, LLC 2010.