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Inversion of substrate stereoselectivity of horse liver alcohol dehydrogenase by substitutions of Ser-48 and Phe-93

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Abstract

The substrate specificities of alcohol dehydrogenases (ADH) are of continuing interest for understanding the physiological functions of these enzymes. Ser-48 and Phe-93 have been identified as important residues in the substrate binding sites of ADHs, but more comprehensive structural and kinetic studies are required. The S48T substitution in horse ADH1E has small effects on kinetic constants and catalytic efficiency (V/K_m) with ethanol, but decreases activity with benzyl alcohol and affinity for 2,2,2-trifluoroethanol (TFE) and 2,3,4,5,6-pentafluorobenzyl alcohol (PFB). Nevertheless, atomic resolution crystal structures of the S48T enzyme complexed with NAD⁺ and TFE or PFB are very similar to the structures for the wild-type enzyme. (The S48A substitution greatly diminishes catalytic activity.) The F93A substitution significantly decreases catalytic efficiency (V/K_m) for ethanol and acetaldehyde while increasing activity for larger secondary alcohols and the enantioselectivity for the *R*-isomer relative to the *S*-isomer of 2-alcohols. The doubly substituted S48T/F93A enzyme has kinetic constants for primary and secondary alcohols similar to those for the F93A enzyme, but the effect of the S48T substitution is to decrease V/K_m for (*S*)-2-alcohols without changing V/K_m for (*R*)-2-alcohols. Thus, the S48T/F93A substitutions invert the enantioselectivity for alcohol oxidation, increasing the *R/S* ratio by 10, 590, and 200-fold for 2-butanol, 2-octanol, and *sec*-phenethyl alcohol, respectively. Transient kinetic studies and simulations of the ordered bi bi mechanism for the oxidation of the 2-butanols by the S48T/F93A ADH show that the rate of hydride transfer is increased about 7-fold for both isomers (relative to wild-type enzyme) and that the inversion of enantioselectivity is due to more productive binding for (*R*)-2-butanol than for (*S*)-2-butanol in the ternary complex. Molecular modeling suggests that both of the *sec*-phenethyl alcohols could bind to the enzyme and that dynamics must affect the rates of catalysis.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the on-line version at <http://dx.doi.org/10.1017/j.cbi.2016.???.???>.

Keywords

Alcohol dehydrogenase; Substrate specificity; Stereoselectivity; Enzyme kinetics; X-ray structure

1. Introduction

Mammalian alcohol dehydrogenases oxidize a broad variety of alcohols and reduce the corresponding carbonyl compounds in the detoxification of primary and secondary alcohols [1,2]. Many ADHs specifically transfer the *pro-R* hydrogen of a primary alcohol to NAD⁺, which can be explained by a close contact of the *pro-S* hydrogen with the benzene ring of Phe-93 where even a methyl group would not easily be accommodated [3]. However, horse liver ADH1E, and human ADH1B and ADH1C are weakly active on secondary alcohols, such as 2-propanol, suggesting that these enzymes are flexible and can accommodate at least a methyl group in place of the *pro-S* hydrogen in the larger substrates [4,5]. Human and monkey ADH1A are much more active on secondary alcohols than are ADH1B and ADH1C [4,5], and it is important to determine the fundamental basis for such activities so that structure-function relationships and metabolic roles can be explained.

From the structures of horse liver and human liver ADHs and model building, it seems that the Ser/Thr-48 and Phe/Ala-93 substitutions are critical determinants of substrate specificities [6-8]. Table 1 indicates that ADH1B has Thr-48 whereas ADH1A has both Thr-48 and Ala-93. Fig. 1 shows that the residues in the active site create a barrel that can bind a variety of hydrophobic substrates with some limitations on size and shape and that residues 48 and 93 constrict the space near the reacting carbon atom of the substrate. Horse liver ADH (*EqADH*) has Ser-48 and Phe-93 and prefers the *S*-enantiomer of secondary alcohols, whereas the ADH1A enzymes from monkey and human liver have Thr-48 and Ala-93 and prefer the *R*-enantiomers [4,5,9,10]. It appears that the F93A substitution is important because the F93A/T94I substitutions in human ADH1B1 inverted the enantioselectivity from favoring *S* to favoring *R* [11], but it is not clear how the enantioselectivity would be affected solely by the F93A or the S48T substitutions. The T48S substitution in human ADH1B1 improves activity on cyclohexanol, suggesting that removal of the methyl group of Thr-48 could better accommodate other secondary alcohols, but the T48S substitution does not produce an exact mimic of ADH1C because differences in residues outside of the active site also affect activity [12]. Thus, the effects of individual substitutions need to be studied in the context of the same background structure, which horse ADH1E provides. Studies of comparable residues in *Saccharomyces cerevisiae* ADH I showed that the T45S substitution did not affect stereoselectivity for isomers of 2-butanol, but the W92A substitution inverted it [13].

Previous steady-state and transient kinetic studies with wild-type *EqADH* on 2-butanol showed that the rate of hydride transfer is faster for (*R*)-2-butanol than for (*S*)-2-butanol, but the catalytic efficiency (V/K_m) is higher for (*S*)-2-butanol [14]. In the present work, we studied the effects of the S48T, F93A and S48T/F93A substitutions in *EqADH1E* on the substrate specificity and stereoselectivity, and we used transient kinetics to address the

question of the connections between substrate binding (affinity) and hydride transfer rates for the reaction of the S48T/F93A ADH on the isomers of 2-butanol.

2. Experimental Procedures

2.1 Materials

LiNAD and Na₂NADH were purchased from Roche Molecular Biochemicals. Benzyl alcohol- α,α -d₂ (98.6% D) was purchased from MSD Isotopes. Alcohols and aldehydes were purchased from Aldrich and were redistilled before use. The fluoroalcohols (98-99%) and pyrazole were purchased from Aldrich and used without further purification. 2-Methyl-2,4-pentanediol (MPD) was obtained from Kodak and treated with activated charcoal before use.

2.2. Enzyme preparation

The plasmid pBPP/EqADH [15] containing the cDNA for horse liver ADH1E (*Equus caballus*, NCBI taxonomy ID 9796, GenBank accession number M64864) was used to create the site-directed substitutions in DNA (see Supplementary Data for details). The enzymes were purified to apparent homogeneity according to the published procedure [15].

2.3. Crystallization

Crystals of the recombinant S48T enzyme complexed with NAD⁺ and 2,3,4,5,6-pentafluorobenzyl or 2,2,2-trifluoroethanol were prepared by the procedure used for wild-type enzyme [16,17]. The enzyme was dialyzed extensively against 50 mM ammonium *N*-[tris(hydroxymethyl)methyl]-2-aminomethane sulfonate, 0.25 mM EDTA, pH 7.0 buffer (pH 6.7 at 25 °C). The enzyme concentration was adjusted to 10 mg/ml ($A_{280} = 4.55/\text{cm}$), and 0.5–1.0 ml was placed in washed ¼ in. diameter dialysis tubing and put into 10 ml of the buffer, to which 2,3,4,5,6-pentafluorobenzyl alcohol (200 mg to make ~100 mM) and LiNAD (8 mg to yield ~1 mM) was added. After an hour (time for the fluorobenzyl alcohol and NAD⁺ to bind to the enzyme), 1.1 ml 2-methyl-2,4-pentanediol (MPD) was added. The concentration of MPD was further increased over some days to about 12%, when crystals formed, and was slowly raised to a final concentration of 25%, which is sufficient for cryoprotection at 100 K. The concentrations of NAD⁺ and pentafluorobenzyl alcohol are sufficient to saturate the S48T enzyme, as the K_d for NAD⁺ is 15 μM , and the K_i for pentafluorobenzyl alcohol is 740 μM . The complex of the S48T enzyme with 2,2,2-trifluoroethanol was prepared similarly except that the TFE concentration was 100 mM ($K_i = 98 \mu\text{M}$). The crystals were mounted on fiber loops (Hampton Research) and flash vitrified by plunging into liquid nitrogen.

2.4. X-ray crystallography

The data for the S48T enzyme complexed with NAD⁺ and PFB were collected at the Advanced Photon Source at Argonne National Laboratories on June 16, 2007, on the GMCA beamline 23ID-B with wavelength of 1.0332 Å at 100 K with a crystal to detector distance of 120 mm, with 0.25° oscillations over 360° total, with 1 s exposures for a high resolution pass, and at 200 mm with 0.5° oscillations over 360° total, with 0.5 s exposure for a low resolution pass on the MAR300 detector.

The data for the S48T enzyme complexed with NAD⁺ and TFE were collected at the Advanced Light Source in Berkeley, CA, on Feb. 1, 2008, on the Molecular Biology Consortium beamline (ALS 4.2.2) with a wavelength of 0.827 Å (15,000 eV) at 100 K and a crystal to detector distance of 95 mm, with 3 s exposures and 0.2° oscillations over 360° total with a Quantum 210 detector.

Data were processed with d*TREK [18]. The structures were solved by molecular replacement with the isomorphous wild-type enzyme (4DWV.pdb) and refined with REFMAC [19]. PFB was removed from the initial model. The dictionary for NAD was modified to relax the restraints on bond angles and distances so that the geometry of the nicotinamide ring in the complex could be properly fitted, and thus the name in the coordinate files is “NAJ” [17,20]. Riding hydrogens were added, and anisotropic displacement parameters were refined for both structures. After each refinement cycle, the models were rebuilt manually using the program “O” using $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ difference maps [21]. Several residues were modeled with alternative rotamers where electron density indicated. Water molecules were included if there was well-formed electron density, *B*-factors were less than about 50 Å², and hydrogen bonds connected them to the protein.

The quality of the refinement was monitored with PROCHECK and SFCHECK [19], MolProbity [22] and PARVATI [23]. Figures were made with the Molray web interface server in Uppsala, Sweden [24].

2.5. Steady-state kinetics

A Cary118C spectrophotometer interfaced to a Data Translation A/D board in an IBM PC/XT was used to determine the change in absorbance at 340 nm due to NADH, and the initial velocities (= *b*) were determined by fitting the time courses to a straight line ($A = a + bt$) or a parabola ($A = a + bt + ct^2$) with a Fortran program. When the K_m for substrate was lower than 0.05 mM, an SLM Aminco 4800 fluorometer ($\lambda_{ex} = 340$ nm and $\lambda_{em} = 460$ nm) was used to determine initial velocities. Data collected in 1 min were fitted to a straight line or parabola with a least squares program provided with the instrument software. The buffer was 33 mM sodium phosphate and 0.25 mM EDTA, pH 8.0, at 30 °C. For surveys of substrate specificity, the concentration of NAD⁺ was fixed at 2 mM. The concentrations of substrates were varied in a 10-fold range around the corresponding K_m values. The data were fitted to the Michaelis-Menten equation with the HYPER program [25]. The kinetic constants for the ordered bi bi mechanism were determined by initial velocity studies with concentrations of both substrates varied in a systematic manner (5 × 5 matrix) and fitting the data to the equation (Eq. 1) for the sequential bi mechanism with the SEQUEN program, where concentrations of reactants ($A = \text{NAD}^+$, $B = \text{alcohol}$) are given in brackets, and the kinetic constants for the respective substrates have the corresponding lower case subscripts. For the reverse reactions, $P = \text{aldehyde or ketone}$ is substituted for B , and $Q = \text{NADH}$ is substituted for A . The initial velocity patterns for varied NADH and acetaldehyde appeared to be parallel, so that the $K_{iq}K_p$ term in Eq. 1 was not defined; thus K_{iq} was determined by competitive product inhibition by NADH against varied concentrations of NAD⁺, where the data were fitted to Eq. 2 with the COMP program [25]. The standard errors for the kinetic

constants were 10–25 % of the fitted values, which indicates that the kinetic constants are well-determined.

$$v = V_1 [A] [B] / (K_{ia} K_b + K_a [B] + K_b [A] + 1) \quad \text{Eq. 1}$$

$$v = V_1 [A] / (K_a (1 + [Q] / K_{iq}) + [A]) \quad \text{Eq. 2}$$

The turnover numbers (k_{cat} or V/E_t , s^{-1}) from initial velocity studies were calculated from the enzyme concentration determined from the titration of active sites and the specific activity in the standard assay [26]. Errors were propagated to account for the errors associated with determination of enzyme concentration and V in the kinetics. As with most steady-state kinetics studies, the reproducibility of the data may vary by 2-fold with different investigators, enzyme and substrate concentrations, estimation of initial velocities, spectrophotometers, etc., which means that the standard errors of the fits underestimate the accuracy of the values.

2.6. Transient kinetics

Transient kinetic reactions at 25 °C in 33 mM sodium phosphate buffer and 0.25 mM EDTA, pH 8.0, were studied in a BioLogic SFM3 stopped-flow instrument with three syringes for variation of the concentrations of reactants. The dead time was determined to be 2.5 ms [27]. The BioKine Software was used for data analysis. For transient kinetics and simulation, an extinction coefficient of $5500 \text{ M}^{-1}\text{cm}^{-1}$ at 328 nm was used for the difference in absorption of NADH and NAD^+ bound to enzyme and its complexes [28,29].

For the transient reactions of substrates, 6–15 μN (active site concentration) enzyme with 2 mM NAD^+ and varied concentration of alcohols for oxidation or with stoichiometric levels (equal to enzyme concentration) of NADH and varied concentration of aldehydes for reduction produced a burst reaction that was fitted by an equation for a first order reaction. The first-order rate constants increased hyperbolically as the concentration of substrates increased.

The binding of NAD^+ was determined by mixing 15–20 μN enzyme with an equal volume of varied concentrations of NAD^+ (0.04–0.3 mM for the F93A enzyme and 0.14–0.70 mM for the S48T/F93A enzyme) and pyrazole (10–50 mM for F93A enzyme and 5–40 mM for S48T/F93A enzyme) at 25 °C. The change in absorbance at 294 nm due to the formation of the enzyme-NAD-pyrazole complex was observed.

The rate of NADH binding was determined by the change in protein fluorescence (quenching with $\lambda_{\text{ex}} = 294 \text{ nm}$, and $310 \text{ nm} < \lambda_{\text{em}} < 384 \text{ nm}$) with 1–4.5 μN enzyme mixed with concentrations of NADH varied from 5–21 μM for the F93A enzyme, or from 12–50 μM for the S48T/F93A enzyme. The transient curves were fitted by an equation for a first-order reaction. The bimolecular rate constant was obtained by fitting the observed rate constants as a function of NADH concentration to a straight line. The rate of dissociation of

NADH was determined by mixing the enzyme-NADH complex with concentrations of NAD^+ varied from 0.05–1.0 mM and 25 mM pyrazole. As the NADH is replaced by NAD^+ , the enzyme- NAD^+ complex is trapped by the pyrazole, and the absorbance at 294 nm was followed. The standard errors for rate constants were less than 10% of the values.

The kinetic simulation program, KINSIM and an automatic fitting routine, FITSIM, were used to estimate rate constants for the overall enzymatic reaction by fitting several progress curves simultaneously [30].

3. Results

3.1. Kinetic characterization

Initial velocity studies in the forward reaction (NAD^+ and ethanol) for all of the enzymes gave intersecting patterns, indicative of a sequential mechanism. In the reverse reaction (NADH and acetaldehyde), patterns were parallel because the dissociation constants for NADH (K_{iq}) are much smaller than the Michaelis constants for NADH (K_q). Product inhibition shows that NADH was a competitive inhibitor against NAD^+ . These data are consistent with the ordered bi bi mechanism. Kinetic constants are summarized in Table 2. The constants appear to be self-consistent because the equilibrium constants for the reaction calculated from the kinetics (Haldane equation) agree with the directly determined value.

Kinetic constants for the S48T enzyme were similar to those for natural wild-type enzyme. The pH dependencies for oxidation of ethanol, benzyl alcohol, and 2-chloroethanol have patterns similar to those for wild-type enzyme, with some differences in values, but the S48T substitution does not alter the proton relay system significantly (Supplemental Data Tables 1S and 5S).

In contrast, the kinetic constants for the S48A enzyme were greatly affected, as affinity for coenzymes decreased 4–40-fold and turnover numbers decreased by 160–350-fold (Table 2). Such a result is expected because the hydroxyl group of Ser-48 interacts with the oxygen of the substrate and the 2'-hydroxyl group of the nicotinamide ribose and participates in the proton transfer mechanism via a low barrier hydrogen bond [3,17,31]. The T48A substitution also inactivates human ADH1B [12]. The results with the Ala-48 enzyme are included here because they support the conclusion that the proton relay system (Fig. 1) is critical for catalysis.

Kinetic constants for the F93A and S48T/F93A enzymes are similar to those for wild-type enzyme, with the notable exceptions that the K_m values for acetaldehyde are increased 50–100-fold, affinity for NADH is increased about 10-fold, and turnover numbers for ethanol oxidation are decreased about 10-fold. Turnover for ethanol oxidation (V_1/E_t) is controlled by the rate-limiting release of NADH in the last step of the ordered bi bi mechanism. Transient kinetic studies determined that the rate constant is 0.34 s^{-1} , whereas the rate constant for binding was essentially the same as for wild-type enzyme, $1.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. The maximum velocity and catalytic efficiency for ethanol oxidation are pH dependent, somewhat different than for wild-type enzyme (Table 2S and 5S, Fig. 1S). Binding of trifluoroethanol is also pH dependent and similar to wild-type enzyme (Table 3S and 5S,

Fig. 1S). The rate constant for the transient oxidation of ethanol determined from the burst phase of the reaction of NAD^+ and ethanol for the F93A enzyme is 23 s^{-1} (Table 4S and 5S, Fig. 1S), which is slower than the rate constant observed for wild-type enzyme of 180 s^{-1} [31]. Thus, the F93A substitution has significant effects on the catalysis of hydride transfer, as was also observed in the reaction with benzyl alcohol and benzaldehyde [32]. Substrate deuterium isotope effects with ethanol and benzyl alcohol confirm that the hydrogen transfer does not limit catalytic turnover (Table 6S).

Trifluoroethanol, heptafluorobutanol and pentafluorobenzyl alcohol (inactive analogues of ethanol, butanol, and benzyl alcohol, respectively) were competitive inhibitors against ethanol. Inhibition constants (equivalent to dissociation constants, K_d) are given in Table 2. (For the S48T/F93A enzyme K_i for heptafluorobutanol was $53 \mu\text{M}$, as compared to a value of $5.3 \mu\text{M}$ for wild-type enzyme [33]). Although the S48T, F93A and S48T/F93A enzymes have Michaelis constants for ethanol (K_b) that are similar to those of the natural enzyme, their affinities ($1/K_i$) for trifluoroethanol are 6–70-fold lower, respectively, than that for the wild-type enzyme, showing that substrate binding sites have been greatly affected by the substitutions. Likewise, the affinities for pentafluorobenzyl alcohol are decreased by 100-fold or more. Trifluoroethanol is an uncompetitive inhibitor against varied concentrations of NAD^+ for the S48T/F93A enzyme, consistent with an ordered bi mechanism with NAD^+ binding first, followed by ethanol. Pyrazole was a competitive inhibitor against varied concentrations of ethanol, with a K_i value of $8.1 \mu\text{M}$, as compared to a value of $0.2 \mu\text{M}$ for wild-type enzyme [34].

3.2. Three-dimensional structures

As a basis for interpreting the kinetic results, the structures for the S48T enzyme complexed with NAD^+ and trifluoroethanol or pentafluorobenzyl alcohol were determined at atomic resolution (Table 3). The structures are almost identical with the corresponding structures for wild-type enzyme [17]. Fig. 2 shows that the S48T substitution has very small effects on the binding of the fluoroalcohols. For TFE binding, the trifluoromethyl group of TFE (Fig. 2A) appears to be rotated because of some interaction with the methyl group of Thr-48. The binding of PFB is almost identical (Fig. 2B), with a slight tilt of the benzene ring because of the interaction with the methyl group of Thr-48, which also prevents Leu-57 from adopting one of the alternative conformations found in wild-type enzyme [17].

A structure for the F93A enzyme previously determined at atomic resolution (1MGO.pdb) is also almost identical to that of the wild-type enzyme, but no clear electron density was apparent for the PFB in the active site [32]. This could be due multiple positions (disorder) because the substrate binding site was enlarged, and NMR results show that the mobility of fluorobenzyl alcohols is increased. Because the affinity for PFB is decreased in this enzyme, 100 mM PFB (~saturating, the same concentration used for S48T ADH) was used for the crystallography, and density for PFB outside the active site was observed. Upon re-examining this structure for the present study, we refined the structure using a dictionary for NAD with relaxed restraints on the nicotinamide ring that allows NADH to be fitted (“NAJ”), and it appears that the F93A ADH structure actually contains NADH, which might have resulted in decreased binding of PFB in the active site. The NADH is identified by a

puckered nicotinamide ring and longer bond distances than those for the nicotinamide ring in NAD^+ , as characterized by structures for various dehydrogenases complexed with NAD^+ , NADP^+ , NADH or NADPH [17]. In contrast, the structures for the S48T enzyme show the strained nicotinamide ring of NAD^+ as found in wild-type enzyme [17]. The NADH can form during the crystallization of the enzyme (over some weeks) when contaminating alcohols or MPD are oxidized by the added NAD^+ and the PFB has relatively low affinity as an inhibitor. Fig. 3 shows the electron density map for the complex of the F93A ADH complexed with NADH .

NADH binds with the same interactions in the active sites of the wild-type and F93A ADHs (Fig. 1 in Ref. 32). Thus, it is not clear why the F93A and S48T/F93A substitutions increase the affinity for NADH (by 10-fold, Table 2). As provided in the Supplemental Data (Fig. 2S), F93A ADH binds NADH (in the presence of isobutyramide) with the same spectral characteristics as does wild-type enzyme, i.e., a shift of the maximum of 340 nm for free NADH to a maximum at about 325 nm for enzyme-bound NADH , so that it appears that the environment around the reduced nicotinamide ring in the ternary complex is not changed by the F93A substitution. Nevertheless, the F93A substitution increases the size of the binding site (Fig. 3), which can account for alteration of substrate specificities observed for the F93A enzymes.

Crystals of the S48T/F93A enzyme suitable for X-ray crystallography were not obtained, but the structure of the enzyme with the double substitution is probably essentially the same as the sum of the individual substitutions because residues 48 and 93 are exposed to solvent in the active sites of the enzymes, and the overall structures of the wild-type, S48T and F93A enzymes are almost identical.

3.3. Substrate specificities

Substrate specificities for a series of primary alcohols were determined (Table 4). The turnover numbers (V_1/E_1) are similar for all the alcohols for each ADH, as expected for rate-limiting release of NADH , but the Michaelis constants (K_m) and catalytic efficiencies (V_1/K_m) show strong dependencies on the chain length. The V_1/K_m values for F93A and S48T/F93A enzymes show a pattern similar to that of *MmADH1A* which suggests that the longer alcohols can fit better into the active site. The removal of the benzene ring at position 93 would give additional space where small substrates, such as ethanol, can freely rotate and fit in nonproductive modes, leading to a decrease of reactivity as seen for similar substitutions in yeast ADH1 [13]. The S48T enzyme was not included in this survey, but it was noted that saturation with ethanol showed substrate activation (“negative cooperativity”) above 2 mM ethanol, which may arise because of the formation of an abortive enzyme- NADH -alcohol complex from which NADH dissociates faster than from the enzyme- NADH complex [35,36]. In contrast, wild-type and S48T/F93A enzymes exhibit substrate inhibition by high concentrations of ethanol, which can result from slower dissociation of NADH from the enzyme- NADH -alcohol complex than from the enzyme- NADH complex.

Kinetic constants for secondary alcohols are presented in Table 5. The turnover numbers (V_1/E_1) for wild-type and S48T enzymes are generally lower than those for primary alcohols, which indicates that hydride transfer is slower than release of NADH . In contrast,

turnover numbers for F93A and S48T/F93A enzymes are very similar for both primary and secondary alcohols, which is consistent with NADH dissociation being the major rate-limiting step in turnover ($\sim 0.34 \text{ s}^{-1}$). The catalytic efficiencies (V_1/K_m) are low for wild-type enzyme and even lower for the S48T enzyme. The catalytic efficiencies for the F93A enzyme for secondary alcohols are significantly larger and increased for both *R* and *S* isomers as compared to wild-type enzyme, consistent with the increased size of the active site. The additional S48T substitution in the S48T/F93A enzyme appears to depress the activity on the *S*-isomers, resulting in enhanced enantioselectivity for the *R*-isomers (Table 6). A similar effect is observed by comparisons of the human enzymes and the F93A/T94I human ADH1B [4,11]. The net effect of the S48T/F93A substitution is to invert the enantioselectivity (*R/S* ratio) by factors of 10, 590, and 190 for 2-butanol, 2-octanol, and *sec*-phenethyl alcohol, respectively, as compared to wild-type enzyme. These results show that both Ser/Thr-48 and Phe/Ala-93 play important roles in stereospecificity, but the effects of the substitutions are difficult to predict from inspection of the enzyme structure.

3.4. Transient oxidation and reduction of substrates

Data for transient oxidation of some alcohols and reduction of the corresponding carbonyl compounds by the S48T/F93A, F93A, and wild-type enzymes are given in Table 7. The F93A and S48T/F93A enzymes have smaller values of k_{max} (rate constant for hydride transfer with saturating substrate concentrations) for ethanol than wild-type enzyme does, consistent with the proposal that the larger active sites do not bind and orient the substrates as well as the smaller site in wild-type enzyme. In contrast, benzyl alcohol is oxidized faster by the S48T/F93A enzyme than by wild-type ADH, consistent with the increased active site size, but the maximum rate constant for reduction for benzaldehyde is dramatically lower for this enzyme as compared to wild-type enzyme. Oxidation of the 2-butanols is faster for the S48T/F93A and F93A enzymes as compared to the rate constants for wild-type enzyme. Reduction of 2-butanone by the S48T/F93A enzyme is very slow, comparable to the rate for wild-type enzyme, and steady-state kinetics for 2-butanone reduction with 0.1 mM NADH (V_2/E_t of $0.081 \pm 0.002 \text{ s}^{-1}$, K_m of $12.7 \pm 0.9 \text{ mM}$ and V_2/K_m of $6.4 \pm 0.3 \text{ M}^{-1}\text{s}^{-1}$) suggests that the hydride transfer to the ketone is rate-limiting for the overall turnover.

Transient progress curves for reactions of (*R*)- and (*S*)-2-butanol show an exponential burst phase followed by a steady-state phase for the alcohols; reduction of 2-butanone was only studied under single-turnover conditions (Fig. 4). These results were analyzed by kinetic simulation in order to estimate microscopic rate constants for each step in the mechanism. The rate constants for binding and release of coenzymes were determined independently, and all of the data from Fig. 4 were fitted together. The solid lines in Fig. 4 represent the fits, and the rate constants are given in Table 8. Rate constants for wild-type enzyme were estimated previously, except that it was assumed that there were two different binding modes for 2-butanone in the reduction reaction with branched paths that can distinguish between *re*- and *si*-faces of the ketone facing towards the *pro*-(*R*) hydrogen on C4N of the dihydronicotinamide of NADH [14]. Although that assumption may be reasonable, we chose to fit a kinetic mechanism with only one E-NADH-2-butanone species because this complex presumably rapidly isomerizes between productive binding modes, and the relative concentrations are implied by the reverse rate constants (k_{-4} and k_{-5}). The rate constants for

the simulations are internally self-consistent as the pH independent equilibrium constant we calculated for the oxidation of 2-butanol is 5×10^{-9} M, as compared to an average value of 3.1×10^{-9} M determined by Adolph et al. [14] and a related value for 2-propanol oxidation of 7.2×10^{-9} M [37].

The major effects of the S48T/F93A substitutions, relative to wild-type enzyme, are that hydride transfer rate constants for oxidation of the 2-butanols (k_4 and k_5) increase 5–9-fold, whereas the reverse reactions (k_{-4} and k_{-5}) decrease 6–7-fold. These changes make the “on-enzyme” equilibrium position 600–1900-fold more favorable for alcohol oxidation than for ketone reduction, as compared to the wild-type position of 15–20-fold. Usually, for wild-type enzyme, the hydride transfer rate constants for reduction of aldehydes are 1.2–1.6-fold faster than for oxidation of primary alcohols, ethanol, propanol, and butanol [31]. With wild-type enzyme, benzaldehyde reduction is 8-fold faster than oxidation of benzyl alcohol [31], but for the S48T/F93A enzyme, oxidation is 120-fold faster (Table 7). This dramatic decrease in the rate constants for reduction must reflect alterations in productive binding modes, which can also be relevant for the inversion of substrate enantioselectivity (for V/K_m values).

Catalytic efficiencies can be estimated from the rate constants in Table 8 (see full equations in Ref. [38] or [39]), and because the rate constant for reduction of 2-butanone is relatively slow, the calculation approximates k_2k_4/k_{-2} for (*R*)-2-butanol and k_3k_5/k_{-3} for (*S*)-2-butanol. The calculated values (k_H/K_d , Table 9) are similar to the steady-state results (V/K_m , Table 5) and confirm the inversion of stereoselectivity of due to the S48T/F93A substitution. However, it is significant that the calculated binding constants show that (*S*)-2-butanol (k_{-3}/k_3) binds more tightly than (*R*)-2-butanol (k_{-2}/k_2) to both wild-type and S48T/F93A enzymes, although the S48T/F93A substitutions increase the binding of the *R*-isomer by 4.9-fold relative to the *S*-isomer. It appears that the binding mode for the *R*-isomer is more productive than that for the *S*-isomer, and some non-productive binding modes increase the affinity for the *S*-isomer.

4. Discussion

With the X-ray structures for the S48T and F93A enzymes available, we sought structural explanations for the changes in activity and stereospecificity for the substrates. The structure of the F93A with bound NADH (1MGO.pdb) is essentially identical to the wild-type and S48T enzymes, and therefore Phe-93 was mutated *in silico* to Ala-93 in the structure of the S48T enzyme complexed with NAD⁺ and PFB. The constraints on the modeling are that the alcohol oxygen is ligated to the zinc, and the transferring hydrogen is directed toward C4N of the nicotinamide ring, such as is observed for the benzyl alcohol analogue, pentafluorobenzyl alcohol (Fig. 1). In wild-type enzyme, the presumed productive binding mode for secondary alcohols places a substituent close to the benzene ring of Phe-93, and the F93A substitution would remove the hindrance, allowing substituents (methyl, propyl, hexyl, or phenyl) to fill spaces near Ala-93 and into the hydrophobic pocket (Fig. 3 and Fig. 5). Thus the catalytic efficiencies for both *R* and *S* isomers of the secondary alcohols can be increased just because the F93A substitution makes more space for binding (Table 5). The energetics of binding of the productive modes for the isomers are about the same.

The additional S48T substitution, to make the S48T/F93A enzyme, has small effects on catalysis for the *R*-isomers, but depresses activity with the *S*-isomers (Table 5). Because the inversion of enantioselectivity for *sec*-phenethyl alcohol due to the S48T/F93A substitution is substantial, we thought that modeling with this substrate would be informative. As shown in Fig. 5, the F93A substitution creates some space for the phenyl group of the *R* isomer, but a contact at 2.9 Å remains with Ala-93. The *S* isomer fits almost into the position observed for pentafluorobenzyl alcohol, with the α -methyl group at 3.2 Å from Cys-174. The *S* isomer may have higher affinity than the *R* isomer for the enzyme, but our modeling is not sufficient to explain the effects on binding energetics or the inversion of the specificities for *sec*-phenethyl alcohol. We note again that the (*S*)-2-butanol binds (3-fold) tighter to the S48T/F93A enzyme than (*R*)-2-butanol does, but the *R* isomer has a 10-fold faster hydride transfer rate. Non-productive binding modes may increase affinity for the substrates but decrease rate constants for hydride transfer, whereas productive binding modes may decrease affinity for substrates and provide for faster rate constants for hydride transfer (“ground state” destabilization).

The F93A substitution increases the affinity for NADH (K_{iq}) while decreasing the rate constants for hydride transfer to aldehydes relative to those for transfer from alcohols. There is no obvious structural explanation for the change in NADH binding because NADH is bound in the same position as found for binding of NADH or NAD⁺ in wild-type enzyme (e.g., compare 1MGO.pdb to 1P1R.pdb or 4DWV.pdb). Perhaps the NADH binds tighter because there is less ground-state destabilization of the NADH (less of the intrinsic binding energy is used to destabilize the NADH). The decreased reactivity of aldehydes can be explained by less productive binding of carbonyl compounds. The Haldane relationship relates the kinetic constants to the thermodynamic equilibrium constant (footnote e in Table 2) and shows that a 10-fold decrease in K_{iq} is compensated for by changes in other kinetic (rate) constants. Thus, for the F93A and S48T/F93A enzymes, V_1/E_t decreases by about 10-fold (due to slower dissociation of the enzyme-NADH complex), whereas K_p increases about 100-fold (Table 2). Accordingly, the catalytic efficiencies with acetaldehyde ($V_2/E_t K_p$) decrease about 100-fold, and may be due to less ground state destabilization of the NADH and less constrained binding of the substrate in the active site. Modeling of the binding mode does not provide a simple explanation. Computations that assess the dynamics of forming productive binding modes and hydrogen transfer may be informative.

This work complements previous studies that show that the T48S substitution in human ADH1B1 (retaining Phe-93) or the F93A substitution (retaining Thr-48) increases activity on secondary alcohols, such as cyclohexanol [11,12]. The F93A/T94I substitution in ADH1B1 (retaining Thr-48) also inverted enantioselectivity on 2-substituted alcohols [11], as found for the horse enzyme. Our results show that the S48T substitution alone decreases activity on secondary alcohols, and somewhat increases enantioselectivity for the *R*-isomers of 2-butanol and 2-octanol, as compared to wild-type human ADH1B1 (with Thr-48 and Phe-93), which favors *S*-2-butanol to *R*-2-butanol by a factor of 6-fold. The combination of the S48T and F93A substitutions in the horse enzyme inverts the specificity. In this regard, the S48T/F93A enzyme resembles, but does not replicate, human ADH1A, which has other differences in the active site, as shown in Table 1 [4]. Overall, it is clear that increasing the size of the substrate binding site can increase catalytic activity for large substrates, while

decreasing activity on small substrates, and that enzyme flexibility and dynamics must contribute to catalysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ADH	alcohol dehydrogenase
Eq	horse <i>Equus caballus</i>
Mm	Monkey, <i>Macaca mulatta</i>
Hs	human
S48T	substitution of Ser-48 with Thr, etc.
TFE	2,2,2-trifluoroethanol
PFB	2,3,4,5,6-pentafluorobenzyl alcohol
MPD	2-methyl-2,4-pentanediol

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Highlights

F93A substitution increases catalytic efficiencies for secondary alcohols.

Enantioselectivity for secondary alcohols is inverted by the S48T/F93A substitutions.

Structures for S48T ADH complexed with NAD⁺ and fluoroalcohols were determined.

Transient kinetic data provide rate constants for the reactions of 2-butanols.

The inversion of enantioselectivity to favor the *R*-isomers reflects productive binding.

Fig. 1.

Active site of horse liver alcohol dehydrogenase complexed with NAD⁺ and 2,3,4,5,6-pentafluorobenzyl alcohol (PFB). The substrate binding site is lined with hydrophobic amino acid residues. The hydrogen-bonded system connecting the oxygen of the alcohol to the catalytic His-51 is shown in dotted lines. The *pro-R* hydrogen of the alcohol is pointed toward C4N of the nicotinamide ring of NAD⁺. Based on 4DWV.pdb.

Fig. 2.

The structure and electron density map for the S48T enzyme complexed with NAD⁺ and fluoroalcohols (shown as ball and stick with atom coloring) superpositioned onto the structures for the wild-type enzyme (shown as green stick). The label “T48” shows the electron density for the inserted methyl group. (A) The complex with TFE (5KCZ.pdb) as compared to the wild-type complex (4DXH.pdb) showing the one position for Leu-57 (due to the displacement of the other conformer by the Thr-48 methyl group) and that the trifluoromethyl group of TFE occupies two positions, with one being slightly rotated relative to the structure for the wild-type enzyme. The map was contoured at about $1.2 \text{ e}^-/\text{\AA}^3$. (B) The complex with PFB (5KCP.pdb) as compared to wild-type enzyme (4DWV.pdb) showing the alternative position of Leu-57 in wild-type enzyme that is prevented by the methyl group of Thr-48 and the slightly-shifted position of the pentafluorobenzyl alcohol away from residue 48 in the S48T enzyme. Note also that Leu-116 adopts different conformations in the two different complexes. The map was contoured at about $0.9 \text{ e}^-/\text{\AA}^3$.

Fig. 3.

The structure and electron density map for the F93A enzyme complexed with NADH. The structure reported as 1MGO.pdb was re-refined with relaxed restraints on the dictionary for NAD, so that NADH could be identified. The electron density map was contoured at about $1.0 \text{ e}^-/\text{\AA}^3$. Ala-93 is clearly identified. The side chains of Leu-57, Leu-116 and Leu-141 have high temperature factors and higher mobility, probably because the substrate binding site is not occupied by any well-ordered substrate analogue (PFB) or solvent molecules. These residues often have alternative conformations for different substrate analogs.

Fig. 4.

The transient and simulated reactions of 2-butanol and 2-butanone for the S48T/F93A enzyme. The buffer was 33 mM sodium phosphate and 0.25 mM EDTA, pH 8.0. Changes in absorbance at 328 nm were measured at 25 °C. (A) Enzyme (13.5 μN) reacted with 1 mM NAD^+ and (*R*)-2-butanol (bottom to top: $\circ$, 0.13 mM; $\square$, 0.5 mM; $\triangle$, 2.5 mM; $\diamond$, 10 mM). (B) Enzyme (13 μN) reacted with 1 mM NAD^+ and (*S*)-2-butanol (bottom to top: $\circ$, 0.18 mM; $\square$, 0.5 mM; $\triangle$, 1 mM; $\diamond$, 2 mM). (C) Enzyme (8 μN) reacted with 8.4 μM NADH and 2-butanone (top to bottom: $\circ$, 10 mM; $\square$, 25 mM; $\triangle$, 50 mM). The points are the data, and the lines are the fits to the mechanism given in Table 8.

Fig. 5.

Stereoview of a model of (*R*)- and (*S*)- α -methylbenzyl alcohols (*sec*-phenethyl alcohols) in the active site of S48T/F93A ADH. *S*-MeBzl and *R*-MeBzl denote (*S*)- α -methylbenzyl alcohol and (*R*)- α -methylbenzyl alcohol, respectively. The coordinates for the enzyme and NAD⁺ are from the refined structure of the S48T enzyme-NAD⁺-pentafluorobenzyl alcohol complex (5KCP.pdb) with the additional “*in silico*” F93A substitution. Both alcohols are positioned so that the hydrogen (H⁻) to be transferred is pointing toward the C4N of the nicotinamide ring while the alcohol oxygen is ligated to the catalytic zinc. The ball and stick model (atom coloring) of (*R*)- α -methylbenzyl alcohol shows that the benzene ring can fit into the space created by the F93A substitution, but with a close contact with Ala-93. In contrast, the benzene ring of (*S*)- α -methylbenzyl alcohol fits into site where pentafluorobenzyl alcohol binds (4DWV.pdb [17]). The modeling shows that there is considerable space to accommodate both isomers, but does not explain why the *R*-isomer is oxidized 190-fold more efficiently than *S*-isomer by the S48T/F93A enzyme.

Table 1

Amino acid residues in the substrate binding sites of ADHs.

Res	<i>Eq1E</i>	<i>Hs1A</i>	<i>Hs1B</i>	<i>Hs1C</i>
48	Ser	Thr	Thr	Ser
57	Leu	Met	Leu	Leu
93	Phe	Ala	Phe	Phe
116	Leu	Val	Leu	Leu
140	Phe	Phe	Phe	Phe
141	Leu	Leu	Leu	Leu
294	Val	Val	Val	Val
318	Ile	Ile	Val	Ile

Table 2Steady-state kinetic constants for ADHs.^a

Kinetic Constant ^b	Wild-type ^c	S48T	S48A	F93A	S48T/F93A
K_a (μM), NAD ⁺	3.9	11	200	3.3	4.4
K_b (mM), ethanol	0.35	0.34	240	0.21	0.26
K_p (mM), acetaldehyde	0.40	0.53	100	41	21
K_q (μM), NADH	5.8	6.3	44	1.9	5.0
K_{ia} (μM), NAD ⁺	27	15	110	44	33
K_{iq} (μM), NADH	0.50	0.57	21	0.036	0.054
V_1/E_t (s ⁻¹)	3.5	1.7	0.01	0.35	0.29
V_2/E_t (s ⁻¹)	47	20	0.29	20	35
$V_1/E_t K_b$ (mM ⁻¹ s ⁻¹)	10	4.9	0.000075	1.7	1.1
$V_2/E_t K_p$ (mM ⁻¹ s ⁻¹)	120	38	0.0028	0.49	1.6
TN ^d (s ⁻¹)	2.4	6.4	0.04	0.24	0.28
K_{eq} ^e (pM)	16	50	49	27	12
K_i trifluoroethanol (μM)	8.4	98	ND	53	600
K_i pentafluorobenzyl alcohol (μM)	0.52	740	ND	51	>10,000

^aBuffer was 33 mM sodium phosphate and 0.25 mM EDTA, pH 8.0. Initial velocities were measured with systematically varied concentrations of both NAD⁺ and ethanol or NADH and acetaldehyde at 25 °C.

^b 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/E_t is the turnover number for ethanol oxidation and V_2/E_t is the turnover number for acetaldehyde reduction. Standard errors of the fitted kinetic constants were less than 15 % of the listed values except for K_a for which the standard deviation was 25 % or less than listed values.

^cFrom Ref. [40].

^dTurnover numbers in standard assay [26] at 30 °C, based on titration of active sites with NAD⁺ in the presence of 10 mM pyrazole [41].

^eThe equilibrium constant was calculated from the Haldane relationship, $K_{eq} = V_1 K_p K_{iq} [H^+] / V_2 K_b K_{ia}$. The experimentally determined, pH-independent equilibrium constant for the reaction, NAD⁺ + ethanol = NADH + acetaldehyde + H⁺, is 10×10^{-12} M [42].

Table 3X-ray data and refinement statistics for S48T ADH complexed with NAD⁺ and fluoroalcohols.

Alcohol	2,2,2-Trifluoroethanol	2,3,4,5,6-Pentafluorobenzyl alcohol
PDB entry	5KCZ	5KCP
cell dimensions (Å)	44.40, 51.38, 92.29	44.44, 51.59, 92.56
cell angles (deg)	91.97, 103.03, 110.28	91.87, 103.06, 110.33
mosaicity	0.71	0.61
resolution range (Å), (shell)	20.0–1.14 (1.17)	19.40–1.10 (1.14)
no. of reflections (total, unique)	910214, 243550	1493516, 269109
redundancy	3.72 (3.20)	5.52 (3.50)
completeness (%) (outer shell)	90.7 (54.6)	89.3 (56.7)
R_{meas} (%) (outer shell) ^b	4.7 (37.2)	6.1 (18.5)
average $\langle I \rangle / \sigma \langle I \rangle$ (outer shell)	13.8 (2.8)	15.5 (5.1)
R_{value} , R_{free} , test (%), number) ^c	12.6, 14.8, 0.5 (1206)	11.8, 13.8, 0.5 (1305)
rmsd for bond distances ^d (Å)	0.015	0.012
rmsd for bond angles ^d (deg)	1.84	1.73
estimated errors in coordinates (Å)	0.019	0.015
mean B -value (Wilson, REFMAC) (Å ²)	9.4, 14.9	10.2, 17.0
non H atoms fitted, total (B value)	6986	6979
protein (with alternative positions)	5784 (13.5)	5733 (15.3)
4 Zn, 2 NAD ⁺ , 2-4 alcohols, 4 MRD ^f	142 (16.5)	176 (20.0)
waters (with alternative positions)	1060 (27.9)	1070 (31.4)
Ramachandran, favored, outliers (%)	97.8, 0	97.5, 0
MolProbity: clash, score, rank	0.58, 98 th ; 0.75, 99 th	1.08, 98 th ; 0.91, 99 th
Matthews coeff., fraction solvent	2.26, 0.46	2.28, 0.46

^a The space group is $P1_1$, with one homodimeric molecule with 748 amino acid residues in the asymmetric unit.^c The data in the following three lines were calculated with the PARVATI server [23].^b R_{meas} , redundancy independent merging.^c $R_{\text{value}} = (\sum |F_o - kF_c|) / \sum |F_o|$, where k is a scale factor. R_{free} was calculated with the indicated percentage of reflections not used in the refinement. [43].^d Deviations from ideal geometry.^f MRD, (4*R*)-2-methyl-2,4-pentanediol.

Table 4

Substrate specificities of wild-type, F93A, and S48T/F93A enzymes on primary alcohols.

Enzyme	Alcohol					
	Ethyl	Propyl	Butyl	Pentyl	Hexyl	Benzyl
$V_i/E_t, s^{-1}$						
<i>EqADH1E</i>	7.3	4.9	4.6	6.1	5.7	3.1
<i>EqF93A</i>	0.25	0.23	0.28	0.23	0.24	0.25
<i>EqS48T/F93A</i>	0.36	0.36	0.43	0.39	0.40	0.57
<i>MmADH1A</i> ^a	1.1	1.4	2.2	1.8	1.6	0.75
$K_m, \mu M$						
<i>EqADH1E</i>	320	150	82	53	24	36
<i>EqF93A</i>	210	19	6.5	2.3	2.3	11
<i>EqS48T/F93A</i>	280	51	15	7.3	9.1	27
<i>MmADH1A</i>	15	2.0	0.19	0.045	0.032	0.036
$V/K_m, mM^{-1}s^{-1}$						
<i>EqADH1E</i>	23	32	56	110	230	86
<i>EqF93A</i>	1.2	12	43	100	100	25
<i>EqS48T/F93A</i>	1.3	7.1	29	53	44	21
<i>MmADH1A</i>	0.73	7.0	12	40	50	21

^aData for monkey (*Macaca mulatta*) enzyme at pH 7.3, 30 °C [5].

Table 5Substrate specificities of the alcohol dehydrogenases on secondary alcohols.^a

Enzyme	2-Propanol	2-Butanol		2-Octanol		sec-Phenethyl alcohol		Cyclo-hexanol
		R	S	R	S	R	S	
V ₁ /E _t , s ⁻¹								
wild-type	1.2	3.1	1.9	0.053 ^b	1.5 ^b	0.038	0.32	9.6
S48T	ND	1.4	0.97	0.041	0.98	ND	ND	ND
F93A	ND	0.33	0.34	0.28	0.27	0.15	0.24	0.30
S48T/F93A	0.45	0.51	0.43	0.56	0.50	0.34	0.096	0.56
K _m , mM								
wild-type	15	7.2	2.0	1.8 ^b	0.19 ^b	22	13	0.87
S48T	ND	28	17	1.8	0.94	ND	ND	ND
F93A	ND	0.063	0.072	0.013	0.0075	1.2	0.60	0.0059
S48T/F93A	0.70	0.088	0.33	0.053	0.094	3.0	11	0.027
V/K _m , mM ⁻¹ s ⁻¹								
wild-type	0.080	0.43	0.94	0.029 ^b	8.4 ^b	0.0017	0.025	11
S48T	0.020 ^c	0.050	0.057	0.023	1.0	ND	ND	0.055 ^c
F93A	ND	5.2	4.7	22	36	0.13	0.40	51
S48T/F93A	0.65	5.8	1.3	11	5.4	0.11	0.0087	21

^a *R* and *S* denote (*R*)- and (*S*)-enantiomers of secondary alcohols. ND denotes not determined.^b Values from Ref. [14].^c Apparent $V/K_m = v/[E][S]$, where v = initial velocity, $[E]$ = enzyme concentration, and $[S]$ = concentrations of substrate below the K_m value.

Table 6

Enantioselectivities of horse ADHs on secondary alcohols.

Ratio of V/K_m for (<i>R</i>)- to (<i>S</i>)-enantiomers			
ADH	2-Butanol	2-Octanol	<i>sec</i> -Phenethyl alcohol
WT	0.45	0.0034	0.067
S48T	0.88	0.023	ND
F93A	1.1	0.62	0.34
S48T/F93A	4.3	2.0	13.

Table 7Kinetic constants for the transient oxidation of alcohols and reduction of carbonyl compounds^a

Enzyme	Substrate	Concentration mM	$k_{\max}$ s ⁻¹	K_m mM
S48T/F93A	Ethanol	1–1000	72 (180)	38 (4.1)
	Acetaldehyde	10–50	58 (390)	150 (2.1)
	Benzyl alcohol	0.1–10	78 (24)	15 (0.03)
	Benzaldehyde	0.1–5	0.67 (320)	0.26 (0.49)
	(<i>R</i>)-2-Butanol	2–100	130 (15)	26
	(<i>S</i>)-2-Butanol	0.6–30	16 (2.5)	4.9
	2-Butanone	10–250	0.14 (0.12)	29
F93A	Ethanol	5–40	22	26
	Benzyl alcohol ^b	0.01–0.24	3.3 [4.0]	
	Benzaldehyde ^b	0.04–0.30	2.2 [3.4]	
	(<i>R</i>)-2-Butanol	0.83–5	44	1.2
	(<i>S</i>)-2-Butanol	1.7–5	29	1.3

^aThe change in absorbance at 328 nm from reactions of 6–15 μ N enzyme with 2 mM NAD⁺ and varied concentration of alcohols for oxidation or under single turnover conditions with NADH concentrations the same as enzyme concentration and varied concentration of carbonyl compounds for reduction were fitted by an equation for a first order reaction. The first-order rate constants increased hyperbolically as the concentration of substrates increased, and the data were fitted to the equation for a hyperbola to obtain the maximum values. Values in parentheses are the corresponding kinetic constants for the wild-type enzyme acting on benzyl alcohol/benzaldehyde and ethanol/acetaldehyde from Ref. [31] or 2-butanol and 2-butanone from Ref. [14].

^bData from Ref. [32]. Substrate deuterium isotope effects are enclosed in brackets.

Table 8Rate constants for the reaction of 2-butanol and butanone with S48T/F93A ADH.^a

step	forward (k_n)	(WT)	reverse (k_{-n})	(WT)
1	$1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$	(1.2×10^6)	59 s^{-1}	(90)
2	$8.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	(1.9×10^4)	2800 s^{-1}	(880)
3	$1.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	(0.54×10^5)	1900 s^{-1}	(170)
4	130 s^{-1}	(15)	0.070 s^{-1}	(1.0)
5	13 s^{-1}	(2.5)	0.020 s^{-1} ^b	(0.12)
6	20 s^{-1}	(2.5 or 4.3)	$1900 \text{ M}^{-1} \text{ s}^{-1}$	(180 or 6.5)
7	0.30 s^{-1}	(5.4)	$7.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$	(1.1×10^7)

^aRate constants for the binding of NAD^+ and NADH were determined by progress curve analysis in separate experiments and were fixed in this simulation. Progress curves (six for (R)-2-butanol, five for (S)-2-butanol, and three for 2-butanone) were combined in one simulation. Standard errors in the estimation of rate constants were 20 % or better, except for k_{-5} which was 110%. Values enclosed with parenthesis are rate constants for the wild-type enzyme at pH 8.5 and 25 °C, where the rate constants for step 6 have separate pathways for binding of “(pro-S)-2-butanone” (oriented to form (S)-2-butanol) given as the first value in parentheses and the binding of “(pro-R)-2-butanone” (to form (R)-2-butanol) as the second value [14]. Rate constants for coenzyme binding for wild-type enzyme were taken from Ref. [44].

^bThis value was estimated by the principle of detailed balance (thermodynamic linkage) for steps 2, 3, 4, and 5, and confirmed by simulations that show equal concentrations of (R)- and (S)-2-butanol at equilibrium.

Table 9

Inversion of stereoselectivity reflects productive binding

Enzyme	R-2-Butanol			S-2-Butanol		
Constant	K_d	k_H	k_H/K_d	K_d	k_H	k_H/K_d
Definition	k_{-2}/k_2	k_4	$k_2 k_4/k_{-2}$	k_{-3}/k_3	k_5	$k_3 k_5/k_{-3}$
Units	mM	s ⁻¹	mM ⁻¹ s ⁻¹	mM	s ⁻¹	mM ⁻¹ s ⁻¹
Wild-type	46	15	0.32	3.1	2.5	0.80
S48T/F93A	31	130	4.2	10	13	1.3

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