The Linear Dependence of Log($k_{cat}/K_m$) for Reduction of NAD$^+$ by PhCH$_2$OH on the Distance between Reactants when Catalyzed by Horse Liver Alcohol Dehydrogenase and 203 Single Point Mutants

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Like most dehydrogenases, with horse liver alcohol dehydrogenase (LADH), a bulky amino acid residue (Val 203) is positioned at the face of NAD$^+$ distal to substrate alcohol in order to restrict the separation of reactants and to control the stereochemistry. Molecular dynamics simulations of native (Val203) and single-point mutants (Leu203, Ala203, and Gly203) of LADH-PhCH$_2$OH-NAD$^+$ provide the close contact distances (CCD) between PhCH$_2$OH and NAD$^+$ reactants. It is found that log($k_{cat}/K_m$) is linearly dependent upon CCD. This linear dependence of log($k_{cat}/K_m$) upon CCD is expected if hydride transfer is the rate-determining step. Since log($k_{cat}/K_m$) has been found to be a linear function of tunneling, ln($k_H/k_T$)/ln($k_D/k_T$), it follows that tunneling in LADH has a linear dependence upon CCD.

INTRODUCTION

Horse liver alcohol dehydrogenase (LADH; EC 1.1.1.1) is a well-characterized enzyme which catalyzes reversible NAD$^+$-dependent oxidation of a wide variety of alcohols to corresponding aldehydes (1). The enzyme has a molecular mass of 80 kDa and is a dimer of two identical subunits (2). Each subunit binds one nicotinamide coenzyme and two Zn(II) ions. One zinc is in the active site, while the other is structural. The active site Zn(II) is associated with the hydroxyl oxygen of the alcohol substrate. Complexation of the oxygen of the substrate alcohol by Zn$^{2+}$ lowers the pK$_a$ of the alcohol hydroxyl group. Dissociation of the alcohol hydroxyl proton is the first step in the oxidation of alcohol by NAD$^+$.

\[
\{E-NAD^+-RCH_2OH\} \rightleftharpoons \{E-NAD^+-RCH_2O^-\} + H^+ \\
\{E-NAD^+-RCH_2O^-\} \rightleftharpoons \{E-NADH-RCHO\}
\]

It has been suggested that this proton is passed to water by way of a hydrogen-bond
network terminating with the imidazole of His51 (3). The second step of the reaction involves $H^-$ transfer from the alkoxide to NAD$^+$ (4, 5). By the principle of microscopic reversibility the initial step in aldehyde reduction by NADH involves hydride transfer from NADH followed by proton transfer to the alkoxide product via the proton relay.

Kinetic parameters and isotope effects, measured for a group of site-directed mutants of LADH with benzyl alcohol as substrate, have shown that alteration of the single amino acid Val203 profoundly influences the rate constant for hydride transfer and the degree of hydrogen tunneling (4, 6). Molecular dynamics (MD) simulations of LADH with NAD$^+$ and various substrates have been reported (7–11). In this article we report MD simulations on both LADH-NADH-PhCHO for native enzyme and LADH-NAD$^+$-PhCH$_2$OH for the native and the single-site-directed mutants V203L, V203A, and V203G. Particular attention is paid to the influence of the bulk of the amino acid occupying position 203 on the experimental rates of reaction.

**COMPUTATIONAL EXPERIMENTS**

Both subunits of the enzyme (with NAD$^+$, substrate, and crystallographic waters) have been included in the MD simulations of the native and single-point-mutant enzyme complexes, using CHARMM (12). The starting structure was obtained from the 2.1-Å crystal structure coordinates (2) of LADH-PhF$_5$CH$_2$OH-NAD$^+$. A best-fit superimposition of PhCH$_2$OH on the PhF$_5$CH$_2$OH moiety followed by removal of the latter gave the native simulation system LADH-PhCH$_2$OH-NAD$^+$. The single-point-mutant structures of LADH-PhCH$_2$OH-NAD$^+$ were obtained by a virtual replacement of the Val203 side chain with those of Leu, Ala, and Gly. Equilibrium bond lengths, angles, and dihedral parameters for Zn(II) bonding residues His67, Cys46, and Cys174 as well as partial charges for NAD$^+$ cofactor were treated as in our earlier studies (7, 13). Water molecules were treated as TIP3P residues (14). Steepest descent (200 steps) and adopted basis Newton–Raphson (7800 steps) energy minimizations were carried out prior to dynamics studies. Bonds containing hydrogen were constrained using the SHAKE algorithm (15) during the molecular dynamics run. There are 370 water molecules bound to the surface of the enzyme in the crystal structure. In addition, there are 12 buried waters found in each subunit. These waters remain buried throughout the MD simulations. For the native enzyme, an 80-ps equilibration period was followed by a 1.5-ns production period.

**RESULTS AND DISCUSSION**

A common feature in certain dehydrogenases is the presence of a bulky hydrophobic residue situated at the face of the nicotinamide ring distal to the substrate. In the wild-type LADH, a valine residue (Val203) is found in the NAD$^+$ binding pocket so that one of the methyl groups is within the van der Waals contact distance from the C5 and C6 atoms of the nicotinamide ring (2, 16). Scheme 1, constructed from a molecular dynamics snapshot, shows the pro-R hydrogen of PhCH$_2$O$^-$ poised to transfer hydride to C4 of NAD$^+$ and the positioning of Val203.

The influence of Val203 on the dynamics of the E-S ground state can be best appreciated when observing the MD simulation of the LADH-PhCHO-NADH complex. The steric demand of Val203 induces an anisotropic bending of the dihydronicotinamide ring of NADH to a quasi-boat conformation with the hydrogen to be trans-
ferred in the axial position facing the substrate (13, 17). The quasi-boat conformation contributes to the energetic advantage (13, 17) of enzymatic catalysis and is a required geometry along the pathway to the transition state. Figure 1 displays the anisotropic bending conformation of the NADH in LADH-NADH-PhCHO as extracted from the

**FIG. 1.** The anisotropic bending conformations of the NADH in LADH-NADH-PhCHO complex from MD simulation. The bending angles \( \alpha_C \) and \( \alpha_N \) are defined in Scheme 2.
nanosecond molecular dynamics simulation. The bending angles $\alpha_C$ and $\alpha_N$ are defined in Scheme 2.

The effect of substitution at position 203 was investigated by MD studies of LADH-NAD$^+$-PhCH$_2$O$^-$ complexes. Comparison of the native and mutant complexes (Val203 (wt), V203L, V203A, and V203G) showed that the changes in the volume of side chain at position 203 resulted in different positioning of the nicotinamide ring relative to PhCH$_2$O$^-$. To quantify this positioning we measured the "close contact distance" between C4 of NAD$^+$ and C7 of PhCH$_2$O$^-$ using 2% of MD snapshots exhibiting shortest C4–C7 distance. The MD-derived close contact distances (CCD) were clearly a function of the bulkiness of the side chain at position 203. More interestingly, the close contact distance correlated strongly with experimentally observed catalytic efficiency of native and mutant LADH (Fig. 2). There appears to exist a linear dependence between the log of the experimental $k_{cat}/K_m$ (4, 6) and close contact distance (Eq. [1]):

$$\log(k_{cat}/K_m) = -1.63 \cdot \text{CCD} + 6.65.$$

[1]

These C7 to C4 distances are proportional to the distance that hydride has to travel during the reduction reaction. The distance that hydride has to travel is shorter than the close contact distance but the exact value depends on the angular orientation of two substrates (A linear relationship was also observed using average MD distances instead of the leading 2% of MD snapshots).

In order to understand the significance of this empirical finding, the kinetic and chemical mechanisms of LADH must be considered.
FIG. 2. Catalytic efficiency ($k_{cat}/K_m$) of LADH as a function of close contact distance between C4 of NAD$^+$ and C7 of PhCH$_2$OH. Values of ($k_{cat}/K_m$) are from the experimental literature (4, 6) and the C4–C7 distances are from MD simulations of native and mutant enzymes. Points refer to wild-type enzyme (V203b) and three mutants: Val203Leu (L203b), Val203Ala (A203b), and Val203Gly (G203b).

The $k_{cat}/K_m$ for benzyl alcohol is given by Eq. [2], indicating that catalytic efficiency of LADH depends on both the rate of hydride transfer and the binding equilibrium of benzyl alcohol. In mutant enzymes, the dissociation of benzyl alcohol is fast relative to the hydride transfer step (4, 6) and $k_{cat}/K_m$ reflects the rate of hydride transfer according to Eq. [3]:

$$\log(k_{cat}/K_m) = \log k_5 - \log K_d(ROH).$$

Dissociation constant of benzyl alcohol, $K_d(ROH)$, is likely to be independent of the amino acid at position 203 because this residue is not in the alcohol binding pocket.
but on the other side of the nicotinamide ring (Scheme 1). The experimental observation that tunneling \( [\ln(k_f/k_p)/\ln(k_d/k_T)] \) increases proportionally with \( \log(k_{cat}/K_m) \) also indicates that changes in \( k_5 \) are responsible for the variation of \( \log(k_{cat}/K_m) \) (6).

It has been shown that for a symmetrical rectangular barrier of low permeability, the probability of tunneling is an exponential function of the barrier width (18). It thus follows that the logarithm of a rate constant for a process which occurs by tunneling is a linear function of the distance that the particle must travel.

It is useful to compare the distances between the RCH\(_2\)OH carbon and the C4 of NAD\(^+\) with different alcohols. The CCD from X-ray coordinates of LADH(Val203)\(\cdot\)NAD\(^+\)\(\cdot\)PhCH\(_2\)OH (Ref. 2; abbreviated V203b), LADH(Val203)\(\cdot\)NAD\(^+\)\(\cdot\)CH\(_3\)CH\(_2\)OH (Ref. 6; V203e), and LADH(Ala203)\(\cdot\)NAD\(^+\)\(\cdot\)CH\(_3\)CH\(_2\)OH (Ref. 6; A203c) were compared to the CCD from MD histograms (Figs. 3A–3D) for V203b, A203b, LADH (Leu203)\(\cdot\)NAD\(^+\)\(\cdot\)PhCH\(_2\)OH (L203b), and LADH (Gly203)\(\cdot\)NAD\(^+\)\(\cdot\)PhCH\(_2\)OH (G203b) (the letters b and e refer to benzyl alcohol and ethanol, respectively). The distances between the carbon of RCH\(_2\)OH and C4 of NAD\(^+\) taken from the X-ray structures

![Diagram](image-url)

**FIG. 3.** (A–D) MD histograms of the C4–C7 distances for V203b, L203b, A203b, and G203b, respectively. In A, the X-ray structure C4–C7 and C4–C1 distances for the V203b (2) and V203e (6) are shown as solid lines. In C, the X-ray structure C4–C1 distance of the mutant A203e (6) is shown as solid line. The estimated X-ray static C4–C7 distance for mutant A203b is shown in C as the solid line with the label (A203b, estimated).
are shown as horizontal lines in the MD histograms in Figs. 3A and 3C. In the X-ray structures, the C4–C7 and C4–C1 distances for V203b and V203e are similar, 3.4 and 3.2 Å, respectively (Fig. 3A). The 0.2-Å-shorter C4–C1 distance for V203e compared to the C4–C7 distance for V203b is most likely due to the lessened steric requirement of the smaller substrate. The close contact C4–C7 distances from the MD simulation of V203b (Fig. 3A) are about 0.1–0.2 Å greater than the same distance in the X-ray structure of V203b. Comparing the X-ray structures of A203e (Fig. 3C) and V203e (Fig. 3A) one observes that the C4–C1 distance is greater by 0.8 Å for the former. This is caused by the smaller side chain of residue 203 being associated with the larger cavity. From the X-ray structure, the enzyme backbone of the main chain closes down by ~0.5 Å, when V203e is replaced by A203e; however, a cavity remains in the active site. The close contact C4–C7 distance in the MD histogram of A203b (Fig. 3C) is greater by 0.9 Å than is the like C4–C7 distance for V203b (Fig. 3A). It is interesting to note that the increase in C4–C7 distance determined by MD simulations is much the same as is observed in the X-ray crystal structures V203e and A203e as discussed above.

From the foregoing observations we gain some predictive ability concerning the interatomic distances in mutants of LADH which have not been crystallized. For example, based on the C4–C7 distance for the crystal structure of V203b and MD results, we can predict the C4–C7 distance for A203b. The corresponding distance in V203b is 3.4 Å (2) and MD simulation shows that in the Val203Ala mutant, the distance has increased by 0.8–0.9 Å. As shown by X-ray data, the change in the C4–C1 distance on mutation of V203e to A203e is also 0.8 Å. Therefore, one would predict that the value of the C4–C7 distance in the crystal structure of the mutant A203b would be 4.2–4.3 Å (solid line in Fig. 3C, labeled A203b, estimated). This value may be compared to the distances measured using the X-ray coordinates for A203e (4.0 Å) and the MD histogram for A203b (4.5 Å). Figure 3C shows the estimated X-ray static C4–C7 distance as a line about 0.2–0.3 Å lower than the minimum C4–C7 distances of MD simulation of A203b (4.5 Å) and 0.2–0.3 Å greater than that seen in A203e (4.0 Å) for the smaller crystal alcohol pocket. The histogram and solid lines in Fig. 3C are related in a like manner to those in Fig. 3A. Following the same method, estimations of C4–C7 and C4–C1 distances, with PhCH2OH and CH3CH2OH, respectively, for L203b, L203e, G203b, and G203e, are 3.7, 3.5, 4.5, and 4.3 Å. It is possible that MD simulations may work in concert with crystallography in a combined structure determination (19).

CONCLUSIONS

In the present study a linear relationship is found between the close contact distances of C4 of NAD+ and the C7 of the substrate PhCH2OH vs the log of the rate constant for H− transfer. This supports the concept that in the E-S complex the mole fraction of near attack conformations determines reaction rates (20, 21). In the light of the knowledge that tunneling in LADH is also a linear function of log(kcat/Km) it is possible that part of the catalytic efficiency of wild-type LADH is related to the restriction in the separation of reactants. Restriction in distance between hydride donor and acceptor would facilitate the catalysis by quantum mechanical tunneling. This
work also illustrates that catalytic efficiency in hydride transfer reaction can be achieved without specifically stabilizing enzymatic transition states.

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