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# Impact of Protein Flexibility on Hydride-Transfer Parameters in Thermophilic and Psychrophilic Alcohol Dehydrogenases

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Understanding hydrogen (proton, hydride, or hydrogen atom)-transfer reactions is vital to the understanding of many biological processes. Mounting evidence from theoretical and experimental studies suggests that the interpretation of anomalous kinetic data such as elevated kinetic isotope effects (KIE) and nonclassical temperature-dependencies for the KIE require the incorporation of quantum tunneling and protein dynamics into the model.[1−4] One of the systems that exhibits such anomalous kinetic data is the thermophilic alcohol dehydrogenase (htADH) cloned from *Bacillus stearothermophilus* strain LLD-R. The hydride transfer between substrate and $NAD^+$ catalyzed by htADH has been found to exhibit strong nonlinear Arrhenius behavior in the temperature range of 5−65 °C.[5,6] Competitive and noncompetitive KIE data indicate that hydrogen tunneling becomes more pronounced in the temperature range of 30−65 °C. These results have been rationalized by an environmentally coupled tunneling model in which the hydride transfer is coupled to protein vibrational modes that only become accessible at elevated temperature.[5,7] A recent hydrogen−deuterium exchange (HX) study provides evidence for the activation of collective motion within the substrate-binding domain of htADH above 30 °C.[8]

The thermophilic ADH is highly homologous to the mesophilic ADH from *Escherichia coli* (eADH) and the newly cloned psychrophilic ADH (psADH) from the Antarctic *Moraxella* sp. TAE 123. All three enzymes are homotetrameric, and their structures appear almost superimposable.[9−11] In psADH and eADH, positions 86 and 147 are occupied by Ala and Leu (or Thr), but the residues change to Pro and Phe in htADH (Figure 1, also see Figure S1). We reasoned that these residues may contribute to the local rigidity observed in htADH at reduced temperature and that mutation of these two residues would affect hydride transfer if this step is indeed coupled to the vibrational modes of the surrounding protein medium. Herein, we show how the mutation P86A or F147L in htADH affects the hydride-transfer properties. Additionally, we show that the structurally homologous psADH exhibits markedly different activation parameters from htADH, likely to reflect significant differences in intrinsic flexibility between the two enzyme systems.

The mutations of htADH reduce $k_{cat}$ by several-fold for F147L and ∼15-fold for P86A across the entire temperature range, such that the mutants continue to exhibit nonlinear temperature-dependent behavior although the kinetic difference between the low- and high-temperature regimes is diminished (Figure S2). The primary H/D KIE ($^Dk_{cat}$) values listed in Table 1 indicate that the hydride-transfer step remains largely rate-limiting for the wild type and the two mutants and, thus, that the effect of mutations is reflected in the hydride-transfer step. In the case of P86A, it appears that a second step has also been affected, reducing $^Dk_{cat}$ from 5.0 to 2.9 at 20 °C.

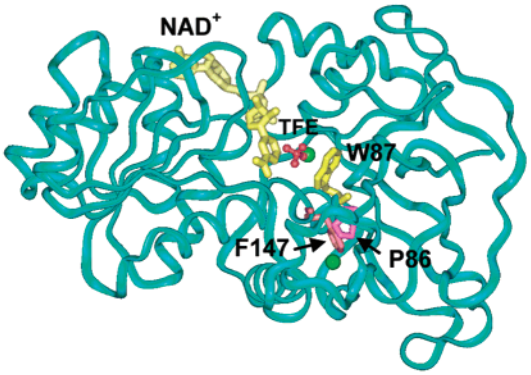


***Figure 1.*** Structure of htADH with substrate analogue trifluoroethanol (TFE) and "modeled-in" $NAD^+$. The distances between the catalytic zinc ion (in green) and the α-carbon of P86 and F147 are 9.1 and 10.1 Å, respectively.

***Table 1.*** Kinetic and Thermodynamic Activation Parameters for the Oxidation of Benzyl Alcohol[a]

| | $k_{cat}$ (40 °C) ($s^{-1}$) | $^Dk_{cat}$ (40 °C) | $\Delta H^‡$ (kcal $mol^{-1}$) | $T\Delta S^‡$ (kcal $mol^{-1}$) |
|---|---|---|---|---|
| | | Above 30 °C | | |
| htADH | 51.0 (2.1) | 2.3 (0.4) | 14.5 (0.4) | −1.4 (0.8) |
| F147L | 9.0 (0.8) | 3.3 (0.7) | 14.5 (1.0) | −1.8 (2.3) |
| P86A | 3.4 (0.4) | 2.0 (0.3) | 12.4 (0.9) | −5.2 (2.2) |
| | $k_{cat}$ (20 °C) ($s^{-1}$) | $^Dk_{cat}$ (20 °C) | $\Delta H^‡$ kcal $mol^{-1}$) | $T\Delta S^‡$ (kcal $mol^{-1}$) |
| | | Below 30 °C | | |
| htADH | 8.6 (0.2) | 5.0 (0.2) | 21.2 (1.0) | 5.2 (2.4) |
| F147L | 1.4 (0.2) | 5.3 (0.3) | 18.4 (1.1) | 1.5 (1.1) |
| P86A | 0.5 (0.1) | 2.9 (0.2) | 21.5 (1.9) | 3.9 (2.2) |
| psADH | 4.1 (0.3) | 4.0 (0.3) | 9.2 (0.6) | −7.3 (1.5) |

[a] The errors in $\Delta H^‡$ and $\Delta S^‡$ were generated by fitting the data to the nonlinear form of Arrhenius equation. Fitting the data to the linear form of Arrhenius equation gave much smaller apparent errors for $\Delta S^‡$ by factors of 2−13-fold.

The activation parameters derived from the Arrhenius plots show that the reduction in $k_{cat}$ for the mutants is due primarily to changes in the entropy of activation ($\Delta S^‡$) for the mutants. For example, at temperatures below 30 °C, the mutation F147L causes a small *decrease* in $\Delta H^‡$ ($\Delta\Delta H^‡ = -2.8 \pm 1.4$ kcal $mol^{-1}$), together with a less positive value for $\Delta S^‡$ ($\Delta\Delta S^‡ = -12.8 \pm 8.8$ cal $mol^{-1}$ $K^{-1}$). The net result is a 6-fold reduction in $k_{cat}$. Note that the sign of $T\Delta S^‡$ for the htADH and the two mutants changes to negative in the temperature range of 30−65 °C, in contrast to the positive $T\Delta S^‡$ values in the temperature range of 5−30 °C. In this case, comparison of a mutant (P86A) with WT indicates a small decrease in $\Delta H^‡$ accompanied by a more negative value for $\Delta S^‡$.

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Despite a sequence similarity of 76%, psADH exhibits very different activation parameters from WT htADH (Table 1). The comparison between psADH and htADH can only be made in the range of 5−35 °C because the psychrophilic ADH loses activity quickly at temperatures above 35 °C. In this temperature range, the primary KIE for psADH varies from 2.9 to 5.3, indicating that hydride transfer is largely the rate-limiting step. The kinetic and thermodynamic parameters for psADH show that psADH and WT htADH exhibit similar $k_{cat}$ values at around 10 °C. However, psADH exhibits a significantly smaller enthalpy of activation ($\Delta H^{\ddagger}$) of 9.2 ± 0.6 kcal mol$^{-1}$, compared with 21.2 ± 1.0 kcal mol$^{-1}$ for htADH. At the same time, psADH exhibits a negative entropy of activation ($\Delta S^{\ddagger}$) of −24.5 ± 5.2 cal mol$^{-1}$ K$^{-1}$, in sharp contrast to the 17.9 ± 8.2 cal mol$^{-1}$ K$^{-1}$ for htADH ($\Delta\Delta S^{\ddagger} = -42.4 \pm 9.7$ eu). These statistically meaningful differences in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are difficult to rationalize by a change in mechanism or active-site geometry since the majority of the residues in the active site are conserved.

Considering that P86 and F147 in htADH are distal from the active site, the changes in kinetic and activation parameters caused by these mutations are unlikely to arise from changes in the electrostatic or solvation environment in the active site, although a small structural perturbation cannot be completely ruled out. On the reasonable assumption that the WT and mutants assume a similar structure at the transition state, the changes in $\Delta S^{\ddagger}$, relative to WT, implicate a change in the degree of disorder (flexibility) at the ground state. The structural basis for an increase in protein flexibility in mutants can be understood by considering that Pro86 is located in the turn between a loop and a $\beta$-strand and is adjacent to Trp 87, one of the residues in direct contact with the substrate. The replacement of the proline residue with the more flexible residue Ala is expected to make the local structure more "floppy". Phe147 is located behind the Pro86, and the mutation F147L is also expected to create a packing defect near the active site that can lead to increased mobility.

The differences in activation parameters for psADH and htADH are especially striking. The change in the sign of $\Delta S^{\ddagger}$ for psADH relative to htADH ($\Delta\Delta S^{\ddagger} = -42.4 \pm 9.7$ eu) points toward significant changes in protein/solvent structure that must accompany H-transfer. Analogous trends in $\Delta\Delta S^{\ddagger}$ have been observed in comparing homologous psychrophilic and mesophilic enzymes, though the impact on $\Delta\Delta S^{\ddagger}$ previously seen is less and has not been correlated with a discrete chemical step.[12] The positive value of $\Delta S^{\ddagger}$ for htADH below 30 °C is consistent with the need for an increase in disorder for efficient catalysis, while the negative value of $\Delta S^{\ddagger}$ for psADH implicates increased structuring at the H-transfer crossing point. Recently, a hydrogen−deuterium exchange (HX) study has shown that a large region of htADH is in a rigid state at temperatures below 30 °C and that the enzyme adopts a more dynamic structure at elevated temperature.[8] In a separate comparative HX study of htADH and psADH, specific regions involved in $NAD^+$ and substrate binding are found to be more flexible in psADH than in htADH at 10 °C, presumably due to a reduced packing density and the existence of packing defects in psADH.[11] Therefore, although it is generally dangerous to draw definitive conclusions based solely on changes in the activation parameters, the HX studies provide support for the interpretation that differences in $\Delta S^{\ddagger}$ between htADH and psADH arise from differences in their degree of ground-state flexibility. This view is also consistent with the observation that psADH has much greater Michaelis−Menten constants ($K_M$) for substrate (see data in Supporting Information).

There is a growing view that protein dynamics may be tightly coupled to the H-transfer coordinate during enzyme catalysis. In the context of the importance of quantum tunneling in htADH and other ADHs,[5,13,14] a realistic model incorporates contributions from two classes of heavy atom coordinates. First, the polar groups in active site must undergo reorientation through thermal fluctuation to generate a transient isoenergetic configuration prior to H-transfer; this is analogous to the solvent reorganization process described by Marcus in the treatment of electron-transfer reactions.[15] Second, because the average equilibrium hydrogen donor−acceptor distance is usually much longer than the H-transfer distance, protein motions are also required to bring the donor and acceptor into close proximity to make effective wave function overlap possible.[4,16] For the same enzyme or a family of structurally similar enzymes, the relative H-transfer rate is determined by the Boltzmann probability ($P$) of simultaneously reaching the transient isoenergetic configuration and the short tunneling distance. Because enzymes are such complex systems, the probability $P$ can, in principle, be controlled by either entropy or enthalpy. The availability of thermodynamic activation parameters for highly homologous enzymes that function within very different temperature regimes allows us to make a distinction in the present case. As shown here, comparison of htADH to psADH or htADH above and below 30 °C indicates $\Delta S^{\ddagger}$ values that undergo significant changes in their signs. We attribute these effects to differences in their ground-state conformational flexibility, that affect the ability of each enzyme to achieve the required heavy-atom configuration for the transfer of hydrogen.

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**Supporting Information Available:** This material is available free of charge via the Internet at http://pubs.acs.org.

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