Effect of Active Site Mutation Phe 93 → Trp in the Horse Liver Alcohol Dehydrogenase Enzyme on Catalysis: A Molecular Dynamics Study

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We have studied the effect of the site-directed mutation, Phe 93 → Trp, in the horse liver alcohol dehydrogenase enzyme as it relates to a specific protein motion that plays a crucial role in catalysis. Results obtained from the study suggest that the protein dynamics as it couples to reaction catalysis is not affected and in fact the coupling between the protein oscillation and the reaction coordinate is similar to that seen in the wild-type. This supports the view that this mutation distal from the NAD^+ cofactor does not change the way protein dynamics influences chemistry.

Enzyme-catalyzed reactions are ubiquitous in biological chemistry. Understanding the mechanism of enzyme action continues to be an active field of research. There have been a number of important mechanisms proposed over the years.[1−3] For instance, nearly five decades ago, the transition state stabilization concept was introduced by Pauling.[4] According to this concept, when the reactants bind to an enzyme, they are postulated to be at or near the top of the uncatalyzed reaction barrier. This selective binding releases energy that stabilizes the transition state and hence lowers the barrier to the reaction. A second mechanism involves ground state destabilization.[5] Accordingly, the enzyme plays the role of making the reactants less stable rather than stabilizing the transition state. Therefore, the energy required to climb the barrier is reduced. A third mechanism suggests lowering of the dielectric environment due to exclusion of water as enzyme binds to the substrates; this alters the energetics and makes it conducive for the reaction.[6−8] In a fourth mechanism proposed by Bruice and co-workers,[9,10] the enzyme positions the substrates in certain conformations, known as the near attack conformations, such that the thermal fluctuations easily take the reactants over the barrier. While all of these mechanisms consider a static view of the enzyme catalysis, more recently, Schwartz and co-workers[11−16] proposed a dynamic view. According to this view, when the reaction coordinate is symmetrically coupled to the protein oscillations (vibrations), the reaction rate is enhanced. Symmetric coupling reduces the distance between donor and acceptor and mathematically arises when a symmetric function can model the coupling in the Hamiltonian. These protein vibrations are called the promoting vibrations.

There have been a number of experimental studies that support this dynamic view. For instance, Petsko et al.[17] have studied hydrogen/deuterium exchange experiments on thermophilic and mesophilic enzymes that have the same catalytic target. They found that the thermophile is significantly less flexible than the mesophile at room temperature; in fact, the thermophile is hardly active at room temperature. Because active sites of both enzymes are quite similar, it was conjectured that conformational flexibility has a significant effect on the enzyme function. Other evidence correlating protein flexibility with catalysis comes from the tunneling rate studies carried out by Klinman and co-workers.[18] The authors have studied the alcohol dehydrogenase (ADH) from *Bacillus stearothermophilus*, a thermophilic protein (ADH-hT) that normally functions at 65 °C, and found that hydrogen tunneling makes a significant contribution at this temperature; this result was analogous to previous findings[19] with mesophilic ADH at 25 °C. What is interesting is that the tunneling with ADH-hT decreases at and below room temperature, contrary to predictions for tunneling through a rigid barrier. Data at 30−60 °C seem to show that tunneling increases with temperature and that the kinetic isotope effect (KIE) is small and temperature-independent. Furthermore, at 5−30 °C, the KIE is larger and exhibits a clear temperature dependence. This change in activity of ADH-hT appears to support the view that internal protein oscillations coupled to the reaction coordinate facilitate tunneling by changing the geometry of the barrier and thus the tunneling distance. At reduced temperatures, the amplitude or coupling strength of these rate-promoting vibrations decreases below a level required for effecting tunneling, because the enzyme becomes conformationally more rigid; therefore, the reaction begins to exhibit more classical behavior. In addition, Kohen and Klinman have found in H/D exchange studies that ADH-hT is in fact more rigid.[20]

In addition, Goldstein, Klinman, and co-workers[21,22] have studied two specific mutations of horse liver alcohol dehydrogenase (HLADH) enzyme, Val203 → Ala and Phe93 → Trp, and these mutations have been shown to affect the enzyme kinetics significantly. The HLADH enzyme catalyzes the oxidation of alcohol to aldehyde, and the cofactor, nicotinamide adenine dinucleotide (NAD^+), acts as the hydride acceptor during the oxidation. Both Val203 and Phe93 residues are located at the active site. A methyl group of the Val203 residue is within van der Waals distance to the cofactor. This residue plays an important role by pushing the NAD^+ closer to the alcohol. However, when this residue is replaced by a smaller Ala residue, the catalytic efficiency of the enzyme is lowered significantly and the indicators of tunneling are also lowered significantly. Replacement of Phe93, which is located in the alcohol binding pocket, with the larger Trp kinetically unmasks

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the indicators of tunneling. Klinman and co-workers hypothesize that the kinetic complexity is reduced because of mutation. In fact, another interesting comparison would be to the double mutant F93W-V203A. In addition, there is evidence from a molecular dynamics (MD) study[23] on HLADH that Val203 forces the nicotinamide ring of NAD^+ into closer proximity to the alcohol, thereby facilitating hydride transfer to produce the corresponding aldehyde. Further support for the role of Val203 was provided by a recent theoretical paper of Hammes-Schiffer et al.[24] We mention, however, that Gao, Truhlar, and co-workers[25] found only small amplitude motion of the residue and thus questioned its importance in the mechanism.

Recently, Caratzoulas and Schwartz[14] developed a computational method to identify the presence of a promoting vibration. They have shown that the friction kernel of the GLE bears a signature of the promoting oscillator. In a subsequent paper, Caratzoulas et al.[15] demonstrated the application of this method for the HLADH enzyme. They have identified a low frequency ($\approx$125 cm^{-1}) vibration between donor and acceptor carbon atoms, which acts as the promoting vibration. In addition, they have also studied the mutation Val203 $\rightarrow$ Ala and found that the spectral density, a measure of the coupling between the reaction coordinate and the promoting oscillator, to be a factor of 2 less than in the wild-type enzyme.[16]

As we mentioned earlier, the Phe93 residue is located in the alcohol binding region. When it is replaced by a larger Trp residue, Klinman and co-workers[21,22] observed a decrease in the size of the alcohol binding pocket and a decrease in the kinetic complexity. In this work, we address whether the coupling between the reaction coordinate and the promoting vibration is affected by this mutation. In the next section, we briefly describe the theory and computational method used and discuss the results obtained from the present work.

The theory applied to the present work has been well-documented in the literature.[14] Therefore, we describe only the essential steps here. Consider a reaction coordinate s, which is symmetrically coupled to an oscillator through a term Cs^2Q, where C is the coupling strength and both the reaction coordinate and the oscillator are bilinearly coupled to the harmonic bath; then, it was shown that the reaction coordinate follows generalized Langevin dynamics as described by

$$m\ddot{s}(t) = -\frac{\partial V(s)}{\partial s} - \int_0^t \gamma_s(t-t')\,\dot{s}(t')\,dt' + F(t) \qquad (1)$$

where $F(t)$ is the random force due to bath. $\gamma_s(t)$ is the memory friction kernel that quantitatively describes the forces acting on the reaction coordinate. Caratzoulas and Schwartz[14] showed that the $\gamma_s(t)$ exhibits an explicit spatial dependence as described by

$$\gamma_s(t-t') = \gamma_{bath}(t-t') + \frac{4C^2}{M\Omega^2}s(t)e^{-\zeta(t-t')/2} \times \left[\cos\tilde{\Omega}(t-t') + \frac{\zeta}{2\tilde{\Omega}}\sin\tilde{\Omega}(t-t')\right]s(t') \quad (2)$$

where $\gamma_{bath}(t)$ is the memory friction solely due to the interaction of reaction coordinate with bath degrees of freedom, Ω is the bare frequency of oscillator Q (the promoting vibration), $\tilde{\Omega} = \sqrt{\Omega^2 - \zeta^2/4}$ is the effective frequency of the oscillator, and ζ is the friction parameter due to coupling of Q to the thermal bath. From $\gamma_s(t)$, one obtains the spectral density, $J_s(\omega)$, which is a measure of the rate of energy transfer to and from the environment and Q. $J_s(\omega)$ is computed by taking the Fourier transform of $\gamma_s(t)$. Because $\gamma_s(t)$ contains a cosine, the Fourier transform will result in a δ-function or a strong peak at the effective frequency $\tilde{\Omega}$. Thus, $J_s(\omega)$ acts as the diagnostic tool to identify the promoting vibration. One typically computes $\gamma_s(t)$ from the force autocorrelation function, $C_F(t)$; these two quantities are related through the second fluctuation−dissipation theorem.[26,27] However, Caratzoulas et al.[15] pointed out that the high frequency oscillations in the protein tend to swamp out strongly coupled low-frequency oscillations when we compute $\gamma_s(t)$ this way. Therefore, they suggested instead to compute $\gamma_s(t)$ from the velocity autocorrelation function. This way, the spurious peaks at very high frequencies are deemphasized.

To carry out the MD simulation, we used the X-ray crystal structure reported by Klinman and co-workers[22] (Brookhaven PDB ID: 1AXE). The 2 Å resolution crystal structure contains HLADH dimer with Phe93 residue in both subunits replaced by Trp residue, and the enzyme is complexed with NAD^+, trifluoroethanol, and Zn. The PDB file was modified by substituting the reactive substrate, benzyl alcohol (in order to compare directly to our previous work on this substrate), for the inhibitor trifluoroethanol. This was done by first importing the benzyl alcohol structure and then superimposing it onto the inhibitor molecule using the Biopolymer module in Insight2000 (MSI, San Diego, CA). While making this change, we ensured that the geometry of the methylene group of the alcohol was not affected. CHARMM[28] (MSI, San Diego, CA) MD simulations were run on a Silicon Graphics work station. Both subunits of the enzyme (with NAD^+, substrate, and crystallographic waters) have been included in the MD simulations. Hydrogens, polar and nonpolar, were added to all of the molecules prior to the simulation. The crystallographic waters were treated as TIP3P residues.[29] Following earlier studies,[15,30] we maintained the geometry of Zn(II) and His67, Cys46 and Cys174 ligands using a bonded approach and the Zn(II)−substrate ligand interaction was treated solely as a nonbonded interaction. In the present work, we present results obtained in the reactant configuration. The benzyl alcohol exists in the deprotonated form as $PhCH_2O^-$.[31] The partial charges on each atom have been set according to the templates in the CHARMM force field.

All assemblies were minimized prior to the dynamics runs. Initially, the minimization was done for 1000 steps using steepest descent with a force criterion of 0.001 kcal per steps and later for 8000 steps using adapted-basis Newton−Raphson with tolerance 1×10^{-9} per 10 steps. The cutoff for nonbonding interactions was set to 14 Å. The equations of motion were solved using Verlet integration with a time step 1 fs. The following dynamics protocol was used, heating for 2 ps during which the temperature was raised to 300 K at a rate of 3 K every 20 integration steps; equilibration for 10 ps; and, finally, 30 ps of observation during which coordinates and velocities were collected for every five steps, resulting in 6000 frames of structures. All bonds involving hydrogen atoms were constrained using the SHAKE algorithm.[32] Time series and correlation functions were generated from the stored structures. The spectral density plots reported here have been smoothed using the Savitzky−Golay method.[33]

As we mentioned earlier, one of the methyl groups in the Val203 is within van der Waals distance to the NAD^+. Earlier studies on the wild-type[22,15] have shown that this residue pushes the nicotinamide ring of NAD^+ closer to the hydride donor benzyl alcohol. Thus, the donor and acceptor carbon atoms vibrate in synchronization with the acceptor and Val203 carbon atoms. One way to find out whether such a concerted process is present in the Trp mutant is to see whether any similarity

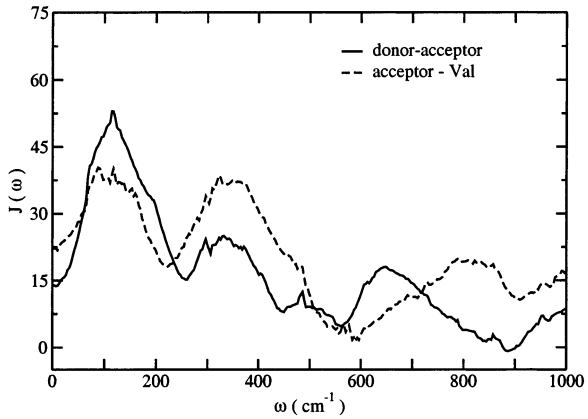

Figure 1. Spectral densities corresponding to the donor and the acceptor carbon atom relative velocities projected onto donor−acceptor direction (solid line) and the acceptor carbon and the C_γ carbon atom relative velocities projected onto donor−acceptor direction (dashed line).

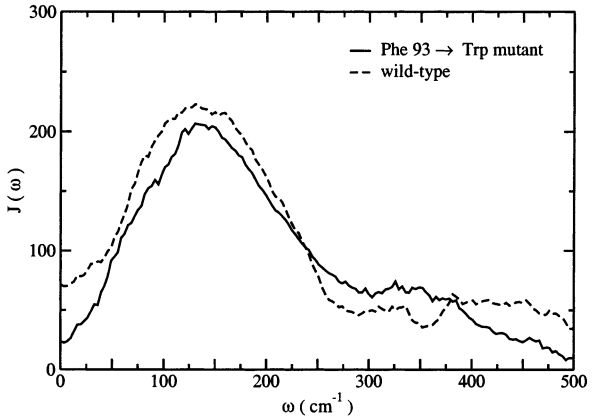

Figure 2. Spectral density corresponding to the reaction coordinate, Trp mutant (solid line) and wild-type (dashed line).

exists between the relative motions of donor and acceptor carbon atoms and the acceptor carbon atom and the C_γ carbon atom of Val203 residue.

In Figure 1, we have shown the spectral densities corresponding to the donor and acceptor carbon atoms relative velocities projected onto the donor−acceptor axis, $J_{DA}(\omega)$ (solid line), and acceptor carbon and C_γ carbon atoms relative velocities projected onto the donor−acceptor axis, $J_{AV}(\omega)$ (dashed line). There is a strong similarity between these atom movements. In accordance with Caratzoulas et al.[15] studies on the wild-type enzyme, we notice a clear low-frequency-promoting vibration peak at ≈125 cm^{-1}. Furthermore, the amplitudes of these peaks are also in agreement with their study. This clearly suggests that the dynamics at the active site region is not affected by the Phe93 → Trp mutation.

The promoting vibration that we analyze is the vibration between donor and acceptor carbon atoms. We show the spectral density of the reaction coordinate, $J_s(\omega)$, in Figure 2. The low-frequency peak at ≈125 cm^{-1} that we observed in $J_{DA}(\omega)$ (Figure 1) is present in $J_s(\omega)$, a clear indication of the existence of coupling between the reaction coordinate and the donor−acceptor relative motion (vibration). Furthermore, the amplitude of the spectral densities for the Trp mutant agrees well with the wild-type (shown in dashed line in Figure 2). This shows that the strength of the coupling between the reaction coordinate and the promoting vibration is not affected by the mutation.

In conclusion, we have demonstrated, using an MD simulation, that the site-directed mutation of the Phe93 residue by a larger Trp residue in HLADH enzyme does not alter the protein dynamics that is crucial for the catalysis.

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