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Structural determinants of stereospecificity in yeast alcohol dehydrogenase

(stereochemistry/nicotinamide cofactors/evolution/site-directed mutagenesis)

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ABSTRACT **Replacing Leu-182 by Ala in yeast alcohol dehydrogenase (YADH; alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) yields a mutant that retains 34% of its k_{cat} value and makes one stereochemical "mistake" every 850,000 turnovers (instead of ≈1 error every 7,000,000,000 turnovers in native YADH) in its selection of the 4-*Re* hydrogen of NADH. Half of the decrease in stereochemical fidelity comes from an increase in the rate of transfer of the 4-*Si* hydrogen of NADH. The mutant also accepts 5-methylnicotinamide adenine dinucleotide, a cofactor analog not accepted by native YADH. The stereospecificity of the mutant is lower still with analogs of NADH where the carboxamide group of the nicotinamide ring is replaced by groups with weaker hydrogen bonding potential. For example, with thio-NADH, the mutant enzyme makes 1 stereochemical "mistake" every 450 turnovers. Finally, the double mutant T157S/L182A, in which Thr-157 is replaced by Ser and Leu-182 is replaced by Ala, also shows decreased stereochemical fidelity. These results suggest that Si transfer in the mutant enzymes arises from NADH bound in a *syn* conformation in the active site and that this binding is not obstructed in native YADH by side chains essential for catalysis.**

Cryptic stereospecificity presents three interesting problems to the biochemist (1). First, although it is clear that enzymes are highly stereospecific, it is not clear how high this stereospecificity is. Second, as cryptic stereochemical choices are, by definition, detectable only by isotopic substitution, it is not clear why evolution should have produced enzymes with high cryptic stereospecificities. Finally, the stereospecificities of enzymes catalyzing analogous reactions in different organisms often display patterns, and it is important to learn whether these reflect natural selection or randomly fixed historical accidents.

Dehydrogenases dependent on nicotinamide cofactors offer an interesting system for addressing these questions. Dehydrogenases are approximately equally divided between those transferring the *Re* (pro-*R*, or A type) hydrogen at the 4 position of NADH and those transferring the *Si* (pro-*S*, or B type) hydrogen (2–5). Also, many classes of dehydrogenases performing similar catalytic functions in different organisms have either convergently evolved identical stereospecificities or conserved stereospecificity over geological times in excess of a billion years (1, 5–7), time during which neutral biochemical traits should have diverged (8).

Any correlation between stereospecificity and catalytic role suggests that stereospecificity serves a selected function, one that either constrains divergent or encourages convergent evolution (8), and several functional theories explain stereospecificity in dehydrogenases in these terms (9, 10). However, because the distinction between the *Re* and *Si* hydrogens of NADH appears subtle, many commentators (5, 11, 12) have rejected functional models for stereospecificity in dehydrogenases in favor of "historical" models (13, 14) that assume that stereospecificity serves no selected function, but rather is a "frozen accident," a vestige of a random choice made early in the evolution of individual classes of dehydrogenases that, once made, is difficult to change.

The last assumption is surprising. Which hydrogen is transferred from NADH appears to depend simply on which side of the nicotinamide ring faces the substrate. Thus, it should be possible to reverse stereospecificity simply by rotating the nicotinamide ring 180° around the glycosidic bond, a rotation that converts the glycoside from the *anti* to the *syn* conformer (or vice versa) to present the opposite face (Fig. 1) of the ring to the substrate.

To explain why stereospecificity, once chosen, is highly conserved despite the existence of an apparently simple mechanism for reversing it, proponents of historical models argue that evolution from a dehydrogenase that binds cofactor *anti* to one that binds it *syn* necessarily involves major reorganization of the active site, reorganization that proceeds via intermediate enzymes with disrupted catalytic activity. For example, in alcohol dehydrogenase from horse liver (HLADH; alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) (which binds nicotinamide *anti* and transfers the 4-*Re* hydrogen) (15), Oppenheimer *et al.* (16) argued that the 180° rotation around the glycosidic bond necessary for the transfer of the 4-*Si* hydrogen requires superimposition of the carboxamide group of the nicotinamide on the side chain of the Cys-174 and Gly-175. Cys-174 coordinates the active site zinc essential for catalysis. Oppenheimer therefore concluded that the rate of transfer of the *Si* hydrogen cannot be increased by point mutation, as the mutations needed for NADH to bind in the *syn* conformation necessary for *Si* hydrogen transfer will also dislocate a group essential for catalysis.

Closer examination of the crystal structure suggests, however, that the binding of the *syn* conformer of NADH is more likely to be obstructed by the side chains of Val-203 and Thr-178 (17). Neither residue appears important for catalytic activity. If these residues, rather than Cys-174, create the obstacles that prevent the nicotinamide ring of NADH from binding *syn*, it should be possible by mutating these residues to decrease stereospecificity by increasing the rate of transfer of the *Si* hydrogen. If so, mutation of Val-203 or Thr-178 could conceivably be the first step in a path for converting a

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Abbreviations: YADH, alcohol dehydrogenase from *Saccharomyces cerevisiae*; HLADH, alcohol dehydrogenase from horse liver; PAD$^+$, pyridine adenine dinucleotide; L182A, mutant YADH with Leu-182 replaced by Ala; T157S/L182A, double mutant YADH with Leu-182 replaced by Ala and Thr-157 replaced by Ser; DTT, 1,4-dithiothreitol.

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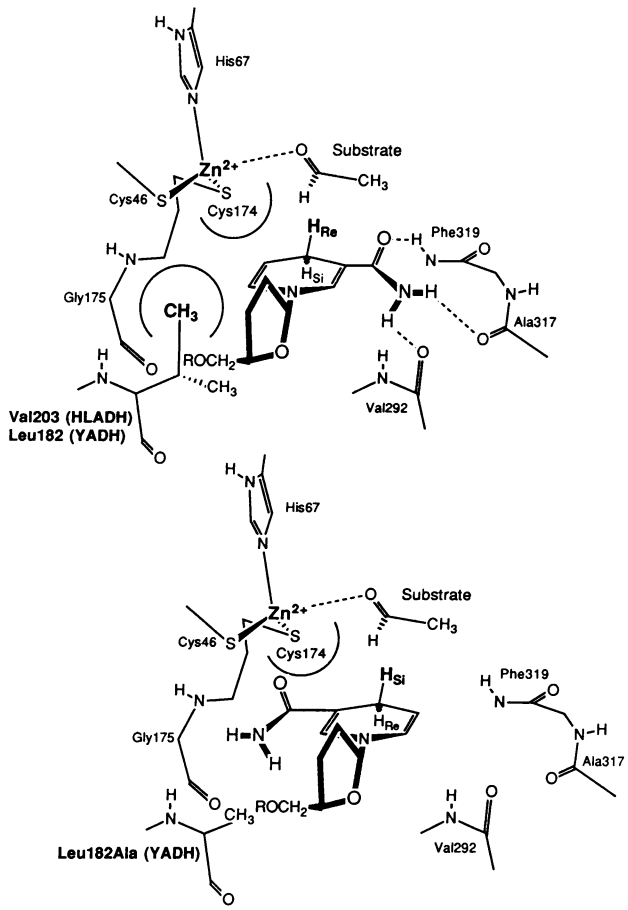

FIG. 1. Stereospecificity in ADH appears to be determined by the conformation of the bound cofactor. Sequence numbers are for HLADH except where noted. Normally (*Upper*), NADH binds *anti*, presenting *Re* hydrogen (H_{Re}) to acetaldehyde lying above cofactor in this diagram. Rotation 180° around the glycosidic bond presents the opposite face in the *syn* conformer, and therefore *Si* hydrogen (H_{Si}), to the substrate (*Lower*), reversing stereospecificity. For clarity, Thr-178 (Thr-157 in yeast) is not shown; the methyl group of this side chain lies below and to the left of the nicotinamide ring behind Val-203. The catalytically important Zn^{2+} lies above the ring.

Re-specific enzyme into a *Si*-specific enzyme via intermediates having catalytic activity.

The experiments described here were undertaken to learn whether it is possible to obtain an increased rate of *Si* hydrogen transfer catalyzed by ADH from yeast (YADH) by changing these residues.

MATERIALS AND METHODS

Mutagenesis and Expression. A gene for YADH-1 was a generous gift of E. Young and B. D. Hall (University of Washington, Seattle). Mutations were introduced in *Escherichia coli* by the method of Kunkel (18), following a protocol in the Mutagene (Bio-Rad) kit. After being fully sequenced, mutant genes were expressed from a modified 2-μm circle in yeast strain RS1-1, where the chromosomal genes for YADH-1 and -2 are disrupted by the *Leu2* and *Ura3* genes (19). YADH activity in crude extracts of this strain is <1% of the wild type, and mutant ADHs were isolated free of wild-type enzyme. Construction and properties of vector and strain are described elsewhere (19).

Enzyme Purification. Yeast were grown to midlogarithmic phase on minimal medium plus histidine, arginine, and lysine; harvested by centrifugation; and disrupted by grinding with glass beads in a 100 mM sodium phosphate buffer (pH 7.0; 2.5 ml per g of cells) containing 1 mM 1,4-dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, and 2-propanol (1%). The supernatant was treated with protamine sulfate (0.15%), and the precipitate was removed. YADH was purified by affinity chromatography on Affi-Gel Blue (Bio-Rad; binding 24.5 mg of albumin per ml) eluted with a gradient of NADH (wild type, 0–0.2 mM; mutants, 0–2 mM) in 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM DTT. The enzymes were further purified by HPLC (LKB; Glass-pak TSK-3000 column) and eluted with "storage buffer" (10 mM sodium phosphate, pH 7.0/300 mM Na_2SO_4/1 mM DTT). The yields of enzymes were wild type, 39%; L182A mutant (Leu-182 replaced by Ala), 53%; T157S/L182A double mutant (Thr-157 replaced by Ser and Leu-182 replaced by Ala), 44%. Enzyme concentrations were determined by UV at 280 nm (ε = 1.26/0.1% cm^{-1}) (20).

Initial Rate (Steady State) Measurements. Ethanol (300 mM) and different concentrations of purified cofactor (NAD^+, 0.5–5 mM; 5-MeNAD^+, 0.2–2 mM) in 32 mM sodium pyrophosphate buffer (pH 8.2; total vol, 1 ml) containing 100 mM Na_2SO_4 were preequilibrated at 25°C for 5 min. Enzyme was then added (0.03 unit using NAD^+ and 1.0 unit using 5-MeNAD^+). Double reciprocal plots yielded k_{cat} and K_m using the extinction coefficients (340 nm) 6220 $M^{-1}\cdot cm^{-1}$ (NADH) and 5800 $M^{-1}\cdot cm^{-1}$ (5-MeNADH) and the specific activities 503 units/mg (wild type), 132 units/mg (L182A), and 52 units/mg (T157S/L182A) under standard assay conditions at 25°C in the same buffer containing 8 mM NAD^+ and 300 mM ethanol.

β-3-Acetylpyridine adenine dinucleotide (3-acetyl-PAD^+; Sigma) and β-thionicotinamide adenine dinucleotide (thio-NAD^+; Sigma) were purified by HPLC [RP-18; TSK ODS-120T 7.8 × 300 mm (LKB) and RP-8; Aquapore Octyl 10 × 250 mm (Brownlee Lab) eluted with 0–20% CH_3CN in 10 mM HCOOH]. β-3-Cyanopyridine adenine dinucleotide (3-cyano-PAD^+) was prepared pure by analytical HPLC (RP-8 HPLC column, RP-300, 4.6 × 220 mm from Brownlee Lab) from purified thio-NAD^+ (21). 5-MeNAD^+ was prepared from 5-methylnicotinamide (22) (45.8 mg; 336 μmol) and NAD^+ (57 mg; 83.9 μmol) with NADase (porcine brain, 169 mg, 1.52 units; Sigma; 37°C, 3 h) (23).

[4-^{3}H]NADH was prepared from NAD^+ (20.1 μg; 30.3 nmol), ATP (138 μg; 250 nmol; Pharmacia), D-[1-^{3}H]glucose (5.0 μg; 27.7 nmol; 230 μCi; 1 Ci = 37 GBq; Amersham), hexokinase (yeast, 3.7 units; Sigma), and glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*, 56 units; Sigma) in 5 mM potassium phosphate buffer (pH 8.0; 0.5 ml), followed (20 min) by oxidation of (4*S*)-[4-^{3}H]NADH with CH_3CHO (440 μg; 5 μmol) using YADH (20 units; Fluka). After ultrafiltration (Centricon 10), [4-^{3}H]NAD^+ (9.8 μg; 14.8 nmol; 112 μCi; 48.7%) was purified using a DEAE-HPLC column (TSK DEAE-5PW, 8 × 75 mm; LKB) eluted (10 min) isocratically (10 mM HCOOH; 1 ml/min) and then with a gradient (0–0.5 M NaCl in 10 mM HCOOH; 20 min). Tritiated cofactor analogs were prepared analogously. As 5-MeNAD^+ is a poor substrate for glucose-6-phosphate dehydrogenase, only 0.05% of the label was incorporated. After purification by DEAE-HPLC, [^{3}H]5-MeNAD^+ (9.7 μg; 13.9 nmol; 0.11 μCi; 45.8%) was further purified by using an RP-18 HPLC column as described above.

Stereochemical Infidelity: Measurement of the Rate of Transfer of *Si* Hydrogen. Stereochemical infidelity was assayed by measuring the release of ^{3}H from [4-^{3}H]NAD^+ to ethanol under conditions of dynamic exchange (24, 25). YADH (L182A and T157S/L182A, 3.9–7.8 μM; native 54–270 μM active sites) was incubated at 20°C in 10 mM potassium phosphate buffer (pH 7.0) containing 300 mM Na_2SO_4, 0.44 mM DTT, 10 μM cofactor [5–10 μCi; except for 5-MeNAD^+ (0.08 μCi)], 100 mM ethanol, 10 mM CH_3CHO, and ADH. The ratio of absorbance at 280 and 260 nm and the absence of absorbance at 340 nm under reducing conditions indicated

that the enzymes were free of cofactor. Aliquots (0.5–1.0 μCi) were removed at intervals and the volatile components isolated by lyophilization. One-tenth was counted. All runs were in parallel with at least two concentrations of enzyme, with blanks lacking enzyme (in addition to other controls; see below), and four to six points per concentration for each cofactor analyzed. The enzymes were stable for 40 h.

Equilibrium Exchange Rate. ADH (L182A, T157S/L182A, or YADH; native) was incubated in ^2H_2O at 20°C in 10 mM potassium phosphate buffer (p^{2}H 7.0) containing 300 mM Na_2SO_4 and 0.44 mM DTT, with the appropriate cofactor, $C^2H_3CH_2OH$, and CH_3CHO. Solutions were concentrated (Centricon 10) to 100 μl, diluted with buffer (2 ml) in ^2H_2O, and concentrated again. Typical concentrations were 10 μM cofactor, 100 mM $C^2H_3CH_2OH$, 10 mM CH_3CHO, and ADH (L182A, 1.1–3.8 μM; T157S/L182A, 1.4 μM; YADH, 0.025 μM active sites). At intervals, aliquots (700 μl) were removed and frozen. Volatile compounds were isolated by distillation and analyzed by ^{1}H NMR. Since the exchange was followed to only 30% of isotopic equilibrium, corrections for the reverse reaction did not significantly alter the measured rates. The ratio of the integrals at δ = 1.08 ppm (carbon-2 of ethanol) and δ = 3.55 ppm (carbon-1) was multiplied by 2/3 and plotted against time.

RESULTS AND DISCUSSION

Wild-type YADH is extremely stereospecific for hydride transfer, making approximately 1 stereochemical "error" every 7 billion turnovers under equilibrium conditions. This estimate is based on a comparison of the rates of two processes—a slow process (the release of ^{3}H to ethanol presumably occurring by a stereochemical error in the active site of YADH) and a fast process [the "normal" transfer of the 4-*Re* hydrogen of NADH to acetaldehyde and the 1-*Re* hydrogen (26, 27) of ethanol to the *Re* face of NAD$^+$].

The faster stereochemically normal rate is easier to quantify. Incubation of 2,2,2-trideuteroethanol and undeuterated acetaldehyde with enzyme and cofactor under equilibrium conditions yields undeuterated ethanol at a rate that can be measured from the rate of increase of the integral of the proton NMR signals from the protons of carbon-2 of ethanol. Protons at carbon-1 provide an internal standard in this experiment, all relevant signals are resolved, and the emerging triplet corresponding to protons at carbon-2 of ethanol is not complicated by geminal ^{2}H coupling, indicating essentially no exchange of acetaldehyde protons with solvent. Thus, rate constants are accurate to between 4% and 23% (Table 1).

Release of ^{3}H to ethanol is much slower. Nevertheless, total counts appearing in the volatile fraction over 24 h were large (300–1000 cpm) relative to the blank (30–40 cpm), increased linearly with time over this period, and were dependent on the concentration of enzyme, the structure of the enzyme (different mutants produce different rates), and the structure of the cofactor. Solid derivatives recrystallized to constant specific activity showed that >95% of the ^{3}H was in the nonexchangeable positions of ethanol.

Several control experiments suggest that ^{3}H appears in ethanol via stereochemical errors made during redox reactions involving ethanol. First, the rate of transfer of ^{3}H to ethanol increases with the concentration of enzyme, both native and mutant, and with both natural and unnatural cofactors. Were a nonenzymatic transhydrogenation between unbound NAD$^+$ and NADH (28) the cause of the release of ^{3}H, the rate should be slower at high concentrations of enzyme (with most of the cofactor bound) and independent

Table 1. Stereochemical infidelity of wild type and variants of ADH

Cofactor	Stereochemical infidelity	Rate constant of transfer of *Si* hydrogen, $\times 10^{-9}$ s^{-1}	Equilibrium reaction rate constant, $\times 10^{-3}$ s^{-1}
		ADH*	
NADH	1:7,000,000,000	2.9 ± 0.2	10,400 ± 2450
Thio-NADH	1:1,500,000	9.7 ± 2.5	7.5‡
		L182A mutant†	
NADH	1:850,000	263 ± 12	113 ± 5
3-Acetyl-PADH	1:40,000	512 ± 189	11 ± 0.6
3-Cyano-PADH	1:<5,000	>1000	2.9‡
Thio-NADH	1:450	117 ± 19	0.026‡
5-MeNADH	1:200,000	36 ± 5	3.4 ± 0.5
		T157S/L182A mutant†	
NADH	1:720,000	423 ± 34	152 ± 6
Thio-NADH	1:200	2140 ± 140	0.21‡

Assays at 20°C at equilibrium with 100 mM ethanol, 10 mM acetaldehyde, 10 μM cofactor, 300 mM sodium sulfate, 0.44 mM DTT, and 10 mM phosphate buffer (pH 7.0). SD values are from plots of integrals vs. time and counts in volatile fractions vs. time. Because stereochemical errors leading to the release of ^{3}H to ethanol are possible in both directions of the redox reaction (see Fig. 2) and equilibrium rates are measured in only one direction, equilibrium rates are multiplied by 2. Rate of *Si* hydrogen transfer is underestimated by a primary ^{3}H isotope effect of unknown magnitude. With an infinite ^{3}H isotope effect, the understatement is 2-fold (see Fig. 2). Data are reported uncorrected. Equilibrium rates of interconversion of ethanol and acetaldehyde in ^2H_2O with $C^2H_3CH_2OH$ as substrate are given. Solvent ^{2}H isotope effects were wild type (1.99) and L182A (1.10); β-secondary ^{2}H kinetic isotope effects were unity. Only for wild type was the concentration of enzyme used to measure infidelity (250 μM) greatly different from that used to measure equilibrium rate (0.025 μM); independent experiments showed <10% nonlinearity over this range.

**Si* rates measured at 250 μM active sites. Observed rates were linear as a function of enzyme concentration from 0 to 250 μM active sites.

†*Si* rates measured at two concentrations of enzyme (typically 3.9 and 7.8 μM active sites) were proportional to concentration. An essentially linear relationship between the rate of release of ^{3}H and enzyme concentration is expected up to 250 μM enzyme. A small deviation from linearity is seen with 3-acetyl-PAD$^+$ at 7.7 μM, the highest concentrations of enzyme used with this cofactor.

‡Based on steady-state parameters at pH 7.0.

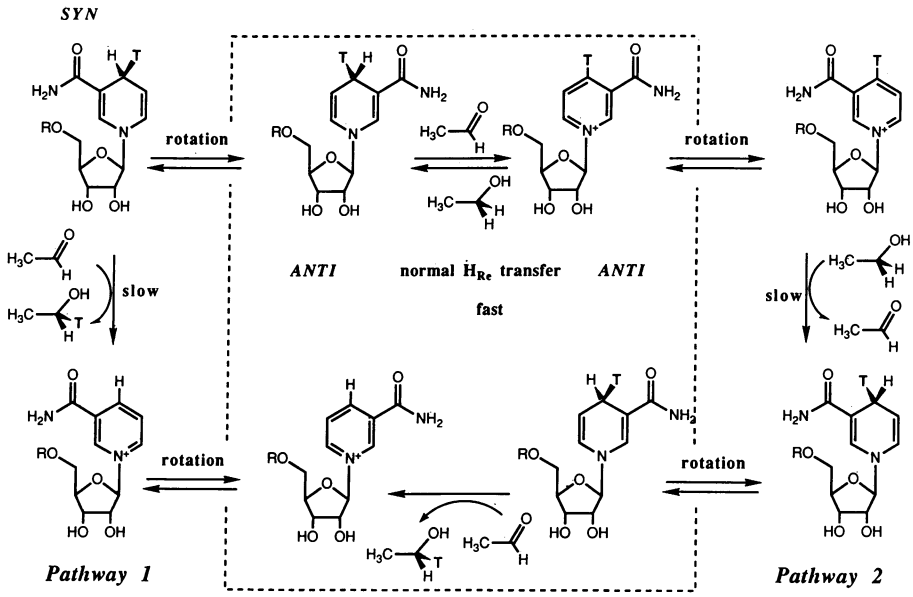

FIG. 2. Two mechanisms for transferring ^3H from NAD^+ to ethanol via a reoriented cofactor. Stereochemically normal (inside the dashed box) and abnormal (outside the dashed box) transfer of hydrogen. H_{Re}, *Re* hydrogen.

of the concentration of YADH at lower concentrations, provided that the equilibrium rate for the redox reaction is faster than the rate of release of ^3H to ethanol.

While these results argue that the release of ^3H to ethanol is catalyzed by a component of the solution containing YADH, this component need not be YADH itself. For example, if this solution contained a transhydrogenase or a contaminating dehydrogenase (and its substrate) that catalyzes the formation of (4*R*)-[4-^{3}H]NADH from [4-^{3}H]NAD^+, transfer of ^3H to acetaldehyde could be catalyzed by YADH in a stereochemically normal reaction. Experimental data make this unlikely as an explanation for the release of ^3H to ethanol. First, the rate of appearance of ^3H in ethanol decreases in parallel with the total ADH activity under conditions where YADH undergoes partial inactivation. Second, the ratio of the rate constants for *Si* hydrogen transfer with NADH and thio-NADH is not the same with the two mutants L182A and T157S/L182A (Table 1). If a contaminating enzyme were responsible for the transfer of ^3H, the ratio of the rate constants for *Si* hydrogen transfer with NADH and thio-NADH should be independent of the structure of YADH.

Finally, the rate of ^3H release was measured with hexadeuterioethanol as substrate. Two pathways involving reorientation of the nicotinamide ring can transfer ^3H to ethanol (Fig. 2). First, ^3H might be transferred directly to acetaldehyde from (4*S*)-[4-^{3}H]NADH bound in the *syn* conformation (pathway 1). Alternatively, protium might be transferred from ethanol to the *Si* face of carbon-4 of [4-^{3}H]NAD^+ bound in the *syn* conformation to form (4*R*)-[4-^{3}H]NADH, which transfers ^3H to acetaldehyde by a rapid, stereochemically normal reaction in the next turnover (pathway 2). While replacing ethanol with d_6-ethanol leaves pathway 1 (essentially) unaffected, it should slow the second pathway via a primary kinetic isotope effect. We found that with native enzyme, d_6-ethanol, and NAD^+, the rate of transfer of ^3H to ethanol was 65% that of the rate with unlabeled ethanol as substrate. It is difficult to explain these results unless the transfer of ^3H to ethanol arises by a process that involved the oxidation of ethanol.

To understand how YADH might catalyze *Si* hydrogen transfer from NADH, mutants were designed by using a model for YADH built by extrapolation (29, 30) of the crystal structure of the homologous dehydrogenase from horse liver (31). In this model, Leu-182 of YADH corresponds to Val-203, Cys-153 corresponds to Cys-174, and Thr-157 corresponds to Thr-178 in HLADH. Two mutants of YADH were prepared, one with Leu-182 changed to Ala, the other with Leu-182 changed to Ala and Thr-157 changed to Ser.

The possibility exists that *Si* hydrogen transfer might occur because acetaldehyde finds a new binding site on the opposite face of the nicotinamide ring still bound in an *anti* conformation, rather than because the nicotinamide ring rotates 180° to bind in a *syn* conformation. Although this possibility would require a more drastic reorganization of the active site, it cannot be ruled out a priori.[§] Several results make this possibility unlikely, however.

First, the L182A and T157S mutations have a small impact on the steady-state kinetic parameters of YADH (Table 2). The k_{cat} (with NAD^+) is decreased by only a factor of 3 in L182A and by another factor of 3 in T157S/L182A. This suggests that these mutations do not greatly alter the structure of the active site.

Second, 5-MeNAD^+ has no detectable activity as a substrate for native YADH. However, 5-MeNAD^+ is accepted by both mutants with a k_{cat} value ≈3% that of NAD^+. Some of the reduced rate is undoubtedly due to the more negative (−25 mV) redox potential of 5-MeNAD^+; the k_{cat} for oxidation of 5-MeNADH with L182A is ≈16% that of NADH. The K_m values for both mutants are smaller for 5-MeNAD^+ than for NAD^+, perhaps indicating that 5-MeNAD^+ has more favorable binding interactions with the mutant than NAD^+. Nevertheless, these results make a convincing case that reducing the size of the side chain at position 182 creates space in the active site that can be occupied by a substituent at the 5-position of nicotinamide bound in the *anti* conformation or, following 180° rotation around the glycosidic bond, the carboxamide of NADH bound in a *syn* conformation.

If this hypothesis is correct, the mutant enzymes should have lower stereospecificity, not necessarily expected were *Si* transfer to arise by a change in the position of binding of acetaldehyde. In fact, L182A and T157S/L182A make ≈1 stereochemical error every 850,000 turnovers with NADH. In both cases, the rate constants for the transfer of the *Si*

[§]Acetaldehyde bound to the opposite face of the nicotinamide ring would almost certainly not be coordinated to Zn^{2+}. It is difficult to estimate the consequences of this on the reaction rate, as the reaction between NADH and acetaldehyde free in solution is unknown, and as slow intramolecular transfer in models requires metal ions.

Table 2. Steady-state kinetic parameters of YADH1 and variants of ADH with different cofactors

Enzyme	Cofactor	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, $mM^{-1}\cdot s^{-1}$
ADH-1	NAD^+	211 ± 19	308 ± 11	1460 ± 80
ADH-1	5-$MeNAD^+$	ND	<0.03	ND
L182A	NAD^+	1890 ± 50	106 ± 2.0	56.4 ± 0.4
L182A	5-$MeNAD^+$	976 ± 152	2.85 ± 0.40	2.92 ± 0.16
T157S/L182A	NAD^+	520 ± 16	31.9 ± 0.6	61.3 ± 0.8
T157S/L182A	5-$MeNAD^+$	195 ± 39	1.20 ± 0.13	6.13 ± 0.68

Assays were at 25°C in a mixture containing 300 mM ethanol, 100 mM sodium sulfate, and 32 mM pyrophosphate (pH 8.2) and various concentrations of cofactors. ND, not determined.

hydrogen are ≈2 orders of magnitude larger than in native YADH, which in turn is ≈4 orders of magnitude more stereospecific than the mutant enzymes.

If the lower stereospecificity in the mutants arises because space is now available to allow NADH to bind in the *syn* conformation (as opposed to allowing acetaldehyde to bind to the opposite face of NADH bound in the *anti* conformation), then some of the stereospecificity remaining in the mutants should arise from attractive interactions, presumably hydrogen bonds, between the carboxamide group of NAD^+ in the *anti* conformation and residues in the active site (Fig. 1). Thus, stereospecificity should be still lower with cofactor analogs having the carboxamide replaced by groups with weaker hydrogen bonding potential.

All cofactor analogs examined are poorer substrates in the transfer of the *Re* hydrogen, in both native and mutant YADH, under both V_{max} and equilibrium conditions (Table 1). In all cases, stereochemical fidelity is lower (Table 1). For example, with the acetyl and cyano analogs of NAD^+ in L182A, ^3H appears in ethanol once every 40,000 and 5000 turnovers, respectively. With thio-NADH, ^3H is transferred once every 450 turnovers. Much of the decrease in stereospecificity comes from a slower rate of transfer of the 4-*Re* hydrogen, consistent with the hypothesis that changes in the structure of the carboxamide group change interactions between this group and the active site.

Furthermore, in the double mutant T157S/L182A, *Si* transfer is only 1.6 times faster than in L182A with NADH as cofactor, yet it is nearly 20 times faster with thio-NADH. This suggests that the space made available by reducing the size of residue 182 is sufficient to accommodate the smaller carboxamide group of NADH, but additional space is needed to accommodate the larger thiocarboxamide group of thio-NADH, and this additional space can be obtained in the active site by reducing the size of Thr-157. Were *Si* transfer arising by a change in the position of binding of acetaldehyde, stereoselectivity need not be different with different cofactor analogs, nor would the double mutant be expected to show lower stereoselectivity relative to the single mutant with thio-NADH when compared with NADH.

Thus, freeing space in the active site where the carboxamide group of NADH would fit if the cofactor binds in a *syn* conformation, and removing interactions between the enzyme and the carboxamide group in NADH bound as the *anti* conformer both yield enzymes with lower stereospecificity. These results suggest that stereospecificity is determined by the conformation of the nicotinamide ring with respect to the glycosidic bond, determined in turn both by repulsive interactions between side chains in the active site (primarily residue 182) and the carboxamide of the nicotinamide ring when bound *syn*, and attractive interactions (hydrogen bonding) between the carboxamide group and residues in the active site when cofactor is bound *anti*. Furthermore, the higher *Si* hydrogen rate in the mutants suggests that *syn* binding of the cofactor does not require the dislocation of groups essential for catalysis.

These results have obvious implications regarding theories concerning the evolution of stereospecificity in dehydrogenases. If a change in the conformation from *anti* to *syn* of the cofactor is not obstructed by a residue essential for catalysis, this removes one explanation for why stereospecificity is highly conserved. This increases the possibility that other explanations are correct—in particular, that stereospecificity itself directly serves a selected function (9, 10, 13, 14). Together with extensive data ruling out simple historical models as viable explanations for dehydrogenase stereospecificity (14), these results strengthen the case that stereospecificity in dehydrogenases serves a selected function.

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