Substitutions of Isoleucine Residues at the Adenine Binding Site Activate Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The contributions of isoleucine residues 224 and 269 of horse liver alcohol dehydrogenase to binding of the adenine moiety of NAD and to catalysis were studied by replacing Ile-224 with glycine (I224G) and Ile-269 with serine (I269S). The kinetic mechanisms of wild-type and both mutated liver enzymes were ordered. Affinities for several adenosine derivatives were decreased 5−50-fold by both substitutions. The I269S mutation differentially destabilized binding of the complete coenzyme, as affinities for NAD^+ and NADH were decreased about 60-fold with the I224G enzyme and 350-fold for the I269S enzyme. The I269S substitution increased the rate constants for the conformational change that occurs when NAD^+ binds. The maximum velocities for ethanol oxidation increased 7-fold with the I224G enzyme and 26-fold with the I269S enzyme due to the faster release of NADH. Hydride transfer limits the rate of oxidation of ethanol by the I269S enzyme. Inhibition constants for the substrate analogues, 2,2,2-trifluoroethanol and *N*-methylformamide, and catalytic efficiencies (V/K_m) for ethanol and acetaldehyde were not changed by the mutations, indicating that binding of the adenosine moiety of the coenzyme is not necessarily coupled to the subsequent reaction of substrates.

X-ray crystallography shows that NAD binds in the cleft between the catalytic and coenzyme binding domains of horse liver alcohol dehydrogenase (*Eq*ADH)[1] with the adenine moiety sandwiched between the side chains of isoleucine residues 224 and 269 in the coenzyme binding domain (Ramaswamy et al., 1994). Yeast alcohol dehydrogenase I (*Sc*ADH1) is homologous to *Eq*ADH, but glycine and serine residues replace the isoleucines at the corresponding positions (Jörnvall et al., 1978; Sun & Plapp, 1992). These substitutions might explain why *Sc*ADH1 binds coenzyme 100-fold less tightly and has higher turnover numbers than does *Eq*ADH (Dworschack & Plapp, 1977; Ganzhorn et al., 1987). The present studies support this proposition, and detailed kinetic studies also show that the I269S substitution in *Eq*ADH affects the rates of conformational change when NAD^+ binds without affecting the rates of reaction of substrates.

EXPERIMENTAL PROCEDURES

Materials. LiNAD and NADH were purchased from Boehringer Mannheim Biochemicals. Ethanol-d_5 was from MSD Isotopes. Adenosine derivatives were from Sigma. 2,2,2-Trifluoroethanol and *N*-methylformamide came from Aldrich. Ethanol and acetaldehyde were redistilled before use.

Mutagenesis. The phagemid pBPE/*Eq*ADH (5.2 kilobase pairs) was used for the expression of *Eq*ADH in *Escherichia coli* strain XL1-Blue from Stratagene (Park & Plapp, 1991). Deoxyribonucleotide mutagenic oligomers were synthesized on an ABI394 DNA synthesizer. Mutamer TTGGGGTC-GACGNCAACAAAG (N = A, T, G, and C in equal molar ratios; underlining marks the changes from the wild-type sequence) was used to change the ATC codon for Ile-224 to codons for Gly, Ala, Val, or Asp and to introduce a second site for restriction enzyme *Sal*I (GTCGAC) in plasmid pBPE. The mutamer TTCCTTTGAAGTCTCGGGTCGGCT was used to change ATT (Ile-269) to TCG (Ser) and generate a fourth site for restriction enzyme *Ava*I (CTCGGG) in the plasmid vector. The Doubletake Double-Stranded Mutagenesis kit from Stratagene was used for mutagenesis (Felts et al., 1992). Mutations were identified initially by digestion of plasmids by a restriction enzyme and confirmed by complete sequencing of the cDNA (Sanger et al., 1977) using the Sequenase kit from United States Biochemicals.

Kinetics. The concentration of enzyme active sites was determined by titration with NAD^+ in the presence of pyrazole (Theorell & Yonetani, 1963). The concentrations of nucleotides were determined by absorbance at 260 or 340 nm. Initial velocity kinetics was studied in 33 mM sodium phosphate and 0.25 mM EDTA buffer (pH 8.0 and 25 °C), unless stated otherwise, as described previously (Ganzhorn et al., 1987; Park & Plapp, 1992).

A BioLogic SFM3 stopped-flow instrument with a dead-time of 2.5 ms was used to measure transient reactions. Coenzyme binding was followed by the quenching of protein fluorescence (λ_{ex} = 294 nm, λ_{em} = 310−384 nm) by mixing 0.7−1.5 μN enzyme in the presence of 10 mM pyrazole with NAD^+ varied from 0.17 to 1.0 mM or with 0.2 M isobutyramide and NADH varied from 7 to 70 μM. The BioKine Software was used to fit the first-order transient reactions. The rates of NAD^+ binding were also determined by following the increase in absorbance at 294 nm in the presence of 10 mM pyrazole (Sekhar & Plapp, 1988, 1990).

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[1] Abbreviations: ADH, alcohol dehydrogenase; *Eq*ADH, alcohol dehydrogenase from horse liver (*Equus cabalus*, EE isoenzyme); *Sc*ADH1, alcohol dehydrogenase I from yeast *Saccharomyces cerevisiae*; I224G represents the substitution of Ile-224 with Gly-224; I269S, substitution of Ile-269 with Ser-269; ADP-ribose, adenosine diphosphoribose.

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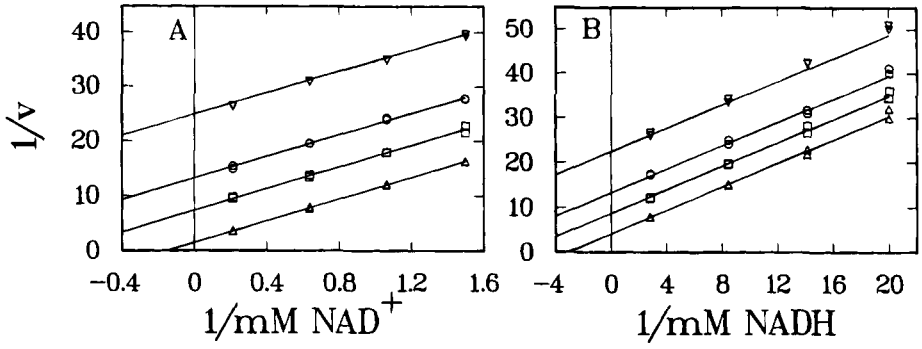


FIGURE 1: Dead-end inhibition patterns for I269S enzyme. Velocities have units of ΔA_{340}/min for (A) and ΔA_{366}/min for (B). (A) Inhibition by trifluoroethanol (△, 0 mM; □, 0.05 mM; ○, 0.1 mM; ▽, 0.2 mM) against NAD^+ (0.667, 0.94, 1.57 and 4.7 mM) at 10 mM ethanol and 41 nN enzyme. (B) Inhibition by *N*-methylformamide (△, 0 mM; □, 10 mM; ○, 20 mM; ▽, 40 mM) against NADH (0.05, 0.071, 0.118 and 0.353 mM) at 10 mM acetaldehyde and 1.8 nN enzyme.

Coenzyme binding was first-order in coenzyme concentration (Shearer et al., 1993).

RESULTS

Protein Properties. The I224G and I269S mutant enzymes were purified by the same procedure used for wild-type enzyme (Park & Plapp, 1991) and appeared to be homogeneous by SDS−polyacrylamide and agarose gel electrophoresis (Laemmli, 1970; Park & Plapp, 1991). The mutant enzymes retained more than 90% of their original activity after storage for 1 month in 20 mM sodium phosphate and 0.25 mM EDTA buffer (pH 8.0) at 4 °C.

Enzyme Mechanism and Kinetic Constants. The initial velocity patterns with both mutant enzymes fit a sequential bi mechanism for ethanol oxidation or acetaldehyde reduction. The kinetic constants were changed significantly (Table 1). The turnover numbers for ethanol oxidation or acetaldehyde reduction (V_1 or V_2) increased up to 7-fold with the I224G enzyme and 30-fold with the I269S enzyme. The dissociation constants of enzyme−coenzyme complexes (K_{ia} and K_{iq}) increased about 60-fold with the I224G enzyme and about 350-fold with the I269S enzyme. Michaelis constants for the mutated enzymes also generally increased. The kinetic constants of the I269S *Eq*ADH were more similar to those of *Sc*ADH1 than to those of the wild-type *Eq*ADH.

Remarkably, catalytic efficiencies calculated from V_1/K_b and V_2/K_p were about the same with all three liver enzymes. However, the overall catalytic efficiency for an enzyme with two substrates is given by $V_1/K_{ia}K_b$ or $V_2/K_{iq}K_p$, which represent the termolecular rate constants for reaction of the enzyme with very low concentrations of substrates (Plapp, 1994). Using this criterion, wild-type *Eq*ADH is the best catalyst, followed by *Sc*ADH1, I224G, and I269S *Eq*ADH. The ratio of overall catalytic efficiencies, which is the Haldane expression for the equilibrium constant, agreed well with the experimentally determined value, indicating that the kinetic constants were self-consistent.

Product and dead-end inhibition studies were used to define the kinetic mechanisms. The I224G and I269S enzymes showed similar inhibition patterns. Oxidized and reduced coenzymes were mutually competitive against each other. Trifluoroethanol, an analogue of ethanol, was a competitive inhibitor against ethanol and an uncompetitive inhibitor against NAD^+ (Figure 1A). *N*-Methylformamide, an analogue of acetaldehyde, was competitive against acetaldehyde and uncompetitive against NADH (Figure 1B). The dissociation constants calculated from the competitive and uncompetitive inhibition studies agreed well. These substrate analogues bound as well to the mutant enzymes as to the wild-type enzyme (Table 1), indicating that the substrate binding site was not significantly affected by the substitutions. The inhibition pattern of *N*-methylformamide against acetaldehyde with the wild-type enzyme appeared to be noncompetitive. This could be due to the formation of both E−NADH−*N*-methylformamide and E−NAD^+−*N*-methylformamide dead-end complexes, as suggested previously for inhibition by tetramethylene sulfoxide (Chadha et al., 1983). The uncompetitive inhibition patterns suggest that the kinetic mechanisms for both mutant enzymes are ordered in both directions.

Isotope effects were determined using protio- or deuterioethanol as substrates (Table 2). For the wild-type enzyme, the rate of ethanol oxidation is limited by NADH release, so that no isotope effect on V_1 was observed. A small isotope effect on V_1 was observed with the I224G enzyme. The I269S enzyme displayed large isotope effects on V_1 and V_1/K_b, suggesting that the hydride transfer step was rate-limiting for the oxidation of ethanol. The lack of isotope effects on V_1/K_a and K_{ia} with both mutant enzymes is consistent with the ordered bi bi mechanism.

Binding of Coenzyme Fragments. Inhibition by adenosine derivatives was used to estimate the magnitudes of the contribution of the isoleucine residues to the binding of the adenosine moiety and to define the subsites that are affected by the mutations (Anderson & Kaplan, 1987). The coenzyme fragments were competitive inhibitors against NAD^+ or NADH, and the K_d values generally increased by a factor of 5−50 with the mutations (Table 3). This is consistent with the loss of hydrophobic interactions with the adenine ring. All three enzymes showed similar patterns of changes in K_d values due to additional interactions when the sizes of the derivatives increased from adenosine to ADP-ribose. AMP and ADP-ribose bind with similar affinities, but ADP binding was relatively weaker, perhaps because of the higher charge density and greater hydration of the ADP molecule (Andersson et al., 1979).

The K_i values determined in this study for wild-type *Eq*ADH at pH 8.0 and 25 °C generally agree with previous data (Reynolds et al., 1970; Yonetani & Theorell, 1964; Li & Vallee, 1963), except that we found that the inhibition constant for adenosine is smaller. NADH binds much more tightly than AMP or ADP-ribose to the wild-type and I224G

Table 1: Kinetic Constants of the Wild-Type and Mutant Alcohol Dehydrogenases[a]

kinetic constants	*Eq*ADH[b]	I224G	I269S	*Sc*ADH1[c]
K_a (mM)	0.0039	0.81	1.0	0.16
K_b (mM)	0.35	1.3	10.7	21
K_p (mM)	0.4	0.15	11[d]	0.74
K_q (mM)	0.0058	0.054	0.57[d]	0.095
K_{ia} (mM)	0.027	1.5	9.5	0.95
K_{iq} (mM)	0.0005	0.033	0.18	0.031
V_1 (s^{-1})	3.5	23	90	360
V_2 (s^{-1})	47	45	1500[d]	1800
V_1/K_b (mM^{-1} s^{-1})	10	18	8.4	17
$V_1/K_{ia}K_b$ (mM^{-2} s^{-1})	370	12	0.88	18
V_2/K_p (mM^{-1} s^{-1})	120	300	140[d]	2400
$V_2/K_{iq}K_p$ (μM^{-2} s^{-1})	0.24	0.009	0.00078	0.08
K_{eq} (pM)[e]	16	10	12	12
activity (s^{-1})[f]	2.4	6.4	40	400
R[g]	0.034	0.069	0.72	0.35
$K_d^{CF_3CH_2OH}$ (μM)[h]	8.4[i]	4.5	7.8	2500
$K_d^{CH_3NHCHO}$ (mM)[j]	1.4[k]	1.5	3.0	0.2[l]

[a] Initial velocity studies were performed at pH 8.0 and 25 °C in 33 mM sodium phosphate and 0.25 mM EDTA. K_a, K_b, K_p, and K_q are the Michaelis constants for NAD^+, ethanol, acetaldehyde, and NADH, respectively. K_i values are inhibition constants. V_1 and V_2 are the turnover numbers of ethanol oxidation and acetaldehyde reduction, respectively. The coenzyme and substrate concentrations were varied in a range from 0.5 to 5-fold of the K_m values in initial velocity studies with the horse enzyme. Standard errors of fits with the SEQUEN program (Cleland, 1979) were less than 15%. [b] Data from Dworschack and Plapp (1977). [c] Data from Gould and Plapp (1990) at pH 7.3 and 30 °C in 83 mM potassium phosphate, 40 mM KCl, and 0.25 mM EDTA. [d] The kinetic constants for acetaldehyde reduction of the I269S enzyme were determined by monitoring absorption changes at 366 nm. The NADH concentrations were varied from 0.042 to 0.39 mM. [e] Equilibrium constant was calculated from $K_{eq} = (V_1K_pK_{iq}[H^+])/(V_2K_bK_{ia})$. The experimentally determined value is 10×10^{-12} M (Sund & Theorell, 1963). [f] Turnover number in standard assay at 25 °C (Plapp, 1970), based on the titration of active sites with NAD^+ in the presence of 10 mM pyrazole. [g] Fraction of enzyme in the ternary complex, calculated from $R = [(1 - K_a/K_{ia}) + (1 - K_q/K_{iq})]/(1/V_1 + 1/V_2)$ (Janson & Cleland, 1974). [h] Dissociation constants of trifluoroethanol, calculated from $K_{ii}/(1 + K_b/[\text{ethanol}])$ in uncompetitive inhibition against NAD^+ or $K_{is}/(1 + K_{ia}/[NAD^+])$ in competitive inhibition against ethanol. [i] Data from Park and Plapp (1991) at pH 7.3 and 30 °C in 83 mM potassium phosphate, 40 mM KCl, and 0.25 mM EDTA. [j] Dissociation constants of *N*-methylformamide calculated from $K_{ii}/(1 + K_p/[\text{acetaldehyde}])$ in uncompetitive inhibition against NADH or $K_{is}/(1 + K_{iq}/[\text{NADH}])$ in competitive inhibition against acetaldehyde. [k] Slope inhibition constant. NADH was fixed at 84 μM (saturating). [l] Corrected slope inhibition constant determined with 0.1 mM NADH.

Table 2: Deuterium Isotope Effects for the Oxidation of Ethanol[a]

enzyme	$^DV_1$	$^DV_1/K_a$	$^DV_1/K_b$	$^DK_{ia}$
wild type[b]	1.1	ND[c]	2.9	ND
I224G	1.5	1.0	4.0	1.1
I269S	4.0	1.0	4.0	0.9

[a] Initial velocity studies were performed at pH 8.0 and 25 °C in 33 mM sodium phosphate and 0.25 mM EDTA. The concentration of ethanol or ethanol-d_5 was varied from 0.55 to 5 mM for I224G enzyme and from 5.5 to 50 mM for I269S enzyme; NAD^+ was varied from 0.3 to 2.7 mM for I224G enzyme and from 0.57 to 5.3 mM for I269S enzyme. V_1, K_a, K_b, and K_{ia} were obtained from a fit with the SEQUEN program (Cleland, 1979). The nomenclature of Northrop (1982) was used. The propagated errors for the isotope effects were <10%. [b] Data from Park and Plapp (1992) at pH 7.3 and 30 °C in 83 mM potassium phosphate, 40 mM KCl, and 0.25 mM EDTA. [c] Not determined.

enzymes, reflecting additional favorable interactions with the nicotinamide ring. For the I269S enzyme, in contrast, the inhibition constants for the complete coenzymes were 400-fold higher than those for the wild-type enzyme. Thus, the

Table 3: Dissociation Constants of Coenzymes and Coenzyme Fragments[a]

	K_d (mM)			ratio	
inhibitor	wild type	I224G	I269S	I224G/WT	I269S/WT
adenosine	3.9	40[b]	19	10	4.9
AMP	0.041	3.2	0.79	78	19
ADP	0.35	16	2.6	46	7.4
ADP-ribose	0.013	6.5	0.61	500	47
NAD^+	0.027	1.5	10[c]	56	370
NADH	0.0005	0.029	0.21	58	420

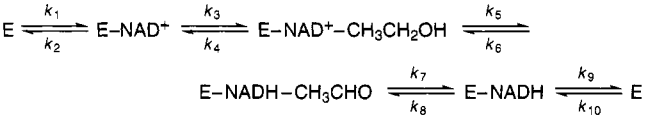
[a] Inhibition studies were performed in 33 mM sodium phosphate and 0.25 mM EDTA buffer (pH 8.0 and 25 °C). Concentrations of ethanol or acetaldehyde were fixed at about their K_m values. NAD(H) concentrations were usually varied from 5/7 to 5 times the K_m value. The concentrations of coenzyme fragments ranged up to the K_d value or higher. Data were fitted to COMP (Cleland, 1979). Standard errors were less than 10% except where indicated. [b] Due to the low solubility of adenosine (about 20 mM), this value has a standard error of about 20%. [c] NADH was varied from 0.053 to 0.37 mM, and the absorption changes at 366 nm were monitored.

Table 4: Rate Constants for Binding of Coenzymes[a]

rate constant	wild type[b]	I224G	I269S
k_1 (μM^{-1} s^{-1})	0.90 (1.2)	0.028 (0.018)	0.090 (0.047)
k_2 (s^{-1})	24	56	900
k_9 (s^{-1})	4.0	28	470
k_{10} (μM^{-1} s^{-1})	8.1 (11)	0.83 (0.80)	2.6 (1.4)

[a] Rate constants were calculated from the kinetic constants in an ordered bi bi mechanism (Cleland, 1963) or measured by stopped flow (values in parentheses): $k_1 = V_1/K_a$, $k_2 = K_{ia}V_1/K_a$, $k_7 = K_{iq}V_2/K_q$, and $k_8 = V_2/K_q$. Standard errors of the values were less than 10%. [b] Steady state data from Dworschack and Plapp (1977); stopped-flow data from Sekhar and Plapp (1990).

Scheme 1



substitutions interfered with coenzyme binding in different ways. Either substitution decreased the binding of adenosine, but the I269S substitution also appeared to interfere with the interactions at both adenosine and nicotinamide portions of the coenzyme.

Association and Dissociation of Coenzymes. Rate constants for coenzyme binding were calculated for the ordered bi bi mechanism (Scheme 1) and determined by stopped-flow experiments (Table 4). Both substitutions decreased the association rate constants for coenzyme. The I224G substitution decreased k_1 by 30-fold, and the I269S substitution decreased k_1 by 10-fold. Similarly, k_{10} was 10- and 3-fold smaller with the I224G and I269S enzymes, respectively. The rate constants for dissociation of coenzymes increased with both mutant enzymes. The I224G substitution increased k_2 and k_9 by 2−7-fold, whereas the I269S change increased k_2 by 40-fold and k_9 by 120-fold. Thus, the higher equilibrium dissociation constants of NAD(H) complexes with the I224G enzyme were mainly due to slower association, whereas both faster dissociation and slower association account for the changes with the I269S enzyme. The increased rates of coenzyme release correspond to the increased turnover numbers (within a factor of 2), except for the I269S enzyme, where k_9 is 5 times larger than V_1. For this enzyme, NADH release is faster than the overall

reaction rate, and hydride transfer becomes the rate-limiting step, as shown by the isotope effects.

DISCUSSION

The I224G and I269S substitutions decrease the affinities (K_{ia} and K_{iq}) for coenzymes by 50−400-fold and increase the turnover numbers (V_1 and V_2) by up to 30-fold. Nevertheless, the mutant enzymes retain the ordered bi bi mechanism and have the same catalytic efficiencies (V_1/K_b and V_2/K_p) as the wild-type enzyme for ethanol and acetaldehyde. It is surprising that alteration of coenzyme binding does not affect the subsequent reaction of substrate, as the adenosine diphosphoribose moiety is expected to help position the nicotinamide ring. The results will be explained in terms of the rate constants for coenzyme binding, conformational changes, and catalytic efficiencies.

The increased activity of the mutant enzymes is due to faster release of coenzyme, which is rate-limiting with the wild-type enzyme (Theorell & Chance, 1951; Wratten & Cleland, 1963; Dalziel, 1963; Sekhar & Plapp, 1990). The I224G substitution increased both k_{10} (Scheme 1) and V_1 by 7-fold. It appears from the stopped-flow data, the small isotope effect on V_1, and the relatively low R value that NADH release controls the turnover of ethanol. V_2 remains unchanged as the I224G substitution has little effect on k_2. Ethanol oxidation catalyzed by the I269S enzyme appears to be controlled by hydride transfer, since there is a large isotope effect on V_1 and the R value is high. Furthermore, k_{10} increased to 470 s^{-1}, which is larger than the turnover number (90 s^{-1}). The turnover number for acetaldehyde reduction, V_2 (1500 s^{-1}), for the I269S enzyme approaches the value for the yeast enzyme and must result from increases in the rate constants k_2, k_4, and k_6 as compared to wild-type enzyme (Sekhar & Plapp, 1990).

The weaker binding of adenosine derivatives to the mutant enzymes is due to the decreased hydrophobic interactions at the adenine binding site, but binding of the complete coenzyme is also coupled to a conformational change. X-ray crystallography shows that the holoenzyme complexes with NAD^+ or NADH and various substrates or analogues are in a closed form due to rotation of the catalytic domain of the protein toward the coenzyme binding domain (Eklund et al., 1981, 1984; Eklund & Brändén, 1987; Ramaswamy et al., 1994). Complexes with AMP and ADP-ribose, and probably adenosine and ADP, and NAD analogues with substitutions for the nicotinamide ring bind to the open apoenzyme conformation (Zeppezauer et al., 1975; Abdallah et al., 1975; Eklund et al., 1976; Samama et al., 1977; Cedergren-Zeppezauer et al., 1982; Li et al., 1994). Complexes of enzyme with NADH in the presence of imidazole or with enzyme carboxymethylated on Cys-46 are also in the open form (Cedergren-Zeppezauer, 1983, 1985). It appears that the conformational change requires binding of the complete coenzyme and is sensitive to small structural alterations.

The inhibition constants for coenzyme fragments (ADP-ribose and smaller) should reflect the interactions with the open (apoenzyme) conformation of the enzyme (Table 3). In this regard, the I224G substitution decreases affinity for the adenosine moiety more than I269S does, which can be explained by the larger change in the side chain. The I224G substitution uniformly affects the binding of coenzyme fragments, NAD^+ and NADH (about 50-fold, except for binding of ADP-ribose), and thus does not appear to affect the conformational change. In contrast, the I269S substitution differentially and significantly disrupts the binding of the complete coenzyme, as evidenced by the 400-fold decreased affinity for NAD^+ and NADH. The carbonyl oxygen of Ile-269 in wild-type enzyme forms a hydrogen bond with the 3′-hydroxyl group of the nicotinamide ribose (Eklund et al., 1984; Ramaswamy et al., 1994). The I269S substitution may perturb the local structure, affecting the hydrogen bond and thereby the conformational change of the enzyme−coenzyme complex.

Scheme 2

$$E \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E\text{–}NAD^+ \text{(open)} \underset{k_{22}}{\overset{k_{21}}{\rightleftharpoons}} {}^*E\text{–}NAD^+ \text{(closed)}$$

The conformational isomerization of the enzyme−NAD^+ complex during binding has also been described kinetically (Wratten & Cleland, 1963; Plapp et al., 1986). Rate constants were estimated using stopped-flow data and kinetic simulations (Sekhar & Plapp, 1988, 1990). The binding of NAD^+ reached a maximal limiting rate of about 600 s^{-1} as the concentration of NAD^+ approached saturation, which was explained by the two-step mechanism shown in Scheme 2. For wild-type enzyme, $k_{11} = 4.5 \times 10^7$ M^{-1} s^{-1}, $k_{12} = 2.3 \times 10^4$ s^{-1}, $k_{21} = 620$ s^{-1}, and $k_{22} = 64$ s^{-1} at pH 8.0 and 25 °C.

When the isomerization step is included in the ordered bi bi mechanism, the rate constant for association (k_{on} or k_1 in Scheme 1) is $k_{11}k_{21}/(k_{12} + k_{21})$, which can be approximated by $k_{11}k_{21}/k_{12}$ since k_{12} is large relative to k_{21}. The apparent binding constant (k_1) appears to be diminished by the partitioning between the E−NAD^+ complexes. The expression for the rate constant for dissociation of coenzyme (k_{off} or k_2 in Scheme 1) for the mechanism in Scheme 2 is $k_{12}k_{21}k_{22}/(k_{12} + k_{21})(k_{21} + k_{22})$. By using the simplifying relationships $k_{12} >> k_{21}$ and $k_{21} >> k_{22}$, k_2 approximates k_{22}. Thus, the turnover number in the reduction of aldehyde (47 s^{-1}) is controlled by the isomerization step.

For the mutated enzymes, the steady state and stopped-flow data provide estimates of the overall rate constants for binding and dissociation of NAD^+, but rate constants for the isomerization could not be determined since no slow step was observed in the formation of the E−NAD^+ complex. Nevertheless, an analysis of the data shows that for both altered enzymes the decrease in the rate constant for binding of NAD^+ (k_1) is due to an increase in the dissociation rate (k_{12}) from the initial E−NAD^+ complex (open conformation). This interpretation is consistent with the weaker binding of coenzyme fragments (adenosine, AMP, ADP, and ADP-ribose). The initial binding (k_{11}) should be diffusion-limited and not be greatly affected by mutations.

On the other hand, the isomerization appears to be affected differently by the two amino acid substitutions. The I224G substitution may have little effect on the isomerization equilibrium. If k_{12} is increased by 50-fold (proportional to the increase in dissociation constants for coenzyme fragments), then k_{21} can be estimated from the approximations given earlier to be 680 s^{-1}, and k_{22} is estimated to be 60 s^{-1}. This means that NAD^+ release (k_2, Table 4) and turnover for aldehyde reduction would be controlled by k_{22}. Thus,

the I224G enzyme is similar to the wild-type enzyme with respect to the conformational change.

The I269S substitution also appears to have increased k_{12} by at least 50-fold, and k_{22} must be increased by at least 25-fold to account for the turnover number of 1500 s^{-1} for aldehyde reduction. On the basis of the assumptions that k_{11} is 4.5×10^7 M^{-1} s^{-1} and that k_{12} is larger than k_{21} (as for wild-type enzyme), the equation given earlier for k_{off} (k_2) shows that both k_{21} and k_{22} must be larger than 1500 s^{-1}. Thus, it is clear that the rates of isomerization have increased, but the equilibrium position for the isomerization (given by the ratio k_{21}/k_{22}) cannot be estimated directly. Nevertheless, since the catalytic efficiencies (V_1/K_b) of the I269S and wild-type enzymes are similar (Table 1), it appears that k_{21} is larger than k_{22}, so that the closed form of the enzyme predominates. If the isomerization equilibrium were shifted toward the open E−NAD^+ form, catalytic efficiency would be decreased due to a decreased concentration of the reactive enzyme form. (By analogy, changes in pH can decrease V/K by producing unreactive forms of enzyme.) This analysis assumes that the closed E−NAD^+ complex most rapidly binds the alcohol, as is suggested by the ordered reaction mechanism. Alternatively, the I269S enzyme could favor the open form in the conformational equilibrium, and binding of substrate would rapidly shift the equilibrium to yield the closed conformation of the ternary complex, in which the nicotinamide ring of the coenzyme is positioned properly for hydride transfer. In either case, the I269S substitution appears to allow increased rates of conformational change in the E−NAD^+ complex.

Rate constants for isomerization of the E−NADH complex have not been estimated, but the decreased rate constants for association and increased rate constants for dissociation with the mutant enzymes (Table 4) suggest that changes in NADH binding also involve altered rates of isomerization.

The I224G and I269S substitutions decrease the affinity for coenzymes and increase the maximum velocities. Remarkably, however, the mutations do not change the catalytic efficiencies for substrates, which shows that the binding of coenzyme and the reaction with substrate are structurally separable. Catalytic efficiency for alcohol, defined as $V_1/K_b = k_3k_5k_7/(k_4k_6 + k_4k_7 + k_5k_7)$, does not include rate constants for coenzyme binding and might not be affected by alterations in coenzyme binding. Nevertheless, catalytic efficiencies with coenzymes with a modified adenosine moiety or with nicotinamide mononucleotide are decreased relative to those with NAD^+, suggesting that catalysis requires the cooperative interactions of all parts of the coenzyme (Plapp et al., 1986). In contrast, the present studies show that coenzyme binding and the reaction of substrates need not be tightly coupled.

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