Protein Flexibility Correlates with Degree of Hydrogen Tunneling in Thermophilic and Mesophilic Alcohol Dehydrogenases

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The physical basis for the enormous catalytic power of enzymes is being actively investigated.[1] Structural studies of enzymes have influenced our view of catalysis enormously, but the three-dimensional pictures they provide generally do not incorporate the wide range of protein dynamical motion.[2] Studies have shown a relationship between protein dynamics and the enzymatic events involved in the formation and breakdown of noncovalent complexes.[3−5] A far greater experimental challenge is to explore whether protein dynamics have a role in the catalysis of bond formation and cleavage. Recent kinetic findings on hydrogen transfer with various enzymes could not be explained without invoking both quantum mechanics and enzyme dynamics, but no direct measurements of dynamics were pursued.[6−8] In other studies, the link between protein dynamics and the chemical step(s) has not been obvious.[3,9−11]

One of these studies (ref 11) examined the activity, stability and protein dynamics of 3-isopropylmalate and D-glyceraldehyde-3-phosphate dehydrogenases from thermophilic and mesophilic organisms (organisms that optimally grow at high temperature and at room temperature, respectively). The findings indicated that the two homologous enzymes have similar flexibility at their respective physiological temperatures, and that the thermophile is more rigid at 25 °C. In this and earlier studies, the comparison of dynamics from hydrogen exchange with activity and melting temperature suggested a correlation between protein flexibility and activity.[11,12] However, the enzyme activity measured in these studies was not directly related to a chemical step during enzymatic turnover.

Our previous studies of alcohol dehydrogenase (ADH) from yeast (a mesophile)[13] and *Bacillus stereothermophilus* (a thermophile denoted ADH-hT)[6] indicated that the hydride transfer step has a significant (and similar) degree of quantum tunneling at each protein's physiological temperatures (25 °C and 65 °C, respectively). Contrary to predictions for tunneling through a rigid barrier, the tunneling with the thermophilic ADH decreases at and below room temperature, resulting in a downward curved Arrhenius plot of $\ln(k_{cat})$ vs reciprocal temperature. The observation of both temperature independent kinetic isotope effects (above 30 °C) and a significant enthalpy of activation (15 kcal/mol) could not be explained by a rigid barrier model.[6,14] Rather, these findings are in accordance with theoretical models of vibrationally enhanced hydrogen tunneling.[14−16] These models invoke vibrational modes of the reaction's environment that increase the tunneling probability along the reaction coordinate. It was suggested that below the transition temperature the thermophilic enzyme had become too rigid to support vibrationally enhanced tunneling.

We now report the results from amide protium/deuterium-exchange (H/D exchange) experiments with both a mesophilic and thermophilic ADH.[11,17] We have compared the global flexibility of the mesophile and the thermophile at their physiological temperatures (25 and 65 °C, respectively), and that of the thermophile at 25 °C (below the observed kinetic transition).[6] H/D amide exchange in D_2O was measured by FTIR spectroscopy.[18] A typical series of absorption spectra collected during the course of a reaction is presented in Figure 1. Absorbancies of the amide I and amide II bands were measured at 1641.8 and 1548.1 cm^{-1}, respectively, and corrected with the baseline absorbance measured at 1726.7 cm^{-1} (Figure 1).

The fraction of the unexchanged peptide's hydrogen atoms was determined as

$$X = \frac{\omega(t) - \omega(\infty)}{\omega(0) - \omega(\infty)} \tag{1}$$

where $\omega(t)$ is the ratio of the amide II and amide I absorbancies (A), corrected with the absorbance of the baseline, at time t:

$$\omega(t) = \frac{A_{\text{amideII}}(t) - A_{\text{base}}(t)}{A_{\text{amideI}}(t) - A_{\text{base}}(t)} \tag{2}$$

$\omega(0)$ is the amide II/amide I ratio of the undeuterated protein and $\omega(\infty)$ is the ratio for the fully deuterated protein. Two independent methods were used to measure $\omega(0)$. First, the undeuterated, lyophilized protein was measured in KBr pellet. Second, the protein was prepared in 50 mM potassium dihydrophosphate, pH 4.2, followed by lyophilization and an exchange experiment at 4 °C. $\omega(t)$ data were then extrapolated to $t = 0$. Both methods result in $\omega(0) = 0.51 \pm 0.04$ for enzymes from yeast and from *B. stearothrmophilus*). $\omega(\infty)$ was determined from samples incubated in D_2O at 37 °C for 14 days with the thermophilic ADH, and for 3 days with the mesophilic ADH. Measurements were carried out after centrifugation and resulted in $\omega(\infty) = 0.10 \pm 0.02$ for both enzymes.

X, the fraction of unexchanged peptide's hydrogens, is a function of time, pH, and temperature. The results were interpreted under the assumption that the protein backbone amide hydrogen

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(18) ASI, Applied Systems ReactIR 1000. CaF_2 windows and a path length of 100 μm were used for both the sample and background measurements. The temperature was thermostated to ±0.1 °C. The thermophile enzyme (ADH-hT) was from the same preparation of enzyme used for the kinetic measurements[6] and was dialyzed in protonated buffer (50 mM potassium phosphate, pH 7, as in ref 6) before lyophilization. Two milligrams of protonated, dry enzyme was dissolved in 100 μL of D_2O to initiate the exchange process. A series of IR spectra (1300−1900 cm^{-1}) at 2 cm^{-1} resolution were recorded starting 3 min after initiation. During the experimental period, 5 to 10% of the specific activity (measured as described previously[6]) was lost.

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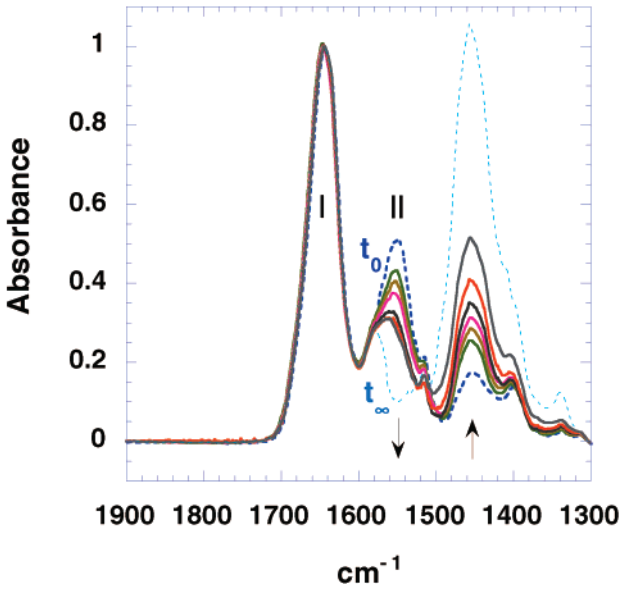

Figure 1. A typical H/D exchange experiment with ADH-hT at 25 °C in the time range of 3 min to 73 h. The amide I band (at 1642 cm^{-1}, mostly a carbonyl stretch) is proportional to the concentration of total amides in solution. The amide II band (at 1548 cm^{-1}, mostly the out of plane N−H bend) shows the decreasing number of amide protons. The broad band at 1450 cm^{-1} reflects the increasing number of N−D and O−D groups and is not useful analytically. The absorbance spectra are normalized to amide I, and the arrows show the direction of changes. Spectra of undeuterated and fully deuterated proteins (dotted lines for t_0 and t_∞, respectively) were measured as described in the text.

bonds form and break faster than the rate of exchange of a free amide (commonly denoted as an EX_2 mechanism).[11,17] The exchange proceeds as a series of simultaneous first-order reactions

$$X = n^{-1} \sum_{i=1}^{n} \exp(-\rho_i k_0 t) \qquad (3)$$

where n is the number of amide hydrogens per protein molecule and ρ is the probability of finding the ith amide exposed to the solvent. k_0 is the exchange rate of an exposed amide proton under the particular conditions of pH and temperature, calculated empirically from

$$k_0 = (10^{-\mathrm{pH}} + 10^{\mathrm{pH}-6}) 10^{0.05(T-25)}\ \mathrm{s}^{-1} \qquad (4)$$

where T is the temperature in °C. This empirical equation, based on a poly-D,L-alanine model, serves as a good approximation when comparing homologous proteins at different temperatures.[11] The parameter of interest with regard to the protein dynamics is ρ which, regardless of the details of the exchange mechanism, is smaller for a more rigid polypeptide. To compare ρ for the two homologous proteins at different temperatures, exchange kinetic data were plotted as X vs log(k_0t), in the form of relaxation spectra (Figure 2).[11,19,20]

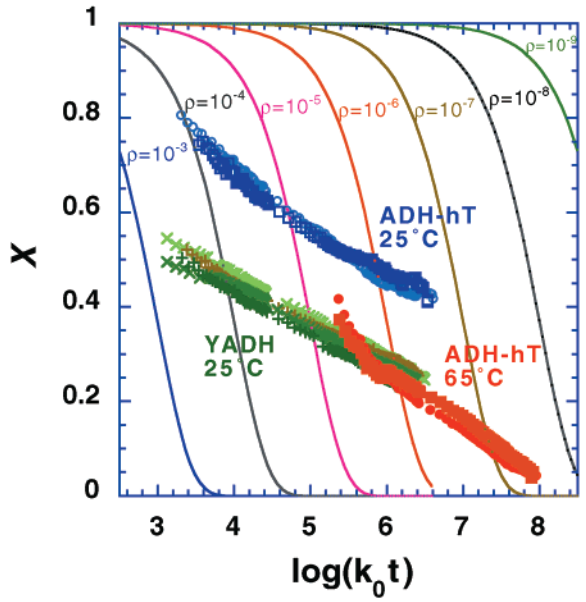

Figure 2. H/D exchange data summarized in the form of relaxation spectra for yeast ADH (green) and ADH-hT (blue) at 25 °C and for ADH-hT at 65 °C (red). X is the fraction of unexchanged peptide hydrogens, t is time in seconds, and k_0 the calculated chemical exchange rate constant. The solid lines represent exchange curves for hypothetical proteins characterized by homogeneous ρ values as indicated in the figure. A shift of the relaxation spectrum toward the right upper corner, i.e., toward smaller ρ values, reflects an increase in the conformational rigidity. Continuous and overlapping spectra reflect similar rigidity.[11]

The relaxation spectra reflect probability distributions. The shift of the relaxation spectrum toward the right upper corner, i.e., toward smaller ρ values, reflects an increase in the conformational rigidity. The upward bend in the plot with ADH-hT at 65 °C during the first 5 min of the experiment is attributed to a temperature equilibration delay.

The curves recorded at the respective optimum temperatures almost coincide and are similar in shape and position, reflecting similar distributions of the conformational fluctuations. As for 3-isopropylmalate dehydrogenase,[11] this observation supports the suggestion that homologous mesophiles and thermophiles have evolved to induce similar dynamic environment at their active sites at very different temperatures. The main observation from this set of experiments is that the thermophilic ADH is significantly more rigid at 25 °C than either itself or the yeast ADH at each protein's physiological temperature.

The results reported herein, taken together with the similar nature of hydrogen transfer at the enzymes' optimum temperatures and the reduced tunneling for the thermophile at 25 °C,[6,13] constitute experimental support for the idea that enzyme dynamical motion has evolved to enhance catalysis of a chemical transformation.

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