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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

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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 $LADH \cdot NAD^+ \cdot PhCH_2OH$ 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.

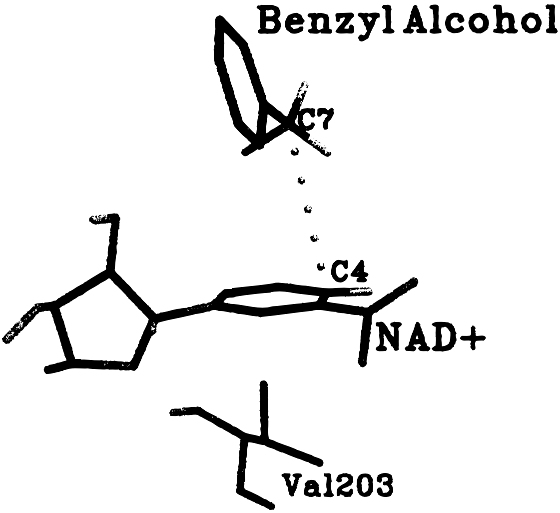
COMPUTATIONAL EXPERIMENTS

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 $LADH \cdot PhF_5CH_2OH \cdot NAD^+$. A best-fit superimposition of $PhCH_2OH$ on the PhF_5CH_2OH moiety followed by removal of the latter gave the native simulation system $LADH \cdot PhCH_2OH \cdot NAD^+$. The single-point-mutant structures of $LADH \cdot PhCH_2OH \cdot NAD^+$ 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 $PhCH_2O^-$ 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-



SCHEME 1

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

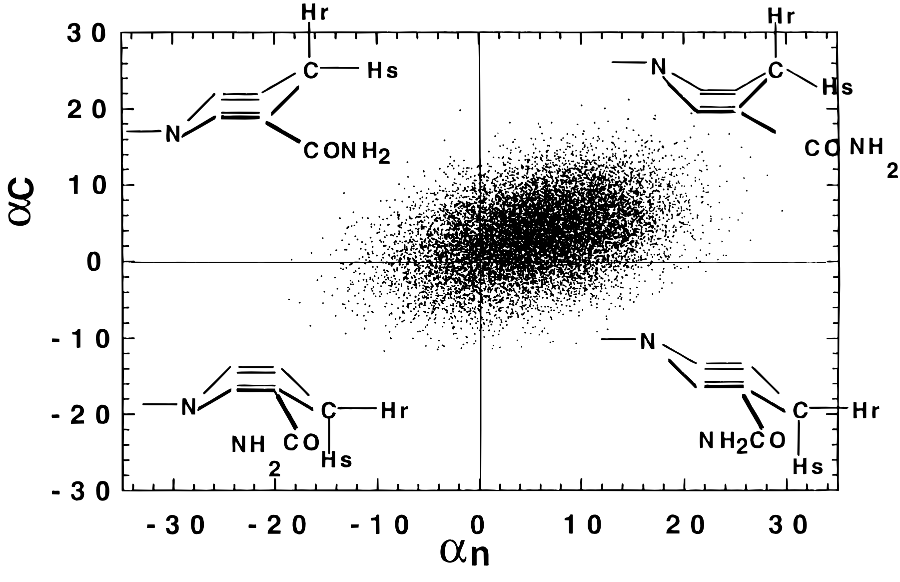


FIG. 1. The anisotropic bending conformations of the NADH in LADH·NADH·PhCHO complex from MD simulation. The bending angles α_C and α_N are defined in Scheme 2.

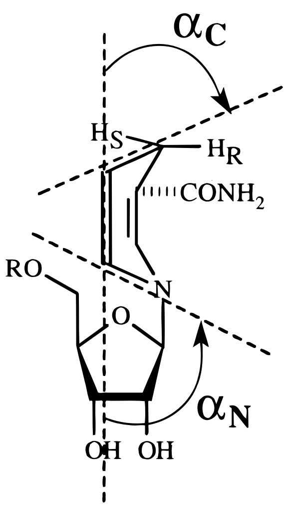
nanosecond molecular dynamics simulation. The bending angles α_C and α_N are defined in Scheme 2.

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]):

$$\log(k_{cat}/K_m) = -1.63 \cdot \mathrm{CCD} + 6.65. \qquad [1]$$

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.



SCHEME 2