Identification of a Long-Range Protein Network that Modulates Active Site Dynamics in Extremophilic Alcohol Dehydrogenases

Zachary D. Nagel‡,1, Shujian Cun‡,¶, and Judith P. Klinman‡,§,¶,2

From the ‡Department of Chemistry, §Department of Molecular and Cell Biology, and the ¶California Institute for Quantitative Biosciences (QB3), University of California, Berkeley, CA 94720

*Running title: Long-Range Protein Dynamics and Catalysis in Alcohol Dehydrogenases

†This work was supported by grants from the National Institutes of Health (GM025765 to J.P.K. and GM008295 to Z.D.N.) and the National Science Foundation (MCB0446395 to J.P.K.).

1Current address: The Samson Lab, Building 56-230, Massachusetts Institute of Technology, Cambridge, MA 02139.

2To whom correspondence should be addressed: Judith P. Klinman, Department of Chemistry, Department of Molecular and Cell Biology, and the California Institute for Quantitative Biosciences (QB3), University of California, Berkeley, CA 94720. Tel: 510-642-2668, Fax: 510-642-8369, Email: klinman@berkeley.edu

The abbreviations used are: ht, high-temperature; ADH, alcohol dehydrogenase; BnOH, benzyl alcohol; FPLC, fast protein liquid chromatography; FRET, fluorescence energy transfer; ps, psychrophilic; KIEs, kinetic isotope effects.

Keywords: extremophiles, convex Arrhenius curves, corresponding state hypothesis, kinetic isotope effects, hydrogen tunneling, conformational sampling, long-range effects, thermo-adaptation, temperature-dependent regulation.

Background: The role of protein flexibility in the C–H activation step, catalyzed by homologous thermophilic and psychophilic alcohol dehydrogenases, is addressed.

Results: Mutation at the substrate-binding site, or at a dimer interface, alters kinetic properties and protein oligomeric structure.

Conclusion: Active site flexibility is correlated with subunit interactions 20 Å away.

Significance: A long-range network of catalytically relevant, dynamical communication is identified.

SUMMARY

A tetrameric thermophilic alcohol dehydrogenase from B. steaothermophilus (ht-ADH) has been mutated at an aromatic side chain in the active site (W87). The ht-W87A results in a loss of the Arrhenius break seen at 30°C for the wild-type enzyme and an increase in cold lability that is attributed to destabilization of the active tetrameric form. Kinetic isotope effects (KIEs) are nearly temperature-independent over the experimental temperature range, and similar in magnitude to those measured above 30°C for the wild-type enzyme. This suggests that the rigidification in the wild-type enzyme below 30°C does not occur for ht-W87A. A mutation at the tetramer interface in a thermostable psychrophilic homologue of ht-ADH, ps-A25Y, leads to a more thermostable enzyme and a change in rate-determining step at low temperature. The reciprocal mutation in ht-ADH, ht-Y25A, results in kinetic behavior similar to
that of W87A. Collectively, the results indicate that flexibility at the active site is intimately connected to a subunit interaction 20 Å away. The convex Arrhenius curves previously reported for ht-ADH (Kohen et al. (1999) *Nature* 399, 496-499) are proposed to arise, at least in part, from a change in subunit interactions that rigidifies the substrate-binding domain below 30ºC, and impedes the ability of the enzyme to sample the catalytically relevant conformational landscape. These results implicate an evolutionarily conserved, long-range network of dynamical communication that controls C–H activation in the prokaryotic alcohol dehydrogenases.

According to the corresponding state hypothesis, proteins evolve to strike a balance between stability and conformational flexibility (1). This idea has been used to explain a large body of literature in which it has been documented that proteins are often just stable enough to operate under physiological conditions (2). It has been suggested that proteins do not evolve to optimal stability because of the requirements of the cell to achieve efficient protein synthesis and turnover, and the need for proteins to undergo rapid conformational sampling to execute their biological function (2-4). The role of conformational sampling in protein function has become the focus of intense research in the field of enzymology, as a large body of experimental data continues to illuminate the link of protein motions to bond cleavage events at enzyme active sites (5-16).

A corollary of the corresponding state hypothesis is that the introduction of stability in excess of that required under physiological conditions, due to a change in temperature or mutation, may adversely affect protein function by impairing functional conformational flexibility. Extensive investigations of proteins as a function of temperature support the expectation that proteins become increasingly rigid with decreasing temperature, and that enzymes evolved to operate at high temperatures exhibit sluggish activity relative to their mesophilic and psychrophilic counterparts at reduced temperature (3,17-23). Evidence continues to emerge that enzymes have evolved in such a way as to optimize the influence of protein dynamics on the chemical steps of catalysis [cf. refs. (24,25)].

Most enzyme reactions can be fit to the Arrhenius equation (Eq 1), which frequently yields a straight line when ln(k) is plotted against 1/T. However, for some enzymes, a transition to more sluggish catalysis has been seen to occur abruptly below a threshold temperature in a manner that is distinct from either a change in rate-determining step or a decrease in enzyme stability (5,24,26). All of these enzymes and most other enzymes that have been shown to exhibit Arrhenius breakpoints are multimeric [cf. refs. (27,28) and references therein], raising the possibility of alterations in subunit interactions or protein oligomeric structure as the origin of the observed behavior.

One of the best-characterized examples of such behavior is an alcohol dehydrogenase from *B. stearothermophilus* (ht-ADH). This enzyme shows a break in its Arrhenius plot at 30ºC that is accompanied by a significant increase in the enthalpy of activation and concomitant rapid decrease in rate with temperature. In addition, the temperature dependence of the KIE is seen to undergo a transition from temperature-independent at elevated temperature to temperature-dependent below 30ºC (5). These changes have been attributed to distinct protein conformational substates above and below the break, with experimental support for this idea coming from hydrogen deuterium exchange that demonstrates a local increase in flexibility above 30 degrees within the substrate-binding domain (21). A psychrophilic ortholog from *Moraxella TAE* sp. 123 (ps-ADH) with very high-sequence identity (61%) has been shown, by contrast, to be highly thermostable (29). The ps-ADH does not show an Arrhenius break, and exhibits local flexibility in the substrate-binding domain at 10ºC that is greater than that measured for ht-ADH at the same temperature (30).

It has been shown previously that both the Arrhenius behavior and KIEs for ht-ADH are sensitive to amino acid substitutions for
conserved bulky hydrophobic residues in the cofactor binding pocket (31,32). We have now analyzed a variant at a conserved residue in the substrate-binding pocket of ht-ADH (ht-W87A) that is shown to abrogate the Arrhenius break in the experimental temperature range. The KIE for ht-W87A at all temperatures resembles that of the wild-type enzyme above 30°C, with a rate that is moderately reduced (ca. 5-fold). Due to the absence of a break in its activity, $k_{cat}$ for W87A approaches that of the wild-type enzyme at reduced temperature. However, the apparent enhancement of activity for ht-W87A relative to wild-type at low temperature is accompanied by a loss of stability that results from dissociation of tetramer into inactive dimers and monomers. A reciprocal relationship emerges from a mutant ps-ADH, in which subunit interactions are strengthened. The ps-ADH (ps-A25Y) variant restores an inter-subunit interaction present at the corresponding position in wild-type ht-ADH; we find that this leads to a marked stabilization of the psychrophilic enzyme that is accompanied by the emergence of an Arrhenius breakpoint at ca. 20°C. Because the ht-ADH active site mutant impacts the subunit interface, and the ps-ADH subunit interface mutation has consequences for catalysis at the active site, we further interrogated ht-ADH by mutating its tyrosine at the subunit interface, Y25A, uncovering kinetic behavior very similar to W87A. It is, thus, possible to identify a ca. 20 Å pathway of dynamical communication between the two targeted residues (Y25 and W87), comprised of the very same peptides that were previously demonstrated to rigidify below 30°C in ht-ADH (21). We conclude that inter-subunit contacts in the family of tetrameric prokaryotic alcohol dehydrogenases can alter the protein conformational landscape (31), thereby controlling the active site configurations that are directly linked to Arrhenius behavior and the properties of hydride tunneling between substrate and cofactor.

**Materials and Methods**

**Materials**—Benzyl alcohol was purchased from Fisher Scientific, and purified by vacuum distillation prior to use. The $\alpha,\alpha$-d$_2$-benzyl alcohol (99.5% D) was purchased from CDN isotopes, found to be chemically pure within the detection limits of GC-MS, and thus, used without further purification. NAD$^+$ and NADH were purchased from Sigma and used without further purification.

**Cloning of ps-ADH Gene**—The DNA sequence for alcohol dehydrogenase from *Moraxella* sp. TAE123 (ps-ADH) was obtained from the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The gene was synthesized commercially, received in a pDrive cloning vector, and subsequently subcloned into a pET-24b expression vector. Correct insertion of the gene was confirmed by DNA sequencing.

**Preparation of Mutants**—Site-directed mutagenesis was carried out as previously described (22), using the the Quikchange Stratagene kit for site-directed mutagenesis, with the following primers and their reverse complements:

ht-W87F, 5’-CCGCGTTGGAATTCTTCTTTATATTTCGCA TGGC-3’;
ht-W87L, 5’-CGCGTTGGAATTCTTTGTTATATTTCGCA TGC-3’
ht-W87A, 5’-CCGCGTTGGAATTCTGCGTTATATTTCGCA TGC-3’
ht-W87A, 5’-CCCGGTGGGAATTCTGCAGTTATATTTCGCA TGC-3’
ht-Y25A, 5’-CCCGGTGGGAATTCTGCCAGTTATATTTCGCA TGC-3’
ht-Y25A, 5’-GAAATGAGAAAACCAACCATTTCAAGCCTGGA GAGATATTAGTCCGC-3’
ps-A25Y, 5’-CCCAACGCGAGTTATGGCGAGATAGTGG-3’

The mutated codon is underlined. Primers were designed using the program PrimerX (33) and purchased from Operon. Plasmids were isolated and sequenced to confirm the mutations and the intactness of the remainder of the gene.

**Expression and Purification of ht-ADH**—Purification and expression of ht-ADH was carried out as previously described (21) with some modifications. For ht-W87A, an AMP
sepharose affinity column (Amersham Biosciences) replaced the Cibicron blue column because it had a higher affinity for ht-ADH. The improved resolution of this column renders subsequent additional purification by size exclusion chromatography unnecessary. The heat treatment step (15 minutes at 60°C) was also eliminated for ht-W87A because this mutant was found to lose significant activity under these conditions (see Results and Discussion). A number of minor changes were introduced into the subsequent preparation of ht-Y25A. These included inoculation of the stock into an auto-inducible medium (Magic Medium @ Invitrogen) with 50 µg/mL kanamycin and 0.25 mM ZnSO₄. The cells were aerobically grown for about 16 h at 37°C, and then collected by centrifuge. The cell pellet was lysed by a mixture of gentle non-ionic detergents (Bugbuster®, Novagen), and the cell extract was heated at 60°C for 15 min. Then the insoluble components were separated from the liquid phase by ultracentrifuge at 4°C, and the supernatant was mounted onto a fast-flow DEAE anion exchange column (GE Healthcare). The column was washed by 60 mM NaCl, and the protein was eluted by 200 mM NaCl. After buffer exchange by centrifugal filters (Amicon®, Millipore), the harvested protein was passed through an NAD+-affinity column (Blue Sepharose®, GE Healthcare) and eluted by 5 mM NAD⁺ from the column. The purified sample underwent buffer exchange and concentration by centrifugal filters.

Purification and Expression of ps-ADH—Because ps-ADH inactivates rapidly above 30°C, E. coli were grown to an OD₆₀₀ of ~0.6 at 37°C, and then cooled to 17°C prior to induction with 0.5 mM isopropyl thiogalactoside (IPTG). Expression of ps-ADH was monitored by gel electrophoresis, and found to reach a maximum within 48 h. Purification of ps-ADH was carried out using the protocol established for ht-ADH with the following modifications. 100 mM NaCl was maintained throughout the purification to prevent aggregation, and the 60°C treatment was eliminated. An empty vector expression control yielded negligible endogenous ADH activity, so that the activity detected under conditions used can be assumed to arise solely from ps-ADH.

Kinetic Assays—Kinetic data were collected for the oxidation of benzyl alcohol (or its αα-d₂ isotope) to benzaldehyde with concomitant reduction of NAD⁺ to NADH, as previously described (5). With the exception of ht-W87A, enzyme stocks were maintained at 4°C with no detectable loss of activity over the course of several hours. W87A stock solutions were pre-incubated at 30°C and an enzyme concentration of 100 µM, in the presence of 1 mM NADH, in 50 mM potassium phosphate buffer, pH 7.0 for 30 min prior to use in kinetic assays (See Results and Discussion below regarding temperature-dependent inactivation and reconstitution). These solutions were then further diluted as needed for kinetic assays, and maintained at room temperature in 1 mM NADH to prevent activity loss. The final dilution of enzyme into the activity assays reduced the concentration of NADH to ≤ 10 uM.

Inactivation Kinetics of ht-W87A—The kinetics of activity loss upon incubation of ht-W87A was measured at multiple concentrations of enzyme and in the presence and absence of cofactor. Aliquots were removed as a function of time and their activity determined under standard assay conditions, which were 2.5 mM NAD⁺ and 5 mM benzyl alcohol, 50 mM potassium phosphate buffer, pH 7.0, at 20°C. For incubation at low enzyme concentration and 0°C (Figure 2, panel A, lowest curve), ht-W87A was incubated under the conditions of the kinetic assay. In this case, the reaction was initiated by the addition of cofactor instead of the addition of enzyme, and the activity was measured at 4°C. Conditions were otherwise identical.

Fluorescence Kinetics — Fluorescence spectra were monitored at 10 and 30°C as a function of time following the addition of 10 µM enzyme to a solution containing 100 mM N-butylformamide, and five equivalents of NADH (50 µM). Samples were excited at 295 nm, and both the excitation and detection slits were set to 1 nm. Fluorescence emission spectra were collected at 1 to 2-min intervals, and the intensity recorded at 338 nm, the
maximum for protein fluorescence, and at 410 nm for enzyme-bound NADH, reflecting fluorescence energy transfer (FRET) from tryptophan to cofactor. Little or no fluorescence signal at 410 nm is expected from free NADH in solution for two reasons. The excitation wavelength (295 nm) gives negligible direct excitation of NADH ($\lambda_{\text{max}}(\text{excitation}) = 340$ nm) and resonance energy transfer from protein tryptophan side chains should be very weak for free NADH due to the $1/r^6$ distance dependence of FRET. Additionally, the fluorescence signal from free NADH is approximately 6-fold weaker than that for protein-bound NADH, due to quenching in solution.

**FPLC Size Exclusion Chromatography**—Protein oligomerization was estimated using a Superdex 200 FPLC column, calibrated with commercially available molecular weight standards (from 6,000 to 690,000 Da). The elution profile was monitored by UV absorption at 280 nm and activity assays carried out for individual fractions. Chromatography and fraction collection were conducted at 4°C. These conditions (50 mM phosphate, pH 7, with 150 mM NaCl, and 500 µg protein) differ from previously reported preparative size exclusion chromatography, which was carried out at higher protein, lower salt, and 1 bar pressure. In all instances, activity was detected solely in the fractions containing tetrameric protein.

**Circular Dichroism (CD)**—Spectra were collected using an Aviv 410 spectropolarimeter in a quartz cuvette with a 1 mm path length and a protein concentration of 0.5 mg/mL, in 50 mM phosphate, pH 7.0. The temperature was controlled by a Peltier temperature controller. Data points were taken every 1 nm. The signal is reported in units of mean residue ellipticity.

**Results**

**Kinetic and Structural Properties of W87A**—Steady-state kinetics, carried out at 30°C for variants of ht-ADH with alternate hydrophobic side chains (at position 87), indicate relatively small changes in $k_{\text{cat}}$ (less than ca. 10-fold) with no obvious trends across the series (Table 1). The measured KIEs are all similar to the value obtained for the wild-type enzyme. A detailed analysis of both rates and KIEs as a function of temperature was carried out for ht-W87A because it represented the most drastic structural perturbation in the series, and was the only variant to exhibit cold lability (see below). An Arrhenius plot of $k_{\text{cat}}$ shows a complete loss of the breakpoint observed at 30°C for the wild-type enzyme (Figure 1). The activation parameters for ht-W87A are similar to those measured above 30°C for the wild-type enzyme (Table 2). In addition, the KIE for ht-W87A is seen to be nearly temperature-independent throughout the experimental temperature range, in contrast to the wild-type enzyme which exhibits a temperature-dependent KIE below 30°C (Figure 1, inset). (Full tabulation of the kinetic data for ht-W87A and the other mutants can be found in Table 3).

In the course of purification, ht-W87A was seen to exhibit cold lability that was not observed in any other variants. In order to establish conditions for steady-state kinetics, the temperature and concentration dependence of this phenomenon was investigated, Figure 2. Wild-type ht-ADH incubated at 0°C and an enzyme concentration of 20 µM showed no activity loss even after 24 h. By contrast, a freshly thawed sample of ht-W87A diluted to 20 µM lost 50% of its activity within ca. 5 h, with more rapid loss at lower enzyme concentration (Figure 2, panel A). When the same sample was incubated at moderately elevated temperature, an increase in activity was observed, with the most rapid reconstitution seen at 30°C (Figure 2, panel B). Slower reconstitution is seen at 40°C, and the enzyme is inactivated upon prolonged incubation at 50°C, (data not shown). It was also found that the presence of cofactor increases the rate of reconstitution, with the most rapid reconstitution seen for a ternary complex with the reduced form of the cofactor (NADH) and the aldehyde analog inhibitor N-butylformamide (Figure 2, panel C). Finally, ht-W87A activity is reconstituted more slowly in the presence of the inhibitor complex at 10°C than was observed at 30°C (Figure 2, panel D). Despite the slightly slower reconstitution than in the presence of N-
butylformamide, NADH alone was found to give the largest absolute increase in specific activity (not shown), possibly due to carryover of inhibitor into the assay. Incubation of ht-W87A at 200 µM enzyme and 1 mM NADH at 30°C for 30 min was, therefore, used to generate stable and reproducible enzyme activity for use in kinetic assays. The presence of NADH was sufficient to prevent activity loss over several hours in 2 to 20 µM stock solutions of ht-W87A maintained at room temperature. Kinetic assays were linear for at least 1 min and linearly dependent on enzyme concentrations between 10 and 55°C, ruling out the possibility of rapid inactivation upon dilution, or during the course of data collection.

Additional characterization of ht-W87A was aimed at determining the mechanism of inactivation and reconstitution. Fluorescence monitored as a function of time under reconstituting conditions at 10°C revealed a time-dependent decrease in fluorescence intensity (Figure 2, panel D). The kinetics of fluorescence quenching were correlated with the kinetics of reconstitution at 10°C (Figure 2, panel D) and 30°C, where both phenomena occur more rapidly. The fluorescence emission spectra are presented in Figure 3A. A CD spectrum for ht-W87A collected for a sample cold-adapted overnight at 4°C, and which showed no detectable activity, was nearly superimposable with a highly active sample that had been incubated under reconstituting conditions (Figure 3B). Size exclusion chromatography indicated that the dominant oligomeric state for wild-type ht-ADH is a tetramer, whereas ht-W87A eluted primarily as a monomer (Figure 4A). A similar experiment conducted in the presence of 1 mM NADH and 1 mM N-butylformamide, which were found to protect the enzyme from cold inactivation (data not shown) resulted in ht-W87A eluting almost exclusively as the tetramer (Figure 4B). In all cases, activity assays performed on fractions eluting from the size exclusion column indicated that only the tetramer was active.

Characterization of a ps-A25Y Variant— Turning to the ps-ADH variants, it was found that these enzymes are also only active as a tetramer. The wild-type enzyme dissociated into an inactive monomeric form when subjected to FPLC size exclusion chromatography at 4°C, and the extreme thermostability reported previously was confirmed by incubation at 50°C (Figure 5). The availability of X-ray structures for both ht-ADH and ps-ADH, together with the high sequence identity between these proteins (61%), afforded the opportunity to search for structural differences at their subunit interfaces. One striking feature is the substitution of a tyrosine at position 25 in ht-ADH by alanine in ps-ADH. We therefore prepared ps-A25Y to contrast its behavior to wild-type ps-ADH. The ps-A25Y mutation significantly stabilized the tetrameric form of the enzyme at 4°C, and led to a dramatic increase in its thermostability at 50°C, Figure 5A. As a result of the increased thermostability, it was possible to measure steady-state kinetics up to 52°C. An Arrhenius plot for ps-A25Y indicates a breakpoint near ca. 20°C. Below this temperature, activity falls more rapidly with temperature, and the KIE on $k_{cat}$ becomes progressively smaller (Figure 6A), in contrast to the wild-type ps-ADH behavior. The activation parameters for ps-A25Y above the breakpoint (between 20 and 52°C) are similar to those measured for wild-type ps-ADH, Table 2. No attempt was made to estimate the same parameters below the breakpoint for ps-A25Y because of the apparent kinetic complexity suggested by the decrease in KIE with temperature.

Contrasting the Behavior of ht-Y25A to ht-W87A—The reciprocal responses of ht-ADH to mutation at its active site and ps-ADH to mutation at its dimer interface suggested that ht-Y25A would produce an enzyme form very similar to ht-W87A. This prediction is confirmed by the kinetic behavior of ht-Y25A, where the breaks at 30°C in rate and the temperature dependence of the KIE for wild-type (Figure 1, inset) are lost in ht-Y25A (Figure 6B). The extreme susceptibility of ht-W87A to dissociation at low temperature is, however, not observed for ht-Y25A, with the fraction of tetramer being highly dependent on the concentration of enzyme analyzed by FPLC. The proportion of the enzyme found in
the tetrameric form varied from ca. 81% at 0.5 μg to 44% at 0.3 μg, to a level below the limit of detection at 0.02 μg of protein analyzed. In the case of ht-W87A, FPLC data (Figure 4A, gray trace) were carried out at extremely high protein concentration (500 μg) in an effort to suppress protein dissociation; nonetheless, the dominant enzyme form remained a monomer. This indicates the much larger effect of an active site mutant on oligomeric protein stability than the subunit interface mutant Y25A.

Discussion

Activation Parameters and KIEs Indicate That A Transition Observed for ht-ADH at 30°C is Absent in ht-W87A and ht-Y25A—Two kinetic signatures define the Arrhenius break for the wild-type ht-ADH. First, an increase in the enthalpy of activation ΔH‡ by ca. 7 kcal/mol is accompanied by an equivalent increase in TΔS‡ below 30°C. Fitting the kinetic data below this temperature to the Arrhenius equation (Eq 1) results in an unusually large Arrhenius prefactor $A_{OBS} \sim 10^{17}$ s$^{-1}$; for the majority of chemical reactions, $A_{OBS}$ falls near $10^{13}$ s$^{-1}$ (34). Recent characterization of ht-ADH variants mutated in the cofactor-binding pocket revealed an enhanced Arrhenius break, with values for $A_{OBS}$ as large as $10^{25}$ s$^{-1}$. A detailed characterization of these enzymes has led to the conclusion that the unusual Arrhenius curves observed for ht-ADH variants reflect a reversible distribution of the protein conformational landscape into inactive, low energy states at low temperature. The present data indicate that the Arrhenius break is completely absent for ht-W87A, as well as ht-Y25A, with values for $A_{OBS}$ throughout the temperature range (between 10 and 52°C) approximating the values measured above 30°C for wild-type. We conclude that ht-Y25A and ht-W87A do not sample the low energy inactive states in the experimental temperature range.

A second signature of the Arrhenius break in the wild-type enzyme is a transition in the temperature dependence of the KIE. Above the 30°C breakpoint, the wild-type enzyme exhibits a nearly temperature-independent KIE: the difference between the energy of activation for protium and deuterium, $E_{a}(D)-E_{a}(H)$ approaches zero (Table 2). By contrast, below the breakpoint temperature, the KIE becomes temperature-dependent, with increasing values for $E_{a}(D)-E_{a}(H)$. The ht-ADH enzyme is thus one of a growing number of enzymes catalyzing hydrogen transfer with KIEs that are nearly temperature-independent under native conditions, but become temperature-dependent upon the introduction of a generic perturbation, such as mutation, protein surface modification, or as in this case, a change in temperature (23). This phenomenon has increasingly been attributed to a failure of perturbed enzymes to sample conformational substates that can support ideal quantum tunneling conditions for hydrogen transfer at the active site. In cases of both ht-W87A and ht-Y25A, the absence of a detectable transition toward more temperature-dependent KIEs at low temperature indicates that conformational sampling remains optimal for hydrogen tunneling throughout the experimental temperature range.

The Catalytic Properties of the ht-W87A Variant More Closely Resemble Those of ps-ADH Than ht-ADH, But The Enzyme is Not Suited For Low-Temperature Catalysis Because of Cold Lability—Extensive comparative studies of the kinetic properties of homologous enzymes from organisms adapted to different temperature niches have revealed several trends that extend to ps-ADH and ht-ADH. Thermophilic enzymes are typically more stable, exhibit smaller $\Delta H^\ddagger$ and more negative $\Delta S^\ddagger$ than their mesophilic and psychrophilic counterparts (22,35-39). In keeping with these trends, ps-ADH shows a smaller $\Delta H^\ddagger$ and more negative $\Delta S^\ddagger$ than ht-ADH (22). Also, whereas the ps-ADH is extremely thermostable, the ht-ADH is stable to extended incubation at 65°C.

These trends in kinetic properties are likely a consequence of co-evolutionary constraints on protein flexibility and stability. Although some studies indicate exceptions are possible (40), enzymes that have evolved to function at elevated temperature tend to be more rigid and
less active at low temperature than their psychrophilic counterparts (3,25,30,41,42). As discussed in the introduction, ht-ADH and ps-ADH can be included in this trend (30), and, importantly, a decrease in local flexibility below 30°C correlates with the Arrhenius break in ht-ADH (21).

The ht-W87A mutation converts ht-ADH to an enzyme with properties intermediate between ht-ADH (>30ºC) and ps-ADH. The mutant retains at all temperatures the lower ΔH‡ and more negative ΔS‡ associated with the wild-type enzyme at high temperature. Thus, ht-W87A activity falls off less steeply with temperature, and ht-W87A and ps-ADH yield similar rate constants at low temperature. However, while it remains more resistant to heat inactivation than ps-ADH, ht-W87A sacrifices thermostability relative to ht-ADH. Collectively, the data suggest that ht-W87A is overall a more flexible catalyst than the wild-type enzyme, failing to undergo rigidification in the experimental temperature range, but paying for increased flexibility via a decrease in stability.

Both ht-ADH and ps-ADH Are Active Exclusively in the Tetrameric Form, and Both The Cold Lability of ht-W87A and The Thermolability of ps-ADH Result From Dissociation of the Active Form of the Enzyme—The cold-lability observed in ht-W87A is concentration-dependent, with dilute enzyme becoming inactivated more rapidly (Figure 2A). These inactivation kinetics are consistent with dissociation of the tetrameric form of the enzyme into inactive, lower order oligomers. Size exclusion chromatography confirmed that the ht-W87A tetramer dissociates to a much larger extent than the wild-type enzyme (Figure 4A), and activity assays indicated that the dimer and monomer are both inactive (not shown). Further evidence that the inactivation of ht-W87A at low temperature arises from reversible dissociation of the tetramer comes from the observation that enzymatic activity can be reconstituted from fractions eluting as inactive monomeric enzyme upon incubation at 30°C in the presence of cofactor under the same conditions used to reconstitute cold-inactivated enzyme in Figure 2 (not shown). Moreover, when size exclusion chromatography was performed at 4°C in the presence of NADH and N-butylformamide, which prevent cold inactivation, ht-W87A eluted almost exclusively as a tetramer, and no activity was associated with minor fractions eluting as monomer or dimer (Figure 4B). Similarly, when ps-ADH was subjected to size exclusion chromatography (also at 4°C), activity was only associated with the tetrameric form of the enzyme. Compared with ht-ADH, a greater proportion of the psychrophilic enzyme elutes as dimer and monomer.

The cold-lability observed in ht-W87A is concentration-dependent, with dilute enzyme becoming inactivated more rapidly (Figure 2A). These inactivation kinetics are consistent with dissociation of the tetrameric form of the enzyme into inactive, lower order oligomers. Size exclusion chromatography confirmed that the ht-W87A tetramer dissociates to a much larger extent than the wild-type enzyme (Figure 4A), and activity assays indicated that the dimer and monomer are both inactive (not shown). Further evidence that the inactivation of ht-W87A at low temperature arises from reversible dissociation of the tetramer comes from the observation that enzymatic activity can be reconstituted from fractions eluting as inactive monomeric enzyme upon incubation at 30°C in the presence of cofactor under the same conditions used to reconstitute cold-inactivated enzyme in Figure 2 (not shown). Moreover, when size exclusion chromatography was performed at 4°C in the presence of NADH and N-butylformamide, which prevent cold inactivation, ht-W87A eluted almost exclusively as a tetramer, and no activity was associated with minor fractions eluting as monomer or dimer (Figure 4B). Similarly, when ps-ADH was subjected to size exclusion chromatography (also at 4°C), activity was only associated with the tetrameric form of the enzyme. Compared with ht-ADH, a greater proportion of the psychrophilic enzyme elutes as dimer and monomer.

Fluorescence and CD Spectra Indicate No Major Changes in Secondary Structure Upon Dissociation of the ht-ADH Tetramer Into Monomers—CD spectra collected for warm-adapted (active) ht-W87A as well as cold-inactivated enzyme were almost superimposable (Figure 3B), indicating that the loss of activity is not a result of significant unfolding of the enzyme upon dissociation of the tetramer. Tryptophan fluorescence spectra collected under conditions that reconstitute activity indicated a time-dependent increase in fluorescence quenching (Figure 2D) that correlates with the restoration of enzymatic activity. Examination of the X-ray structure for ht-ADH indicates that W49 forms a pi-stacking interaction with F272 of an adjacent subunit (Figure 7B). The formation of this interaction upon oligomerization could lead to a portion of the observed protein fluorescence quenching (43). Fluorescence energy transfer to the bound NADH cofactor was detected simultaneously at λemission = 410 nm, and a gradual blue-shift of this signal with a similar time dependence as the quenching of tryptophan fluorescence (Figure 3A) suggests that the formation of subunit interactions directly alters the active site microenvironment. Taken together, the spectroscopic data support the conclusion that ht-W87A retains the overall subunit structure of the wild-type enzyme, and that minor structural changes are responsible for converting the inactive dimer to the active tetramer form.

Increased ps-A25Y Thermostability is Due to Strengthened Inter-Subunit Interactions,
However the Enzyme Becomes Less Suited For Catalysis at Low Temperatures Because of a Change in Rate-Limiting Step—The observed relationship between oligomeric integrity and activity in ht-W87A suggested that thermolability in ps-ADH may arise from weakened subunit interactions. Homology modeling to ht-ADH indicates a ps-ADH structure nearly superimposable with that of the thermophilic enzyme, which we regard as a dimer of dimers (44). Subunits interact strongly at a ‘dimer interface’ in the dimeric eukaryotic alcohol dehydrogenases (45); however, the interactions that join two such dimers at what we term the ‘dimer-dimer interface’ are relatively sparse. Notably, a π-stacking interaction at the tetramer interface between Y25 and its counterpart in an adjacent subunit is lost in ps-ADH, as the psychophilic enzyme contains alanine at position 25 (Figure 7B). We hypothesized that restoration of this interaction in the psychrophilic enzyme would confer significant stabilization. Upon incubation at 50°C, ps-A25Y is seen to be dramatically more stable than the wild-type enzyme, retaining nearly 75% activity after 20 min, at which time ps-ADH is completely inactivated (Figure 5A). Analysis by size exclusion chromatography further shows that the experimental conditions promoting extensive dissociation of the wild-type ps-ADH lead to retention of the tetramer in the ps-A25Y mutant (Figure 5B).

Kinetic characterization of ps-A25Y at 30°C shows this enzyme is only modestly less active than ps-ADH, and similar values for the KIE indicate that the chemical step remains rate-determining (Table 1). In the elevated temperature range for ps-A25Y (between 20 and 50°C), activation parameters are also similar to those measured for ps-ADH between 5 and 35°C (Table 2). Below 20°C, ps-A25Y and ps-ADH exhibit divergent behavior. A breakpoint emerges in the ps-A25Y Arrhenius point, with an apparent increase in the enthalpy of activation, reminiscent of the ht-ADH behavior (Figure 6A). However, examination of the KIE on $k_{cat}$ ($^Dk_{cat}$) reveals a trend toward smaller values at lower temperature, opposite what is seen for ps-ADH (22). As a result, the ‘break’ in the ps-A25Y Arrhenius plot likely reflects increasing contributions to $k_{cat}$ from steps other than hydride transfer; by contrast, the hydride transfer step appears to be fully rate-limiting at all temperatures for ps-ADH. Although ps-A25Y is much more stable than ps-ADH, the mutant is significantly less active than ps-ADH at temperatures below 20°C, consistent with the hypothesis that the need to maintain flexibility at reduced temperature places a limit on the stability of psychrophilic enzymes.

The Mutant ht-Y25A Exhibits Similar Kinetic Characteristics to ht-W87A, However, Its Quaternary Structure is More Resistant Against Cold Dissociation—The π-stacking interaction between Tyr25 residues located on opposing subunits appears to stabilize their association in the ps-A25Y variant and the wild-type ht-ADH. Given these observations, the ht-Y25A variant was expected to exhibit stability and kinetic properties resembling those of ht-W87A. Similar to ht-W87A and the wild-type ps-ADH, the ht-Y25A variant does not yield a statistically significant Arrhenius break at 30°C (Figure 6B). The activation parameters across the experimental temperature range (10 ~ 50°C) resemble those observed for the wild-type ht-ADH above 30°C (Table 2). This observation provides further support for the notion that Arrhenius behavior can be controlled at least in part by inter-subunit interactions. However, unlike ht-W87A, which undergoes a significant loss of quaternary stability at low temperature (Figure 4A), the ht-Y25A tetramer shows a more modest propensity to dissociate in a concentration-dependent manner (not shown). The contrasting stabilities of ht-W87A, ps-ADH and ht-Y25A at reduced temperature indicate that an increase in local flexibility at the active site (expressed as a loss of the Arrhenius break) does not require, a priori, a large destabilization of inter-subunit interactions.

Reciprocal Effects of Mutation to ht-ADH and ps-ADH Implicate a Network of Communication Between the Active Site of the Enzyme and the Subunit Interface—Because the active site of the tetrameric bacterial alcohol dehydrogenases does not lie at a
subunit interface, the exclusive activity of the tetrameric state must reflect subtle structural changes that propagate from the dimer-dimer interface to the active site of the enzyme upon formation of inter-subunit interactions. The ps-A25Y mutant indicates that the effects of strengthened subunit interactions can propagate to the active site in a deleterious manner, by the introduction of a new rate-limiting step. Reciprocally, the ht-Y25A mutant with a weakened subunit interaction appears to prevent the enzyme from falling into a subset of low energy, catalytically inactive conformers that has been invoked previously to account for inflated Arrhenius prefactors in other variants of ht-ADH (31). In the case of ht-W87A, the introduction of a packing defect at the active site results in a significant weakening of subunit interactions at low temperature, indicating that changes in active site geometry likewise propagate to interactions at the subunit interface. Thus, the aggregate data indicate bi-directional communication between the active site and the subunit interface. The five peptides that have previously been shown by way of hydrogen-deuterium exchange experiments to rigidify below 30°C in ht-ADH directly connect Y25 to W87 via a contiguous beta sheet (Figure 7A), presenting pathway by which small perturbations at either position may exert structural and dynamical changes at the other position (21).

The functional link between structural features at the active site and the subunit interface is reminiscent of allostery, wherein the role of ligand binding as the allosteric signal has been replaced by a change in temperature. Furthermore, the lack of major rearrangements in the secondary structure of the protein as a function of temperature (31), or upon disruption of inter-subunit interactions (W87A) indicate that conformational sampling may carry the “allosteric” signal in place of a large change in the average conformation of the protein. Transmission of an allosteric signal via dynamic fluctuations, and in the absence of detectable conformational change has been proposed for other proteins recently, and has been discussed in detail (46). The present data show that prokaryotic alcohol dehydrogenases have evolved to tune the long-range interactions between the subunit interface and the active site in support of optimal catalysis within their respective temperature niches.

Convex Arrhenius Curves Observed For ht-ADH and ps-A25Y Appear to Arise from a Temperature-Dependent Change in Subunit Interactions—Although the Arrhenius break for ps-A25Y arises from kinetic complexity, it contributes to a pattern in which stronger subunit interactions tend to be associated with convex Arrhenius curves. Weakened subunit interactions in ps-ADH and ht-W87A, or the direct removal of an inter-subunit interaction in the case of ht-Y25A, are all associated with the absence of an Arrhenius break. A similar relationship has been observed recently for a thermophilic, dimeric dihydrofolate reductase (27). In those experiments, the wild-type enzyme showed a modest breakpoint at 25 °C below which both $\Delta H^\circ$ and the temperature dependence of the KIE increased, as seen with ht-ADH. The introduction of a mutation at the subunit interface resulted in an active monomeric enzyme, and abrogated the break. However, unlike the ht-ADH and more similar to ps-A25Y, the break in the Arrhenius plot was found to arise from a change in rate-determining step. “Convex” Arrhenius curves in which the hydrogen transfer step remains rate-determining at all temperatures have been proposed to arise from conformational pre-equilibria (28,47,48), and we have shown that impaired conformational sampling can be used to model the enormously inflated Arrhenius pre-factors measured for a series of ht-ADH mutants at reduced temperatures (31). The molecular basis for these Arrhenius breakpoints has been elusive, although a clue may lie in the observation that the transition from low to high-temperature conformational equilibria in ht-ADH requires both a large positive $\Delta H^\circ$, and a large positive $\Delta S^\circ$ (31), as have been measured for the dissociation of protein subunits (49).

The results presented herein, taken together with extensive previous characterization of the
ht-ADH support a model for the observed kinetic properties, presented in Figure 7A. At elevated temperature, ht-ADH adopts a subset of relatively flexible conformational substates that support efficient tunneling. Below 30°C, the enzyme adopts more rigid conformational substates that still support catalysis, however with altered tunneling parameters. The ability of the enzyme to adopt these conformers depends upon inter-subunit interactions at position 25. As the temperature decreases, the rigidified subunits can enter into a second, lower energy interaction with one another that forces the enzyme into a catalytically inactive conformational space via K2. As this occurs, the rate of catalysis decreases rapidly with temperature, resulting in inflated Arrhenius parameters (31). We propose that the packing defect caused by the ht-W87A mutation introduces flexibility into the protein that overwhelms the stabilizing effect of interactions at Y25. This prevents the enzyme from rigidifying at reduced temperature, so that it is able sample a conformational subspace compatible with efficient tunneling at all temperatures (K1 in the model approaches zero). In complementary fashion, the absence of a pi-stacking interaction at the subunit interface in ht-Y25A likewise prevents the enzyme from rigidifying at reduced temperature (K1 in the model again approaches zero). Since ht-Y25A remains more cold-stable than the ht-W87A mutation, the latter appears to weaken additional inter-subunit interactions, possibly through a process of mild deformation of the protein about the resulting cavity in the active site. This process can be prevented or reversed by the binding of NAD cofactor, NADH, or a combination of NADH and the aldehyde analogue inhibitor, N-butylformamide. Cofactor binding to alcohol dehydrogenase is known to induce a conformational change. This could offset the extreme destabilizing effect of the ht-W87A by introducing inter-subunit interactions that are unavailable in the conformational substates adopted in the absence of cofactor. In the case of ps-A25Y, an increase in subunit interactions analogous to the process represented by K2 is suggested to take place below 20°C. Since the enzyme remains catalytically active, the consequence of these interactions must be to slow down a step other than hydride transfer (possibly product release). The observation of Arrhenius breaks in multimeric enzymes may, thus, be a consequence of a temperature-dependent change in the strength of subunit interactions, which imposes restrictions on the conformational landscape, and thereby distorts the active site away from its optimal geometry. The ability of subunit interactions to regulate enzyme activity in a reversible and temperature-dependent manner may also serve a previously unrecognized biological role, presenting a strategy by which organisms can respond rapidly to temperature changes at the level of enzyme activity without the need for cycles of protein degradation and replenishment.

Conclusion
An evolutionary trade-off among stability, local flexibility and catalytic activity is evident in ps-ADH, ht-ADH and their respective mutant variants. The data support a model that involves communication between the dimer-dimer interface and the enzyme active site in prokaryotic alcohol dehydrogenases. The two enzyme forms for which a catalytically deleterious rigidification appears to occur, ht-ADH and ps-A25Y, share the feature of relatively strong inter-subunit interactions. In both cases, subunit interactions propagate to the active site, with ht-ADH exhibiting impairment of hydride transfer, and ps-ADH a change in rate-limiting step. Reciprocally, attenuated subunit interactions in ps-ADH, ht-Y25A, and ht-W87A permit hydrogen tunneling to proceed unimpaired at reduced temperature. These observations provide new insight into the molecular origins of unusual Arrhenius curves seen in ht-ADH and other oligomeric proteins. A more detailed understanding of the molecular basis for subtle, correlated changes between catalysis and protein flexibility may yield novel targets for the disruption of protein-protein interactions and the inhibition of enzymatic activity.
References

1. Somero, G. N. (1978) *Annu. Rev. Ecol. Syst.* **9**, 1-29
2. Somero, G. N. (1995) *Annu. Rev. Physiol.* **57**, 43-68
3. Zavodszky, P., Kardos, J., Svingor, A., and Petsko, G. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 7406-7411
4. Feller, G. (2010) *J. Phys.-Condens. Matter* **22**
5. Kohen, A., Cannio, R., Bartolucci, S., and Klinman, J. P. (1999) *Nature* **399**, 496-499
6. Benkovic, S. J., and Hammes-Schiffer, S. (2003) *Science* **301**, 1196-1202
7. Hammes-Schiffer, S., and Benkovic, S. J. (2006) *Annu. Rev. Biochem.* **75**, 519-541
8. Fan, F., and Gadda, G. (2007) *Biochemistry* **46**, 6402-6408
9. Henzler-Wildman, K., and Kern, D. (2007) *Nature* **450**, 964-972
10. Benkovic, S. J., Hammes, G. G., and Hammes-Schiffer, S. (2008) *Biochemistry* **47**, 3317-3321
11. Klinman, J. P. (2009) *Chem. Phys. Lett.* **471**, 179-193
12. Pudney, C. R., Hay, S., Levy, C., Pang, J. Y., Sutcliffe, M. J., Leys, D., and Scrutton, N. S. (2009) *J. Am. Chem. Soc.* **131**, 17072+
13. Bhabha, G., Lee, J., Ekiert, D. C., Gam, J., Wilson, I. A., Dyson, H. J., Benkovic, S. J., and Wright, P. E. (2011) *Science* **332**, 234-238
14. Schramm, V. L. (2011) Enzymatic Transition States, Transition-State Analogs, Dynamics, Thermodynamics, and Lifetimes. in *Annu. Rev. Biochem., Vol 80* (Kornberg, R. D., Raetz, C. R. H., Rothman, J. E., and Thorner, J. W. eds.), Annual Reviews, Palo Alto. pp 703-732
15. Stojkovic, V., Perissinotti, L. L., Willmer, D., Benkovic, S. J., and Kohen, A. (2012) *J. Am. Chem. Soc.* **134**, 1738-1745
16. Glowacki, D. R., Harvey, J. N., and Mulholland, A. J. (2012) *Nature Chemistry* **4**, 8
17. Tang, K. E. S., and Dill, K. A. (1998) *J. Biomol. Struct. Dyn.* **16**, 397-411
18. Fields, P. A., and Somero, G. N. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11476-11481
19. Vihinen, M. (1987) *Protein Eng.* **1**, 477-480
20. Varley, P. G., and Pain, R. H. (1991) *J. Mol. Biol.* **220**, 531-538
21. Liang, Z. X., Lee, T., Resing, K. A., Ahn, N. G., and Klinman, J. P. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 9556-9561
22. Liang, Z. X., Tsigos, I., Bouriotis, V., and Klinman, J. P. (2004) *J. Am. Chem. Soc.* **126**, 9500-9501
23. Nagel, Z. D., and Klinman, J. P. (2009) *Nat. Chem. Biol.* **5**, 543-550
24. Heyes, D. J., Levy, C., Sakuma, M., Robertson, D. L., and Scrutton, N. S. (2011) *J. Biol. Chem.* **286**, 11849-11854
25. Oyeyemi, O. A., Sours, K. M., Lee, T., Kohen, A., Resing, K. A., Ahn, N. G., and Klinman, J. P. (2011) *Biochemistry* **50**, 8251-8260
26. Anandarajah, K., Schowen, K. B., and Schowen, R. L. (2008) *J. Res. Phys. Chem. Phys. Chem.* **222**, 1333-1347
27. Loveridge, E. J., Rodriguez, R. J., Swanwick, R. S., and Allemann, R. K. (2009) *Biochemistry* **48**, 5922-5933
28. Massey, V., Curti, B., and Ganther, H. (1966) *J. Biol. Chem.* **241**, 2347-&
29. Tsigos, I., Velonia, K., Smonou, I., and Bouriotis, V. (1998) *Eur. J. Biochem.* **254**, 356-362
30. Liang, Z. X., Tsigos, I., Lee, T., Bouriotis, V., Resing, K. A., Ahn, N. G., and Klinman, J. P. (2004) *Biochemistry* **43**, 14676-14683
31. Nagel, Z. D., Dong, M., Bahnson, B. J., and Klinman, J. P. (2011) *Proc. Natl. Acad. Sci. U. S. A.* **108**, 10520-10525
32. Nagel, Z. D., Meadows, C. W., Dong, M., Bahnson, B. J., and Klinman, J. P. (2012) Biochemistry 51, 4147-4156
33. (2008) PrimerX.com.
34. Forst, W. (1973) Theory of unimolecular reactions, Academic Press, New York
35. Lonhienne, T., Gerday, C., and Feller, G. (2000) Biochim. Biophys. Acta-Protein Struct. Molec. Enzym. 1543, 1-10
36. D'Amico, S., Collins, T., Marx, J. C., Feller, G., and Gerday, C. (2006) EMBO Rep. 7, 385-389
37. Feller, G. (2007) Extremophiles 11, 211-216
38. Nagel, Z. D., and Klinman, J. P. (2006) Chemical Reviews 106, 3095-3118
39. Lam, S. Y., Yeung, R. C. Y., Yu, T. H., Sze, K. H., and Wong, K. B. (2011) PLoS Biol. 9
40. Merkley, E. D., Parson, W. W., and Daggett, V. (2010) Protein Eng. Des. Sel. 23, 327-336
41. Kohen, A., and Klinman, J. P. (2000) J. Am. Chem. Soc. 122, 10738-10739
42. Wolf-Watz, M., Thai, V., Henzler-Wildman, K., Hadjipavlou, G., Eisenmesser, E. Z., and Kern, D. (2004) Nature Struct. Mol. Biol. 11, 945-949
43. Nanda, V., and Brand, L. (2000) Proteins 40, 112-125
44. Ceccarelli, C., Liang, Z. X., Strickler, M., Prehna, G., Goldstein, B. M., Klinman, J. P., and Bahnson, B. J. (2004) Biochemistry 43, 5266-5277
45. Eklund, H., Samama, J. P., Wallen, L., Branden, C. I., Akeson, A., and Jones, T. A. (1981) J. Mol. Biol. 146, 561-587
46. Tzeng, S. R., and Kalodimos, C. G. (2011) Curr. Opin. Struct. Biol. 21, 62-67
47. Truhlar, D. G., and Kohen, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 848-851
48. Limbach, H. H., Lopez, J. M., and Kohen, A. (2006) Arrhenius curves of hydrogen transfers: tunnel effects, isotope effects and effects of pre-equilibria.
49. Weber, G. (1995) J. Phys. Chem. 99, 1052-1059
Table 1. Kinetic parameters at 30°C for ADH variants.

|        | $k_{cat}$ (s$^{-1}$) | $K_{M}$. BnOH (mM) | $K_{M}$. NAD$^+$ (mM) | KIE for $k_{cat}$ |
|--------|----------------------|--------------------|-----------------------|------------------|
| ht-ADH$^a$ | 24.9 (±2.5)          | 6.9 (±0.5)         | 1.1 (±0.1)            | 3.2 (±0.2)       |
| ht-W87F  | 6.1 (±0.5)           | 5.2 (±0.8)         | 2.1 (±0.3)            | 3.4 (±0.6)       |
| ht-W87L  | 2.2 (±0.1)           | 18 (±2.2)          | 4.0 (±0.7)            | 3.0 (±0.3)       |
| ht-W87A  | 5.5 (±0.4)           | 1.5 (±0.3)         | 0.2 (±0.05)           | 3.2 (±0.6)       |
| ht-Y25A  | 14.0 (±1.0)          | 7.2 (±1.7)         | 1.0 (±0.5)            | 2.9 (±0.2)       |
| ps-ADH$^b$| 6.9                  | 29.4               | -                     | 3.2              |
| ps-A25Y  | 3.8 (±0.2)           | 16 (±1.5)          | 0.5 (±0.06)           | 3.7 (0.6)        |

$^a$From ref. (5).

$^b$From ref. (22).
Table 2. Activation parameters for wild-type ht-ADH and mutants for the oxidation of benzyl alcohol.

|                  | ht-ADH, 5-30°C\(^a\) | ht-ADH 30-65°C\(^a\) | ht-W87A 12-52°C\(^b\) | ps-ADH 5-35°C\(^c\) | ps-A25Y 20-52°C\(^b\) | ht-Y25A 10°C-50°C\(^b,\(^c\) |
|------------------|-----------------------|-----------------------|------------------------|----------------------|-----------------------|-------------------------------|
| ΔH\(^\ddagger\)  | 21.2 (±1.0)           | 14.5 (±0.4)           | 14.8 (±0.4)            | 9.2 (±0.6)           | 7.2 (±0.3)            | 13.0 (±0.4)                   |
| TΔS\(^\ddagger\) | 5.2 (±2.4)            | -1.4 (±0.8)           | -1.8 (±0.4)            | -7.3 (±1.5)          | -9.6 (±0.3)          | -0.3 (±0.4)                   |
| E\(_a\)(D)-E\(_a\)(H) | 0.9 (±0.05)         | 0.1 (±0.01)           | 0.2 (±0.01)            | 4.5 (±1.5)           | ND\(^d\)             | 0.7 (±0.07)                   |

\(^a\) From ref. (5).
\(^b\) Data were fit to a single line.
\(^c\) From ref. (22).
\(^d\) ND, not determined.
\(^e\) The data were analyzed as a single line or as two separate slopes with a very subtle break. The resulting difference between the separate enthalpies was less than the errors generated for a single slope.
Table 3. Kinetic parameters for ht-W87A, ps-A25Y, and ht-Y25A.

|        | T(ºC) | k\text{cat}(H) | Error | D_{k_{\text{cat}}} | Error |
|--------|-------|----------------|-------|---------------------|-------|
| ht-W87A | 12.5  | 1.5            | 0.1   | 3.2                 | 0.2   |
|        | 15    | 1.6            | 0.1   | 3.1                 | 0.4   |
|        | 20    | 2.5            | 0.2   | 2.9                 | 0.4   |
|        | 28.5  | 5.2            | 0.7   | 3.6                 | 0.5   |
|        | 38    | 14.0           | 1.8   | 3.2                 | 0.6   |
|        | 45    | 23.0           | 1.2   | 2.9                 | 0.2   |
|        | 52    | 34.7           | 2.5   | 3.0                 | 0.3   |
| ps-A25Y | 5     | 0.5            | 0.01  | 2.4                 | 0.1   |
|        | 11.5  | 1.0            | 0.04  |                     |       |
|        | 16    | 1.5            | 0.04  | 2.8                 | 0.1   |
|        | 20    | 2.7            | 0.1   |                     |       |
|        | 25    | 3.2            | 0.2   |                     |       |
|        | 29    | 3.8            | 0.3   | 3.7                 | 0.6   |
|        | 39    | 7.0            | 1.0   |                     |       |
|        | 46    | 7.9            | 0.9   | 3.5                 | 0.6   |
|        | 52    | 13.0           | 1.9   | 3.6                 | 0.6   |
| ht-Y25A | 10    | 3.2            | 0.5   | 2.9                 | 0.3   |
|        | 20    | 7.5            | 0.5   | 3.5                 | 0.4   |
|        | 30    | 14.0           | 1.0   | 2.7                 | 0.4   |
|        | 40    | 27.8           | 5.7   | 2.4                 | 0.6   |
|        | 50    | 58.9           | 6.4   | 2.9                 | 0.3   |

a D_{k_{\text{cat}}} refers to the primary KIE for oxidation of benzyl alcohol relative to its \(a,a\)-\(d_2\)-isotopolog.

b KIEs were only measured at the temperatures indicated.
**Figure Legend**

**Figure 1.** Arrhenius plot for ht-W87A between 10 and 52°C. The temperature dependence of $k_{cat}$ (filled circles, left axis), KIE (open squares, right axis), and the corresponding plot for wild-type ht-ADH (inset) are presented.

**Figure 2.** Inactivation and reconstitution of ht-W87A. **A.** Percentage of activity remaining after incubation at 0°C for 20 µM wild-type ht-ADH (triangles, dashed line), 20 µM ht-W87A (squares, solid line), or 0.5 µM ht-W87A (circles, dotted line). **B.** Percentage of activity remaining for ht-W87A after incubation at 0°C circles, dotted line; 10°C squares, solid line; 20°C up triangles, dashed line; or 30°C down triangles, solid gray line. **C.** Time-dependent reconstitution of activity at 30°C as a percentage of the activity extrapolated at $t = \infty$, with no addition (circles, dotted line) or in the presence of 1 mM NAD+ (squares, solid gray line), 1 mM NADH (up triangles, dashed line), or 1 mM NADH + 1 mM N-butyllformamide (down triangles, solid line). **D.** Time-dependent reconstitution of activity at 10°C in the presence of 1 mM NADH + 1 mM N-butyllformamide (circles, dotted line), superimposed over time-dependent changes in fluorescence emission intensity at 338 nm under the same conditions (squares, solid line).

**Figure 3.** Spectroscopic characterization of ht-W87A. **A.** Time-dependent fluorescence emission spectrum for ht-W87A. Arrows represent the direction of change observed as a function of time. The fluorescence data in **Figure 2, panel D**, were generated from the spectra shown herein. Small, but significant changes were detected in two distinct regions of the fluorescence spectrum. Protein fluorescence ($\lambda_{max} \sim 333$ nm) was seen to decrease as a function of time. Fluorescence due to NADH simultaneously became blue shifted, as seen by an increase in the intensity at 410 nm. **B.** CD Spectra at two temperatures for ht-
W87A. Samples were incubated overnight at 20 µM enzyme at 0°C (heavy black line) or for 1 h at 30°C and 200 µM enzyme, with a spectrum collected immediately following dilution to 20 µM at 30°C (gray line with black dots). Difference spectrum, (fine black line).

**Figure 4.** Analysis of ht-W87A by FPLC size exclusion chromatography. **A.** Elution profile of wild-type ht-ADH (black) and ht-W87A (gray) at 4°C showing peaks associated with the tetramer (ca. 11 mL), dimer (ca. 13 mL) and monomer (ca. 15 mL). **B.** Elution profile of ht-W87A at 4°C in the presence (black) or absence of 1 mM NADH and 100 mM N-butylformamide. High background absorption (ca. 0.85 absorbance units) due to NADH was subtracted from the data collected in the presence of the cofactor in order to superimpose the two traces for comparison.

**Figure 5.** Stability and FPLC size exclusion chromatography of ps-ADH variants. **A.** Inactivation of 20 µM ps-ADH (open squares and gray line) or ps-A25Y (closed squares and black line) at 50°C. **B.** Elution profile at 4°C for wild-type ps-ADH (black) and ps-A25Y (gray) showing peaks associated with the tetramer (ca. 11 mL), dimer (ca. 12.5 mL), and monomer (ca. 14 mL). Peaks between the column dead volume (ca. 7 mL) and the tetramer represent higher order aggregates.

**Figure 6.** Arrhenius plots for ADH variants with amino acid substitutions at the subunit interface. **A.** Arrhenius plots for ps-A25Y between 5 and 52°C. Temperature dependence of $k_{cat}$ (filled circles and solid line, left axis), KIE (open squares, right axis) are shown. **B.** Arrhenius plot for ht-Y25A between 10 and 50°C. The temperature dependence of $k_{cat}$ (filled circles and solid line, left axis) and the KIE (open squares, right axis).

**Figure 7.** **A.** X-ray structure of ht-ADH and a model for the origin of the Arrhenius break. Peptides undergoing a transition in flexibility at 30°C are illustrated in color. Trp 87 (green, near
the end of peptide #4), the active site zinc (crimson sphere), and the alcohol analog
trifluoroethanol (cyan) abut a contiguous beta raft, which forms a contact with the same structural
feature in the adjacent subunit via tyrosine 25 (red) near the end of peptide #1. At right, three
conformational landscapes of the wild-type ht-ADH tetramer are represented in cartoon form. At
elevated temperature (circles), a flexible tetramer exhibits weakly temperature-dependent KIEs.
Below a transition temperature of 30°C (dotted line), a more rigid conformational landscape
(squares) dominates, exhibiting temperature-dependent KIEs. These substates are in equilibrium
with an additional landscape in which strong inter-subunit interactions are proposed as the origin
of low energy, inactive forms of enzyme. B. X-ray structure of ht-ADH. One dimer is colored
black and the other, blue and yellow, to emphasize interactions at the dimer-dimer interface
where Tyr25 is shown in green. The interaction between Trp 49 and Phe 272 is highlighted by a
black circle. Residues from the subunit on the left are colored blue, those from the right subunit
are yellow. On the right side of the figure, the image is rotated 90 degrees. A beta sheet
interaction between the subunits is seen at the center of the image, flanked by the W49:F272
interaction.
Figure 2

A Concentration Dependence

B Temperature Dependence

C Cofactor Dependence

D Activity v. Fluorescence Change
Figure 3

A

B
Figure 4

A

B

Absorbance

Volume Eluted / mL

Absorbance

Volume Eluted / mL
Figure 5

A

% Activity Remaining

Time, Minutes

0 10 20 30 40

B

Absorbance

Volume Eluted / mL

0 5 10 15
Figure 6

A

B
Identification of a Long-Range Protein Network that Modulates Active Site Dynamics in Extremophilic Alcohol Dehydrogenases
Zachary D. Nagel, Shujian Cun and Judith P. Klinman

J. Biol. Chem. published online March 22, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.453951

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts