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Identification of a Protein-Promoting Vibration in the Reaction Catalyzed by Horse Liver Alcohol Dehydrogenase

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Abstract: In this article we present computational studies of horse liver alcohol dehydrogenase (HLADH). The computations identify a rate-promoting vibration that is symmetrically coupled to the reaction coordinate. In HLADH a bulky amino acid (Val203) is positioned at the face of the nicotinamide adenine dinucleotide (NAD^+) cofactor distal to alcohol substrate to restrict the separation of reactants and control the stereochemistry. Molecular dynamics simulations were performed on the dimeric HLADH, including the NAD cofactor, the substrate, and the crystallographic waters, for three different configurations, reactants, products, and transition state. From the spectral density for the substrate-NAD relative motion, and that for the NAD-Val203 relative motion, we find that the two motions are in resonance. By computing the associated spectrum, we find that the reaction coordinate is coupled with the substrate-NAD motion, and from the fact that the coupling vanishes at or near the transition state (demonstrated by the disappearance of strong features in the spectral density), we conclude that the substrate-NAD motion plays the role of a promoting vibration symmetrically coupled to the reaction coordinate.

Introduction

Despite many decades of intense study, aspects of enzyme catalysis are not well understood, and the exact physical mechanism by which enzymes effect catalysis is still a topic for vigorous dialogue.[1−3] The transition state binding concept introduced by Pauling[4] is still the widely accepted approach used to explain the catalytic efficiency of enzymes. This is a static view of enzymatic catalysis according to which, as a chemical substance is being transformed from reactants to products, the species that binds most strongly to the enzyme is, at some intermediate point, postulated to be at or near the top of the solution phase (i.e., uncatalyzed) barrier to reaction. This preferential binding releases energy that stabilizes the transition state and thus lowers the barrier to reaction. A second approach involves ground-state destabilization. In this picture,[5] the role of the enzyme is to make the reactants less stable rather than stabilize the transition state. Thereby the required energy for thermal activation is lowered. Recent calculations[6] suggest that this model may well be dominant for one of the most efficient enzymes known, orotidine monophosphate decarboxylase.

There is another possibility for the action of enzymes in certain reactions: motions within the enzyme itself actually speed the rate of a chemical reaction. These are thermal motions of atoms in the enzyme that are not directly involved in substrate binding and stabilization of the Michaelis complex. There is experimental support[7−12] for such a dynamic view of the catalytic process in certain enzymes. In spirit, there is some relation between this view and that on the formation of near attack conformations (NACs).[3,13,14] It must be stressed, however, that arguments pertaining to the NACs are of chemical/structural nature, whereas the notion of the existence of rate-promoting motions (vibrations) is a dynamic one, since it entails coupling of actual protein motions to a reaction coordinate.

Recent molecular dynamics simulations undertaken by Radkiewicz and Brooks[15] have explored potential links between catalysis and dynamics in the dihydrofolate reductase (DHFR) catalytic pathway. Calculations on three ternary complexes for the DHFR catalytic cycle and comparison of the dynamics of the protein in the different complexes demonstrated that the ligands affected the behavior of the protein even though the ligands only differed by one or two hydrogen atoms. Strong-

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coupled motions that appeared in the reactive complex disappeared in the product complexes, indicating a possible link of these motions to the catalytic process. The same studies also revealed that mutations that had been observed to be damaging to certain chemical steps in catalysis occurred with high correlation in those regions of the protein structure that their simulations had indicated to participate in highly coupled motions.

It has also been previously suggested that such coupling can help account for signatures of hydrogen tunneling in enzymatic reactions.[16] These measurements differ quite significantly from those that are observed in simple one-dimensional reaction coordinates for tunneling reactions in a condensed phase. The characteristic of reactions in which specific vibrations couple strongly to a reaction coordinate is moderate kinetic isotope effects, as opposed to the large one that would be expected for a one-dimensional reaction in the condensed phase. It has been demonstrated[17,18] how such behavior can be ascribed to the *symmetry* of the coupling, which changes the barrier geometry, that is, the barrier height and the transfer distance for the tunneling particle. This concept has also been used to explain hydride transfer kinetic isotope effects in methylamine dehydrogenase.[19]

As mentioned, experimental evidence for the involvement of dynamic protein modes in catalysis is beginning to grow. Petsko and co-workers have carried out studies on thermophilic and mesophilic enzymes that have the same catalytic target.[12] One system studied is 3-isopropylmalate dehydrogenase from the thermophilic bacteria *Thermus thermophilus*. Hydrogen/deuterium exchange experiments showed it to be significantly less flexible than the mesophile at room temperature; in fact, the thermophile is hardly active at room temperature. Because the active sites of the two enzymes are quite similar, it was conjectured that conformational flexibility has a significant effect on enzyme function in this case.

More experimental evidence correlating protein flexibility, and thus dynamics, with tunneling rates has recently been provided by Klinman and co-workers[10] and their studies on hydrogen transfer under physiological conditions. In particular, they have studied the alcohol dehydrogenase from *Bacillus stearothermophilus*, a thermophilic protein (ADH-hT) that normally functions at 65 °C, and found that hydrogen tunneling makes significant contribution at this temperature; this result was analogous to previous findings[20] 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 activity transition for 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 effective tunneling, because the enzyme becomes conformationally more rigid, and thus the reaction begins to exhibit more classical behavior. Additionally, two specific mutations of horse liver alcohol dehydrogenase (HLADH) have been identified, Val203 → Ala and Phe93 → Trp, which significantly affect enzyme kinetics. Both residues are located at the active site. Val203 impinges directly on the face of the nicotinamide ring in the nicotinamide adenine dinucleotide (NAD) cofactor distal to the alcohol substrate. Replacement of this residue with the smaller Ala significantly lowers the catalytic efficiency of the enzyme, as compared to the wild type, and also significantly lowers indicators of hydrogen tunneling.[21,22] Replacement of Phe93, which is in the alcohol binding pocket, with the larger Trp makes it harder for the substrate to bind, but does *not* lower the indicators of tunneling.[23] Additionally, there is evidence from molecular dynamics simulations[24] on HLADH that Val203 forces the nicotinamide ring of NAD^+ into closer proximity to the substrate, thus facilitating the hydride transfer to produce the corresponding aldehyde. The above suggests that the internal motion of the residue is central to the rate-promoting vibration.

The concept that relative motion of reactants in a chemical reaction may significantly affect the dynamics of reaction is not new. For example, in gas-phase dynamics, Benderskii and co-workers[25−29] have shown that tunneling reaction rates can be significantly enhanced if the reaction coordinate is *symmetrically* coupled to a harmonic oscillator. Similar effects have been observed in reactions in model condensed phase studies, as in the Hamiltonians considered by Borgis and Hynes[17,30] and Suarez and Silbey.[31] By generalizing that work, Schwartz and co-workers recently developed a condensed phase model whereby they were able to show the significant contribution to tunneling from excited states,[18,32] and also to reproduce[33] the trends seen in the foregoing experiments reported by Klinman and co-workers. The common feature of all these models is again the issue of the symmetry of coupling. A rate-promoting vibration may be physically thought of as one which periodically narrows (and lowers) the height of a barrier to reaction. In contradistinction, an antisymmetrically coupled vibration does not affect the width of the barrier, but rather the position of the minima at the well bottoms. It is for this reason that antisymmetric couplings are central to the Marcus theory,[34] in which a rate is maximized when well bottoms are at the same energy. These two possibilities for the symmetry of coupling are purely classical dynamics effects − they are seen as large scale motions of atoms in a molecule. As we have described previously, the

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physical effects of symmetric or antisymmetric coupling have corresponding mathematical manifestations in the way we describe the motions of atoms during chemical reactions. Thus, in the previously cited study, Antoniu and Schwartz[18,32] assumed that the reaction coordinate, *s*, which is coupled to a thermal bath with known spectral density, is also coupled symmetrically to a harmonic vibration via a term of the general form Cs^2Q. *Q* is the harmonic vibration of frequency Ω and mass *M*, and *C* is a coupling constant. For example, in a reaction involving atom transfer, *Q* typically describes an oscillatory motion which modulates the distance between the donor−acceptor pair of atoms. Just as a symmetric coupling can be modeled in a mathematical formalism, the classical mechanics of atoms and molecules can provide a unique diagnostic of a motion coupled to a reaction coordinate and which, during oscillation, periodically reduces the separation of the reacting partners. The frequency of all oscillations convolved with the strength of coupling of each oscillation to the reaction coordinate is commonly referred to as the spectral density. We have recently demonstrated[35] that the spectral density of a reaction coordinate coupled to an infinity of oscillators will demonstrate the existence of one or more symmetrically coupled motions (rate-promoting vibrations) in the following manner: *a peak, at the effective frequency of the promoting vibration, whose intensity increases with the reaction coordinate and vanishes at the point of minimal coupling* − typically at the top of the barrier to the reaction. The minimum coupling need not occur exactly at this point, but it is not unreasonable that it does. We shall have the opportunity to return to this point in the following section, where we shall be reviewing the theoretical framework of our methodology.

In this paper, we apply this methodology to the hydride transfer in the oxidation reaction of $PhCH_2OH$ catalyzed by HLADH and present simulation results, for the wild type and the Val203 → Ala mutant, that strongly support the view that in this enzymatic reaction there are protein modes that couple, symmetrically, to the reaction coordinate. HLADH is a well-characterized enzyme which catalyzes reversible NAD^+-dependent oxidation of a wide variety of alcohols to corresponding aldehydes.[36] The enzyme has a molecular weight of 80 kDa and is a dimer of two identical units.[37] Each subunit binds one nicotinamide coenzyme and two Zn(II) ions. One Zn is in the active site and is associated with the hydroxyl oxygen of the alcohol substrate; the other zinc is structural. Dissociation of the alcohol hydroxyl proton is the first step of the oxidation of the alcohol by NAD^+. It has been suggested that this proton is passed to water by way of a hydrogen-bond network terminating with the imidazole of His51.[38] The second step of the reaction involves[23,39] hydride transfer from the alcoxide to NAD^+ and is the step on which we shall focus.

Methods

Theoretical Background. We shall briefly review the theoretical basis of our methodology. The reader is referred to ref 35 for a more detailed analysis.

The method is based on Zwanzig's formulation of the microscopic theory of activated transfer in condensed phases.[40] We extended the Zwanzig Hamiltonian to include the oscillator *Q*, which we symmetrically coupled to *s* via a term of the general form Cs^2Q so that there would be no contribution to the reorganization energy of the environment − it is only the shape of the potential barrier that is affected. The oscillator *Q* was allowed to interact with the harmonic bath. We then showed that the reaction coordinate *s* follows Generalized Langevin dynamics as described by the stochastic differential equation

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

According to this description, at any given moment in time the reaction coordinate is acted upon by a random force $F(t)$ due to the environment. How soon the reaction coordinate is losing memory of its history, that is, of the effect of the forces it has experienced at earlier times, is determined by the extent to which the random forces, at various instances of time, are correlated to one another; namely, it is determined by the force autocorrelation function, $C_F(t)$. $C_F(t)$ is defined as the time average of the product $F(t + t')F(t')$ over a long period of time.[41] Because of the interaction with the environment, *s* experiences friction, which, quantitatively, is described by the so-called memory friction kernel $\gamma_s(t)$. The latter is obtained from $C_F(t)$ via the second fluctuation−dissipation theorem,[40,42] which states that $k_BT\gamma_s(t) = C_F(t)$, where k_B is the Boltzmann constant, and *T* is temperature.

We showed that for a reaction coordinate in an environment of infinitely many antisymmetrically coupled vibrations (we call them the bath), and a single symmetrically coupled vibration (promoting vibration), $\gamma_s(t)$ exhibits explicit spatial dependence, as depicted in the following expression:

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

In eq 2, $\gamma_{bath}(t)$ is the memory friction due to the interaction of the reaction coordinate with the bath degrees of freedom alone; $\tilde{\Omega} = \sqrt{\tilde{\Omega}^2 - \zeta^2/4}$ is the effective frequency of the *Q* oscillator, and ζ is a friction parameter due to the 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 at a certain environmental frequency, ω, and is also the diagnostic of a promoting vibration. $J_s(\omega)$ is computed by taking the cosine transform of $\gamma_s(t)$, and for those familiar with such mathematical operations, the cosine transform of the term due to the promoting vibration − which itself contains a cosine − will result in a δ-function, or a strong peak at the effective frequency $\tilde{\Omega}$. The spatial dependence (i.e., dependence on where the particle is along the reaction coordinate) is in this model only caused by the promoting vibration, and this spatial dependence will naturally carry through to the spectral density as well.

The reaction coordinate experiences forces from the thermal bath − here, the thermal motions in the protein − and a force proportional to *sQ* due to the coupling to *Q*. Thus, for *fixed s*, $J_s(\omega)$ will be the superposition of two spectra: one due to the bath, $J_s^{(bath)}(\omega)$, and another due to *Q*, $J_s^{(Q)}(\omega)$. If *Q* is a harmonic mode, as in the model employed by Antoniu and Schwartz,[18,32] $J_s^{(Q)}$ should consist of a sharp peak at $\omega = \tilde{\Omega}$. More generally, *Q* will undergo quasi-periodic motion, due to anharmonicities in its bare potential and due to its coupling to the thermal bath. Thereby, $J_s^{(Q)}$ should consist of a series of peaks at the various frequencies in which the *Q* motion may be analyzed. The symmetry of the coupling is manifest when *s* is moved. There will be

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a position at which the force on s due to Q will vanish or become minimal, typically at the top of the barrier to the reaction. As already mentioned in the Introduction, the minimum coupling need not occur exactly at this point, but it is not unreasonable that it does. If we imagine fixing a particle anywhere on a potential barrier and then periodically modulate the *width* of the barrier, the only place in which this modulation will not result in a force on s is at the *top* of the barrier. When that occurs, the $J_s^{(Q)}$ contribution to J_s will also vanish.

In contemplating such computations on a protein, one final augmentation is needed to our previously published theory. In a reaction taking place in a simple liquid, there are no high-frequency collective vibrations which effect the reaction. In a bonded system such as a protein, there are many such high-frequency modes. Recall that the spectral density is the distribution of the frequencies of oscillation convolved with the strength of coupling to the reaction coordinate. The difficulty with the protein is that even when high-frequency motions ($2000-3000$ cm^{-1}) are hardly coupled to reaction at all, their huge frequency, not present in a simple liquid, swamps out strongly coupled low frequency motions. For this reason, we actually study the spectral density obtained from the transform of the velocity autocorrelation function rather than the force. It can be proved that the two spectral densities are related by a factor of the square of the frequency of the motion − the velocity spectrum $G(\omega) \propto J(\omega)/\omega^2$. Thus, in the two spectra, the peaks occur at the same positions, but the spurious peaks at the very high frequencies are de-emphasized in the velocity spectrum.

Molecular Dynamics Simulations. For our studies, we used the X-ray structure for dimeric HLADH complexed with NAD, pentafluorobenzyl alcohol, and Zn, at 2.1 Å resolution, solved by Ramaswamy et al.[37] (Brookhaven PDB ID: 1HLD). The PDB files were modified by substituting the reactive substrate, benzyl alcohol, for the inhibitor pentafluorobenzyl alcohol. The replacement was carried out by superimposing the substrate onto the inhibitor molecule using the Biopolymer module in Insight2000 (MSI, San Diego, CA). CHARMM[43] (MSI, San Diego, CA) molecular dynamics 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 the molecules in the assembly prior to the dynamic studies. The crystallographic waters were treated as TIP3P residues.[44]

It has been pointed out by Olson et al.[45] that a key feature to be maintained in the MD calculations is the geometry of Zn(II) and His67, Cys46, and Cys174 ligands; thus, we have adopted the so-called "bonded approach" of ligand atoms to the zinc, except for the substrate ligands, which were allowed to interact solely via nonbonded potentials. Bond and dihedral angle parameters for the residues His67, Cys46, and Cys174 bonded to Zn(II) at the active site were adopted from Olson et al.[45] and are based on the parametrization of the zinc ion in HLADH by Ryde.[46] This parametrization was based on ab initio calculations of model zinc complexes.[47]

For the wild type, we shall present results from simulations for three configurations: reactants (R), products (P), and minimal coupling (MC). The R configuration consists of NAD^+ and the deprotonated benzyl alcohol ($PhCH_2O^-$). The P arrangement consists of NADH and benzaldehyde. It was prepared by carrying out the appropriate bond changes in the R configuration. In addition, the *pro*-R hydrogen in the alcohol was removed and attached to the C4 carbon of the nicotinamide ring in the *axial* position facing the alcohol.[48,49] In the third configuration MC, we have NAD^+ and $PhCH_2O^-$ with the *pro*-R hydrogen restrained so that it is equidistant from the hydroxyl α-carbon (hydride donor) and the C4 carbon (hydride acceptor) in the nicotinamide ring. For the Val203 → Ala mutant, we present results for the reactants configuration, prepared in the same way as the configuration R for the wild type.

The partial charges $q_i^{(R)}$ and $q_i^{(P)}$ for each atom i in the reactant and product states have been set according to the templates in the CHARMm force field. In preparing the MC configuration, we were faced with the difficulty of assigning potential parameters, since classical potentials, and the CHARMm force field in particular, just cannot break bonds. Nevertheless, it should be pointed out that discovery of a promoting vibration is only relatively weakly dependent on the details of the potential along the reaction coordinate. The vibration exerts its force on the reacting atoms, and, while changes in the potential do make a difference in the effect of the vibration, it does not cause the nature of the promoting vibration to appear or disappear. The electrostatic interactions must vary as the hydrogen atom is transferred, and thereby the charges q_i must also vary continuously. It is not a trivial matter to assign partial charges along the reaction coordinate since bonds start to break and form. To achieve this we have followed the current literature and adopted the switching function[50]

$$q_i = 0.5[q_i^{(R)}(1 - \tanh(r/a)) + q_i^{(P)}(1 + \tanh(r/a))] \qquad (3)$$

where r is the position of the hydrogen atom relative to a specified point along the donor−acceptor axis (typically the midpoint), and a is a flexible parameter chosen to properly mimic charges and bond lengths in the R and P states. Following Hurley and Hammes-Schiffer, we have used a value of $a = 0.1$ Å;[51] in MC, the above formula reduces to $q_i = 0.5(q_i^{(R)} + q_i^{(P)})$. We have varied the charge parameters at the point of minimal coupling by a significant amount (up to 30%) around the values determined by eq 3, and, in support of the statement made above, the form of the spectral density was relatively insensitive to this change of parameters − there were only minor quantitative but not qualitative changes in the shape and structure of the computed spectra.

Because the reaction involves atom transfer, we define the reaction coordinate as follows:

$$s = -\left(\frac{m_D}{m_D + m_A}\boldsymbol{R}_D + \frac{m_A}{m_D + m_A}\boldsymbol{R}_A - \boldsymbol{R}_H\right)\cdot\hat{\mathbf{r}}_{DA} \qquad (4)$$

In eq 4, $\boldsymbol{R}_i$ is the position vector of the ith atom: i = D labels the hydride donor, i = A labels the hydride acceptor, and i = H denotes the transferred hydrogen atom; $\hat{r}_{DA}$ is the unit vector in the direction of the donor−acceptor pair. In essence, s follows the motion of the transferring hydrogen relative to the center of mass of the donor−acceptor pair.

All assemblies were minimized prior to the dynamics runs to make sure that there were no overlapping atoms. First, we minimized for 1000 steps using steepest descent with a force criterion of 0.001 kcal per 10 steps and then for 8000 steps using adopted-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 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 we collected coordinates and velocities every five steps, thus creating 6000 structures. During the dynamics, all bonds involving hydrogen atoms were constrained using SHAKE.[52]

Results

As stated earlier, for the wild type we have performed three simulations, each representing a different configuration, reactants

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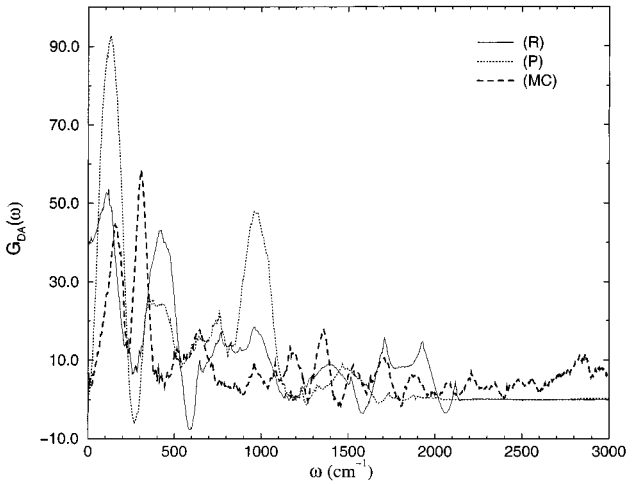

Figure 1. The spectral density $G_{DA}(\omega)$ for the donor−acceptor relative motion in the wild type; it monitors the relative motion between the benzyl alcohol and the nicotinamide ring in NAD. The solid line represents the reactants configuration (R), the dotted line represents the products configuration (P), and the dashed line represents the minimal coupling configuration (MC). The power spectrum is reported in CHARMM units.

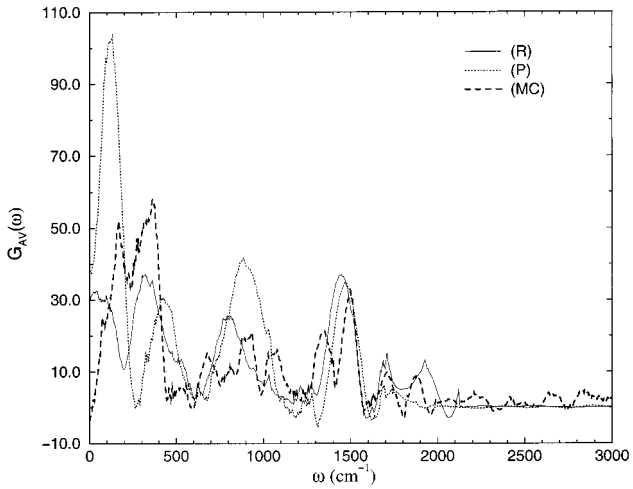

Figure 2. The spectral density $G_{AV}(\omega)$ for the relative motion between the acceptor C atom and the Val203 C_γ atom closest to the nicotinamide ring in NAD, in the wild type. The solid line represents the reactants configuration (R), the dotted line represents the products configuration (P), and the dashed line represents the minimal coupling configuration (MC). The power spectrum is reported in CHARMM units.

(R), products (P), and minimal coupling (MC). Regarding the MC configuration, and as we mentioned earlier, it is likely, on physical grounds, that the point of minimal coupling is in fact the transition state for hydride transfer, but in no way is our methodology and analysis in a position to prove that, and it would be incorrect to imply this. In fact, the point of minimal coupling has been located by numerical experimentation. From a number of configurations that we considered, with the *pro*-R hydrogen restrained at various positions between the donor and acceptor atoms, the MC configuration that was described in the previous paragraph was the one in which the coupling between the reaction coordinate and the promoting vibration was found to be minimal.

Time series and correlation functions for the various observables were generated from the stored structures. All the spectra that we report here have been smoothed using the Savitzky−Golay method.[53]

In HLADH a bulky amino acid (Val203) is positioned at the face of NAD^+ distal to the alcohol substrate. Site-directed mutagenesis (Val203 → Ala) studies[21,22] and numerical simulations[24] suggest that the role of Val203 is not limited to controlling the stereochemistry and restricting the separation of reactants. It appears that the residue's side-chain impinging motion on the face of the nicotinamide ring in NAD induces a promoting vibration, forcing the acceptor carbon into closer proximity to the alcohol. So, in addition to *s*, we have monitored the donor−acceptor relative motion, r_{DA}, and the relative motion, r_{AV}, between the acceptor C atom and the Val203 C_γ atom closest to the nicotinamide ring of NAD.

In Figure 1, we plot $G_{DA}(\omega)$ for all three configurations, obtained from the autocorrelation function of the projection of the relative velocity v_{DA} into the donor−acceptor direction. At low frequencies we have a peak at ca. 125 cm^{-1}, common to both R and P. The corresponding peak in MC is slightly upshifted, appearing at ca. 150 cm^{-1}. This small shift should be ascribed to differences in the electrostatic interactions (due to varying partial charges) as well as to the two harmonic restraints that force the H atom to be equidistant from the donor and acceptor C atoms.

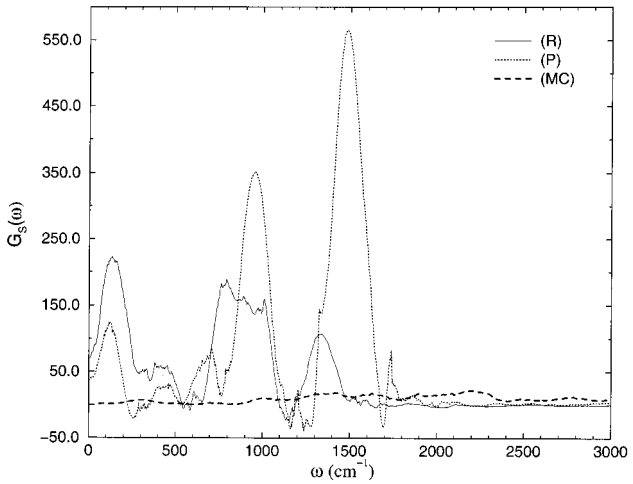

Figure 3. The spectral density $G_s(\omega)$ for the reaction coordinate, in the wild type. The solid line represents the reactants state (R), the dotted line represents the products state (P), and the dashed line represents the minimal coupling configuration (MC). The power spectrum is reported in CHARMM units.

From the component of the relative velocity v_{AV} along the donor−acceptor pair axis we obtain $G_{AV}(\omega)$, which is shown in Figure 2. The similarity with the corresponding $G_{DA}(\omega)$, in Figure 1, is worth noting. More specifically, both R and P have a low-frequency peak at ca. 125 cm^{-1}, as in $G_{DA}(\omega)$. Also, the corresponding peak in MC remains at ca. 150 cm^{-1}, same as in Figure 1. The two motions appear to be in resonance. That was anticipated, since Val203 is found in the NAD^+ binding pocket with one of the methyl groups within the van der Waals contact distance from the nicotinamide ring in NAD.[24,37]

The spectral density, $G_s(\omega)$, associated with the reaction coordinate is shown in Figure 3. Again, the peak at ca. 125 cm^{-1} is common to both R and P. This is a strong indication that the *s* motion is coupled to the relative motion between the alcohol and the nicotinamide ring. Notice, however, that on the

(53) Press, W. H.; Flannery, B. P.; Teukolsky, S. A.; Vetterling, W. T. *Numerical Recipes in FORTRAN*, 2nd ed.; Cambridge University Press: Cambridge, 1992.

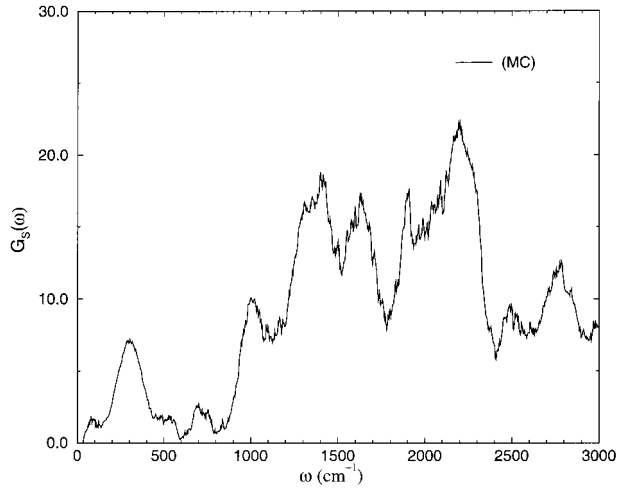

Figure 4. The spectral density $G_s(\omega)$ for the reaction coordinate in the minimal coupling state (MC), in the wild type. The power spectrum is reported in CHARMM units.

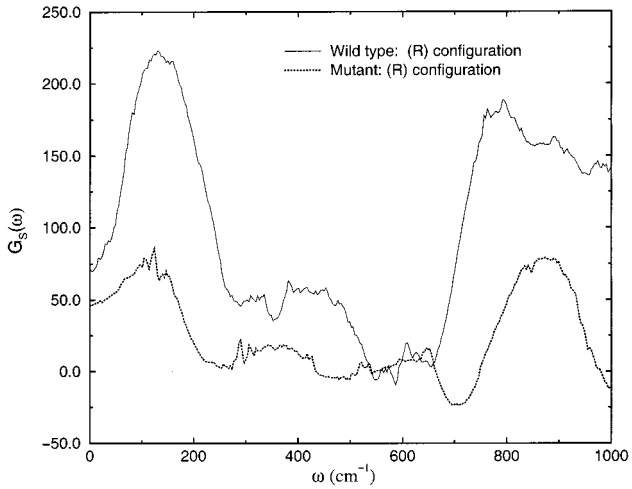

Figure 5. The spectral density $G_s(\omega)$ for the reaction coordinate in the reactants (R) configuration. The solid line represents the wild type; the dotted line represents the Val203 → Ala mutant. The power spectrum is reported in CHARMM units.

scale of the graph, $G_s(\omega)$ for MC appears structureless, almost flat, which clearly shows that $G_s(\omega)$ is position-dependent. In fact, the coupling strength (reflected in the intensity of the spectrum) is about 2 orders of magnitude smaller than that in R or P, as one can clearly see in Figure 4, where $G_s(\omega)$ for MC is plotted by itself. Furthermore, we must stress that although the high frequency peaks are still present, albeit with significantly lower intensity, the peak at ca. 150 cm^{-1} is almost absent in MC. These results strongly suggest that the reaction coordinate is coupled to an oscillation of frequency ca. 150 cm^{-1}. We would like to point out that these observations are not tied to the way we have defined the reaction coordinate. We have also investigated the spectra obtained from the absolute velocity of the H atom and have obtained the same spectra as those shown in Figures 3 and 4.

To test convergence of the results, the simulations were repeated for times up to 100 ps. For these longer simulations, as starting configuration we took the final one from the 30 ps run, that is, no minimization was performed. We then followed the protocol: heating for 2 ps up to 300 K at a rate of 3 K every 20 integration steps; equilibration for 20 K; and, finally, 100 ps of observation during which we collected 6000 structures. The time step was 1 fs, and, as before, all bonds involving hydrogen atoms were constrained using SHAKE to avoid unnecessarily small time steps. Upon comparison with our results from the 30 ps runs, no qualitative changes were observed. That was anticipated, because the promoting vibration appears at about 150 cm^{-1} (about 200 fs time-scale), which implies that a simulation of tens of picoseconds involves many full cycles of the promoting vibration, thus convergence of the results is rapidly reached.

While the foregoing results show that, to the extent that the CHARMm force field is accurate, the natural motion of HLADH includes a symmetrically coupled or promoting vibration, we would like to add a biochemical proof to the mathematical proof already presented. This comes in the form of study of the previously mentioned mutant Val203 → Ala. As mentioned earlier, the site-directed mutagenesis (Val203 → Ala) studies by Klinman and co-workers[21,22] and numerical simulations by Bruice and co-workers[24] suggest that the smaller Ala residue does not "push as hard" on the face of the nicotinamide ring in NAD. To test our methodology, we have also carried out simulations for the Val203 → Ala mutant, and results are presented below. This mutation introduces only a small change in the overall composition of the enzyme matrix, and, as a result, the frequency of the detected promoting vibration is essentially unchanged. This can be seen in Figure 5 where we plot $G_s(\omega)$ for the mutant in the reactants configuration. The dotted line represents $G_s(\omega)$ for the mutant; the solid line represents $G_s(\omega)$ for the wild type and is drawn from Figure 3. The peak at ca. 125 cm^{-1} (recall that for the wild type we had a peak at ca. 125 cm^{-1} in R and P and ca. 150 cm^{-1} in MC) is still present in $G_s(\omega)$ for the mutant, but the strength of the coupling is now smaller − this is clearly manifest in the lower intensity of the peak in the spectral density for the mutant. More detailed computational studies for this mutant are underway, and results will be presented in a forthcoming publication.

In view of our earlier analysis, our results strongly suggest that the reaction coordinate is symmetrically coupled to the alcohol-NAD motion, which in turn is induced by Val203, whose side chain impinges directly on the face of the nicotinamide ring. It is thus shown that there is such a promoting vibration in HLADH, and what is strongly suggested by this work, in concert with cited experimental evidence, is that this dynamic coupling is central to the catalytic process.

Conclusion

In an earlier paper we suggested an MD-based methodology whereby rate-promoting vibrations can be unambiguously identified by the fact that when such motions are symmetrically coupled to the reaction coordinate they will leave a unique signature on the spectral density of the latter. Motivated by recent experimental work by Klinman and co-workers and computer simulations by Bruice and co-workers, we applied this methodology to the oxidation of benzyl alcohol by HLADH and found that the relative motions between the alcohol and NAD and between NAD and Val203 are in resonance. Furthermore, we found that the spectral density obtained from the velocity autocorrelation function of the reaction coordinate has a peak at ca. 150 cm^{-1} which nearly *vanishes* at the point of minimal coupling, and from that we concluded that the alcohol-

NAD relative motion is symmetrically coupled to the reaction coordinate. This relative motion acts as a promoting vibration, since it modulates the distance between the donor and acceptor C atoms, and is induced by Val203, whose side chain impinges on the face of the nicotinamide ring in NAD.

One point to note is that the promoting vibration appears in a time domain orders of magnitude faster than the known chemical step of this enzyme. Thus, it is clearly the case that the enzyme goes through many cycles of the promoting vibration before an effective combination of all motions and atomic positions is produced that will yield catalysis. This should in no way be surprising − not every motion in the protein will yield catalysis. Just what set of atomic rearrangements must accompany this promoting vibration is a complex issue that is currently under study in our group. This question in the biochemistry of the enzyme is equivalent to identification of the actual reaction coordinate in a nonbiological condensed phase chemical reaction. For example, in a proton-transfer reaction in polar medium, reaction can only occur when the solvent is appropriately prearranged. The action of the enzyme in speeding the chemical reaction, however, is postulated to be intimately connected to the directed vibrational motion identified in this paper. Thus, it appears that evolution has designed the protein matrix of an enzyme not just to hold substrates or stabilize transition state formation, but rather to channel energy in a specific chemically relevant direction. It is to be admitted and emphasized that the current methodology locates the existence of a promoting vibration, but not the location, the identification of the exact residues involved in creating the promoting vibration. We are currently developing such a companion algorithm.

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