Inversion of the Substrate Specificity of Yeast Alcohol Dehydrogenase*

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The relationship between the size of the substrate binding pocket and the catalytic reactivities with varied alcohols was studied with the Saccharomyces cerevisiae alcohol dehydrogenase I (ScADH) and compared with the liver enzymes from horse (EqADH, EE isoenzyme) and monkey (MmA DHa, α-isoenzyme). The yeast enzyme is most active with ethanol, and its activity decreases as the size of the alcohol is increased, whereas the activities of the liver enzymes increase with larger alcohols. The substrate pocket in ScADH was enlarged by single substitutions of Thr-48 to Ser (T48S), Trp-57 to Met (W57M), and Trp-93 to Ala (W93A), and a double change, T48S:W93A, and a triple, T48S:W57M:W93A. The T48S enzyme has the same pattern of activity (V/K) as wild-type ScADH for linear primary alcohols. The W57M:W93A enzymes have lowered reactivity with primary and secondary alcohols. The wild-type  and T48S:W93A enzymes resemble MmA DHa in having an inverted specificity pattern for primary alcohols, being 3- and 10-fold more active on hexanol and 350- and 540-fold less active on ethanol, and are as reactive as the liver enzymes with long chain primary alcohols. The three Ala-93 enzymes also acquired weak activity on branched chain alcohols and cyclohexanol.

Alcohol dehydrogenases (EC 1.1.1.1) react with a wide variety of alcohols, aldehydes, and ketones (Brändén et al., 1975). Yeast alcohol dehydrogenases have more restricted specificity than mammalian liver enzymes. For example, the "ethanol-active" horse liver EE isoenzyme (EqADH) and the α-isoenzyme from monkey liver (MmA DHa) can oxidize benzyl alcohol and cyclohexanol (Light et al., 1992), but the constitutive isoenzyme I from yeast Saccharomyces cerevisiae (ScADH) cannot. Moreover, the activity of ScADH decreases as the chain length of the primary alcohols increases (Dickinson and Monger, 1973; Ganzhorn et al., 1987), whereas the activity of the liver enzymes increases (Dalziel and Dickinson, 1966; Light et al., 1992). The yeast and liver enzymes also have quite different activities and stereospecificities on secondary and branched chain alcohols (Dickinson and Dalziel, 1967a, 1967b; Dickinson and Dickinson, 1975; Stone et al., 1989).

The amino acid sequence homologies of the enzymes have allowed three-dimensional models of ScADH and liver enzymes to be constructed from the structure of EqADH (Eklund et al., 1976, 1987; Jörnvall et al., 1978; Ganzhorn et al., 1987). Such modeling suggests that the substrate pocket is smaller in ScADH than in the liver enzymes due to Thr-48, Trp-57, and Trp-93 (Table I). Eklund et al. (1987) proposed that Thr-48 decreased activity with cyclohexanol, and Höög et al. (1992) showed that the T48S substitution made the human β enzyme more active on cyclohexanol. However, HsADHa and MmA DHa have Thr-48 and highest activity on cyclohexanol (Stone et al., 1988; Light et al., 1992). The P93A substitution (along with T94I) in the human β enzyme increased efficiency for secondary alcohols but did not produce the pattern of activity exhibited by the α enzymes on primary alcohols (Hurley and Bosron, 1992).

This work tests the proposal that the lower activity of ScADH on larger alcohols is due to the smaller size of the substrate binding pocket. We show that enlarging the pocket significantly increases catalytic efficiency on long chain primary alcohols.

**EXPERIMENTAL PROCEDURES**

**Materials**—The oligodeoxyribonucleotides used for mutagenesis of the ScADH gene (*"mutamers") were synthesized on a Beckman instrument by the phosphoramidite method (Beaucage and Caruthers, 1981). CTGTCATCTGCTGTTG, CACGGTACATGCAAT TGC, and GTATCGAGCCGTAGAACGG were used to change the codons for Thr-48 to Ser (TCT), Trp-57 to Met (ATG), and Trp-93 to Ala (ATG). The numbering of residues in the yeast enzyme corresponds to the sequence of the horse liver enzyme, based on the alignment of Jörnvall et al. (1978).
Phage, as described by Ganzhorn and Plapp (1988). The W93A mutants were generated from a template containing uracil (Kunkel to Ala (GCG), respectively (underlines mark the mutations). The oligonucleotide procedure of Zoller and Smith (1984) using M13 mp18 Boehringer Mannheim.

were redistilled before use. Ethanol-ds was purchased from Fluka or the United States Biochemical Corp. Alcohols from Aldrich or Fisher MSD Isotopes. NAD+ and NADH were the best grades available from

Sequenase (Schena, 1989) was used for second strand synthesis for oligonucleotides used for sequencing the ScADH gene (Bennetzen and Hall, 1982) were complementary to the noncoding strand (Ganzhorn et al., 1982). Yeast Alcohol Dehydrogenase mutagenesis, and DNA sequencing were purchased from Bethesda
to Ala (GGC), respectively (underlines mark the mutations). The oligonucleotides used for sequencing the ScADH gene (Benen and Hall, 1982) were complementary to the noncoding strand (Ganzhorn et al., 1987). Enzymes and materials for M13 subcloning, mutagenesis, and DNA sequencing were purchased from Bethesda Research Laboratories, Amersham Corp., New England Biolabs, and United States Biochemical Corp. Alcohols from Aldrich or Fisher were redistilled before use. Ethanol-ds was purchased from Fluka or MSD isotopes. NAD+ and NADH were the best grades available from Boehringer Mannheim.

The T48S and W57M mutations were constructed with the double-oligonucleotide procedure of Zoller and Smith (1984) using M13 mp18 page, as described by Ganzhorn and Plapp (1988). The W93A mutants were generated from a template containing uracil (Kunkel et al., 1987) produced by Escherichia coli strains BW313 or CJ236. Sequenase (Schena, 1989) was used for second strand synthesis for the double and triple mutations with W93A. Mutations were con-

To A 10-fold range up to 100 mM at 5 mM NAD+; butanol or butanol-\(d_6\) was varied over a 10-fold range up to 100 mM at 5 mM NAD+. For T48S:W57M:W93A, the acids and NAD+ were varied over a 10-fold range up to 500 and 5 mM, respectively.

For T48S enzyme, the concentrations of ethanol or ethanol-\(d_6\) and NAD+ were varied over a 10-fold range up to 200 mM and NAD+ was varied from 0.1 to 4.0 mM. For W93A and T48S-W93A enzymes, the concentrations of ethanol and NAD+ were varied over a 5-fold range up to 1000 mM at 10 mM NAD+; butanol or butanol-\(d_6\) was varied over a 10-fold range up to 100 mM at 5 mM NAD+. For T48S:W57M:W93A, the acids and NAD+ were varied over a 10-fold range up to 500 and 5 mM, respectively.

TABLE II

Kinetic constants for wild-type and mutant yeast alcohol dehydrogenases

Measurements were made at 30 °C in a buffer of 83 mM potassium phosphate, 40 mM KCl, and 0.25 mM EDTA, pH 7.3.

| Constant* | Wild-type | T48S | W57M | W93A | T48S:W93A | T48S:W57M:W93A |
|-----------|-----------|------|------|------|-----------|----------------|
| \(K_a\) \(\mu\)M | 160 | 91 | 830 | 2300 | 920 | 590 | 5400 |
| \(K_b\) mM | 21 | 17 | 45 | 1600 | 4200 | 33 | 160 |
| \(K_{mNAD}\) mM | 0.74 | 0.74 | 5.7 | ND" | 89 | 5.9 | ND |
| \(K_{mAA}\) mM | 94 | 1180 | 280 | ND | 39 | 130 | ND |
| \(K_{max}\) mM | 950 | 1200 | 2900 | 530 | 320 | 240 | 4000 |
| \(V_i\) s" | 120 | 38 | 46 | ND | ND | ND | ND |
| \(V_i\) pm" | 1.5 | 1.4 | 1.1 | ND | ND | ND | ND |
| \(K_{aAA}\) \(pm\)" | 31 | 37 | 63 | 60 | 30 | 18 | ND |
| \(K_{aAA}\) \(mm\)" | 96 | 42 | 69 | ND | ND | ND | 2.8 |
| \(V_i\) m" | 366 | 200 | 220 | 110 | 140 | 7.8 | 120 |
| \(V_i\) s" | 1800 | 1500 | 1900 | ND | 530 | 120 | ND |
| \(V_i\) pm" | 12 | 9.1 | 11 | ND | 27 | 26 | ND |
| \(V_i\) mm" | 2.8 | 3.6 | 21 | 32 | 14 | 13 |
| Activity, s" | 400 | 220 | 94 | 30 | 16 | 10 |

* \(K_a\), \(K_b\), and \(K_{mNAD}\) are the Michaelis constants for NAD+, ethanol, acetaldehyde, and NADH (or butanol and butyraldehyde for one column). \(K_a\) values are inhibition constants. \(V_i\) and \(V_c\) are the turnover numbers of forward and reverse reaction. Standard errors ranged from 5 to 20%, except for \(K_a\) and \(K_{mNAD}\), which had errors up to 30%.

\(ND\) not determined.

\(T48S:W57M:W93A\) enzymes were calculated to be 1.14 and 0.95, respectively. Enzyme activity, active site concentrations, steady-state kinetic constants, and isotope effects were determined as described previously (Ganzhorn et al., 1987; Ganzhorn and Plapp, 1988). The buffer for enzyme kinetic studies was 83 mM potassium phosphate, 40 mM KCl, 0.25 mM EDTA at pH 7.3 and 30 °C, as an approximation to physiological conditions (Cornell, 1983; den Hollander et al., 1981).

Model Building—The amino acid sequences of ScADH (Jornvall et al., 1978) and MmADHa were aligned to the EqADH sequence. Residues in the ternary EqADH-NAD+-p-bromobenzyl alcohol complex (Eklund et al., 1982) were replaced with the corresponding amino acid residues in MmADHa and ScADH using the molecular modeling program PRODO (Jones, 1978; Jones and Soren, 1986) with an Evans & Sutherland PS300 computer graphics system. The MmADHa and EqADH sequences were readily aligned since both enzymes contain 374 amino acids per subunit and there is 96% sequence identity. Insertions and deletions required to align functionally important residues in ScADH followed the alignment of Jornvall et al. (1978) and did not occur within defined secondary structures. The most prominent deletion is of residues 119–139, which was accommodated by deleting the loop and joining residues 118 and 140 without changing the tertiary structure. The conformation of the ScADH peptide backbone where insertions and deletions were made was modeled from peptide structures of similar sequence from the Protein Data Bank (Jones and Soren, 1986). The models of MmADHa and ScADH conserved those residues that are thought to be important for the structure and mechanism (Eklund et al., 1976, 1982).

RESULTS

Kinetic Mechanisms—Initial velocity studies showed that the mutated yeast enzymes have sequential mechanisms and

| Substrate | Specificity | Yeast Alcohol Dehydrogenase | T793 |
|-----------|-------------|-----------------------------|------|
| Ethanol | 2.5 | 2.3 | 2.9 |
| Butanol | 4.1 | ND | 1.2 |
| Ethanol | 2.3 | 2.3 | 2.9 |
| Butanol | 4.1 | ND | 1.2 |
| Ethanol | 2.3 | 2.3 | 2.9 |
| Butanol | 4.1 | ND | 1.2 |

ND, not determined.

Deuterium isotope effects

For T48S enzyme, the concentrations of ethanol or ethanol-\(d_6\) and NAD+ were varied over a 10-fold range up to 200 mM and NAD+ was varied from 0.1 to 4.0 mM. For W93A and T48S-W93A enzymes, the concentrations of ethanol and NAD+ were varied over a 5-fold range up to 1000 mM at 10 mM NAD+; butanol or butanol-\(d_6\) was varied over a 10-fold range up to 100 mM at 5 mM NAD+. For T48S:W57M:W93A, the acids and NAD+ were varied over a 10-fold range up to 500 and 5 mM, respectively.

| Enzyme | Substrate | Isotope effects | \(V_i\) | \(V_i\) | \(V_i\) |
|--------|-----------|----------------|------|------|------|
| Wild-type | Ethanol | 1.8 | 1.8 | 3.2 |
| T48S | Ethanol | 1.7 | 1.2 | 1.7 |
| W57M | Ethanol | 2.3 | 2.3 | 2.9 |
| W93A | Ethanol | 3.9 | ND" | 3.0 |
| Butanol | 4.1 | ND | 3.4 |
| T48S:W93A | Ethanol | 2.5 | ND | 3.4 |
| T48S:W57M:W93A | Ethanol | 3.8 | 3.1 | 3.0 |

* Nomenclature of Northrop (1982).

\(\gamma\) Gould and Plapp (1990).

\(\gamma\) ND, not determined.

\(\gamma\) Inhibition constant of trifluoroethanol against ethanol.

\(\gamma\) Turnover number in standard assay (Plapp, 1970) at 30 °C, based on titration of active sites with NAD+ and pyrazole.
provided the fundamental kinetic constants given in Table II. The magnitudes of the constants for the wild-type and T48S enzymes were very similar, whereas the W57M enzyme had increased values for the constants associated with acetaldehyde (K_\text{a}) and ethanol. For the reaction of NADH and acetaldehyde catalyzed by the W93A and T48S:W57M:W93A enzymes, the K_\text{a} values were so large that good estimations were not feasible. The inhibition (dissociation) constants for NAD\(^+\) (K_\text{a}) and NADH (K_\text{a}) were similar for all of the enzymes except for the triple mutant, indicating that binding of coenzyme was not greatly affected by the mutations. The equilibrium constants calculated from the kinetic constants (Hal dane relationship) agreed well with the established value of 10 \text{pM}, indicating that the kinetic constants were self-consistent.

The mechanisms were further defined by product and dead-end inhibition studies. For the T48S and W57M enzymes, NAD\(^+\) and NADH were mutually competitive product inhibitors, whereas ethanol and acetaldehyde were mutually non-competitive inhibitors, as observed for wild-type enzyme (Ganzhorn et al., 1987). For the W93A and triple mutant enzymes, acetaldehyde appeared to be a competitive inhibitor against varied concentrations of NADH, indicative of a random addition of NADH and ethanol. The enzyme (Ganzhorn et al., 1987) agreed well with the established value of 5 \text{pM}, indicating that the kinetic constants were self-consistent.

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**TABLE IV**

| Alcohols | Ethyl | Propyl | Butyl | Pentyl | Hexyl | Heptyl | Octyl | Nonyl |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|
| Sc I     |       |       |       |       |       |       |       |       |
| Sc T48S  | 5.4   | 5.3   | 5.3   | 5.4   | 5.3   | 5.3   | 5.3   | 5.3   |
| Sc W57M  | 5.3   | 5.3   | 5.3   | 5.3   | 5.3   | 5.3   | 5.3   | 5.3   |
| Sc T48S:W93A | 5.3 | 5.3 | 5.3 | 5.3 | 5.3 | 5.3 | 5.3 | 5.3 |
| Sc T48S:W57M:W93A | 5.3 | 5.3 | 5.3 | 5.3 | 5.3 | 5.3 | 5.3 | 5.3 |

Activities were measured at 30 °C in a buffer of 83 mM potassium phosphate, 40 mM KCl, and 0.25 mM EDTA, pH 7.3. NAD\(^+\) was fixed at 1 mM for Eq E; 2 mM for Sc I, T48S, and Mm \alpha; 5 mM for Sc W93A and Sc T48S:W93A; and 10 mM for Sc W57M and Sc T48S:W57M:W93A. The concentrations of alcohols were varied over a 10-fold range, whenever possible, around the corresponding K_\text{a} values. The highest concentrations of substrates used for each enzyme are included in the table. Standard errors of the fits to the Michaelis-Menten equation were less than 10%.

*Ganzhorn et al. (1987), except the data for the three longest alcohols.

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*Light et al. (1992).*
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Deuterium isotope effects for ethanol oxidation (Table III) support the conclusion that the mechanisms of wild-type and T48S enzymes are predominantly ordered, with NAD⁺ binding before ethanol (Northrop, 1982; Ganzhorn and Plapp, 1988). For the other mutant enzymes, the larger values for V/ν suggest that the mechanisms approach rapid equilibrium random, with hydride transfer partially limiting turnover. The relatively large values for V/ν/Kₐ show that catalytic efficiency is also partly controlled by the chemical reaction.

**Substrate Specificities**—The activities of the various enzymes on a series of primary alcohols were determined with a fixed saturating concentration of NAD⁺ and varied concentrations of alcohol (Table IV). Results for EqADH and MmADHα are presented for comparison (Light et al., 1992). The turnover numbers (Vₐ or kₐ) showed different patterns for the various enzymes. Wild-type ScADH and the T48S, W57M, and W93A, and triple mutant enzymes show decreasing values with increasing chain length; the W93A and T48S:W93A mutants have the highest values with ethanol or long chain alcohols and the lowest values with butanol. The liver enzymes have almost constant values. These results reflect not only the differences in size and shape of the substrate binding pockets but also different rate-limiting steps in catalysis. With EqADH, for instance, release of NADH and isomerization of enzyme-NAD⁺ or ternary complexes can be rate-limiting for these alcohols (Sekhar and Plapp, 1990). Thus, the best measure of catalytic efficiency is Vₐ/Kₐ, which reflects the bimolecular binding and oxidation of the alcohol.

The V/K data are presented in logarithmic form in Fig. 1. Two general patterns emerge. For ScADH and the T48S and W57M mutants, catalytic efficiencies decrease as chain length increases, whereas for the liver enzymes and the W93A or T48S:W93A mutants of ScADH, reactivities increase with chain length. The contrasting behavior may be explained by the differences at residue 93, where enlarging the substrate binding pocket reduces activity with the smaller substrates but enhances activity with larger substrates that fit better in the pocket.

The W93A and T48S:W93A substitutions significantly increased catalytic efficiencies for large primary alcohols. The double mutation increased activity on hexanol, heptanol, and octanol by about 10-fold and made an enzyme that is at least as active on these substrates as the wild-type enzyme is on ethanol. The double mutant enzyme is as active as the horse enzyme on octanol. These results show the potential for engineering enzymes with enhanced activities.

**DISCUSSION**

Interpretation of the results is facilitated by reference to a model of the substrate binding site of ScADH with cyclohexanol as a potential substrate (Fig. 2). Modeling of cyclohexanol binding to the horse liver enzyme can explain the specificity of that enzyme for various derivatives of cyclohexanol (Horjales and Brandén, 1985; Lee et al., 1988). Cyclohexanol has multiple interactions with the horse enzyme, and Fig. 2 shows that Thr-48, Trp-57, Trp-93, and Met-294 would make close contacts (<4 Å) in the yeast enzyme. Since cyclohexanol is not a substrate with the yeast enzyme, some of these interactions may be too close, or some other part of the
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Activity was measured at 30 °C in a buffer of 83 mM potassium phosphate, 40 mM KCl, and 0.25 mM EDTA, pH 7.3. NAD⁺ was fixed at 1 mM for Sc I, T48S, W57M, W93A, T48S:W93A, T48S:W57M:W93A, W93A, W57M, and W93A. The concentrations of alcohols were varied over a 10-fold range around the corresponding Km values. The highest concentrations used are indicated in the table. Standard errors were less than 20%.

| ADH          | 2-Butanol | 2-Propanol | 2-Methyl-1-propanol | 3-Methyl-1-butanol | Benzy1 alcohol | Cyclo-hexanol |
|--------------|-----------|------------|---------------------|--------------------|----------------|--------------|
|              | R         | S          | S                   | RS                 |                |              |
|              | (mM)      | (mM)       | (mM)                | (mM)              |                |              |
|              | V (s⁻¹)   |            |                      |                    |                |              |

| Sc I         | 4.8       | 0.05       | 1.0                 | 0.19               | NA             | NA           |
| Sc T48S      | 4.0       | 0.11       | 1.3                 | 1.7                | 0.14           | NA           |
| Sc W57M      | 2.5       | 0.36       | 0.43                | 0.17               | NA             | NA           |
| W93A         | 1.7       | 0.098      | 0.097               | 0.34               | 0.16           | 0.40         |
| T48S:W93A    | ND⁺       | 0.019      | 0.19                | ND                 | 0.34           | 1.2          | 0.014        | 0.045        |
| T48S:W57M:W93A | 0.15     | 0.047      | 0.33                | 0.12               | 0.18           | 0.14         | 0.12         | 0.009        |
| Eq E         | 1.6       | 0.42       | 1.4                 | 0.62               | 0.80           | 0.77         | 2.1          | 0.78          | 3.0          |
| Mm ADHa      | 0.58      | 1.7        | 1.2                 | 5.3                | 5.7            | 5.3          | 2.8          | 3.6           | 6.1          |

Km (mM)

| Sc I         | 190       | 61         | 55                  | 25                 | NA             | NA           |
| Sc T48S      | 170       | 89         | 48                  | 42                 | 51             | 31           | 52           | NA           |
| Sc W57M      | 219       | 200        | 131                 | 68                 | NA             | NA           | NA           | ND           |
| W93A         | 1100      | 46         | 55                  | 110                | 58             | 43           | 46           | 15           | 43           |
| T48S:W93A    | ND        | 135        | 870                 | ND                 | 21             | 21           | 26           | 2.8          | 40           |
| T48S:W57M:W93A | 190     | 94         | 79                  | 32                 | 54             | 60           | 44           | 13           | 52           |
| Mm ADHa      | 3.5       | 0.42       | 1.4                 | 1.2                | 0.25           | 0.20         | 0.10         | 0.036        | 0.024        |
| Eq E         | 24        | 16         | 4.1                 | 0.40               | 0.31           | 0.52         | 0.15         | 0.046        | 1.1          |

V/K (μM⁻¹ s⁻¹)

| Sc I         | 25        | 0.8        | 18                  | 7.6                | NA             | NA           | NA           | NA           |
| Sc T48S      | 24        | 1.2        | 27                  | 40                 | 2.7            | 4.2          | 14           | NA           |
| Sc W57M      | 12        | 1.4        | 3.3                 | 2.5                | NA             | NA           | NA           | ND           |
| W93A         | 1.5       | 0.61       | 0.49                | 3.1                | 2.8            | 9.3          | 52           | 17           | 3.041        |
| T48S:W93A    | ND        | 0.14       | 0.22                | ND                 | 16             | 57           | 230          | 5.0          | 1.1          |
| T48S:W57M:W93A | 0.79     | 0.50       | 4.2                 | 3.8                | 3.3            | 2.3          | 2.7          | 9.2          | 0.17         |
| Mm ADHa      | 4.0       | 4.500      | 1000                | 520                | 3000           | 3500         | 21,000       | 21,000       | 130,000      |
| Eq E         | 24        | 110        | 290                 | 13,000             | 18,000         | 10,000       | 19,000       | 78,000       | 3500         |

Highest alcohol concentrations (mM)

| Sc I         | 500       | 200        | 200                 | 100                | 100            | 100          | 40           | 100          |
| Sc T48S      | 500       | 200        | 40                  | 100                | 100            | 100          | 40           | 100          |
| Sc W57M      | 500       | 100        | 100                 | 100                | 100            | 100          | 40           | 100          |
| W93A         | 1000      | 155        | 155                 | 150                | 40             | 40           | 40           | 40           | 150          |
| T48S:W93A    | ND        | 155        | 155                 | ND                 | 40             | 40           | 40           | 10           | 50           |
| T48S:W57M:W93A | 300     | 155        | 160                 | 150                | 40             | 40           | 40           | 40           | 43           | 150          |
| Mm ADHa      | 20        | 1          | 2                   | 1                  | 1              | 1            | 1            | 0.1          | 0.1          |
| Eq E         | 50        | 100        | 100                 | 1                  | 1              | 1            | 1            | 0.1          | 3            |

*NA, no measurable activity. In general, NA means that the activity was less than 1/10⁶ of that with the standard assay. Typically, concentrations of enzyme were 1000 times higher (4–100 μg/ml) than those used for assays with ethanol. If the rates of change in absorbance did not vary with varied substrate concentration and were approximately the same as without added substrate (typically <0.001 ΔA₄₅₀/min), it was concluded that there was no measurable activity. ND, not determined.

A substrate binding pocket may restrict access of cyclohexanol to the site. Mutagenesis studies provide a test of this model. Removal of one methyl group with the T48S substitution produced relatively small effects on the specificity for straight chain primary alcohols but significantly increased activity on the branched chain alcohols 2-methyl-1-propanol, 2-methyl-1-butanol, and benzy1 alcohol (Table V). Molecular modeling suggests that the T48S substitution should favor binding of the methyl group on the branched alcohols. The T48S enzyme was not active on cyclohexanol or 3-pentanol. Thus, the size of the pocket was further enlarged with the W57M and W93A substitutions. Mm ADHa has these residues and has highest activity on cyclohexanol (Light et al., 1992).

The W93A substitution substantially affected activity. Catalytic efficiency decreased on ethanol, but increased on hexanol, relative to wild type enzyme (Fig. 1). The single and double (with T48S) mutant yeast enzymes with Ala-93 resembled the monkey enzyme in the pattern of activity. These results can be explained simply by the increased volume in the substrate binding pocket. For ethanol, activity is probably decreased due to additional space in which the substrate can rotate into positions that cannot transfer hydrogen. Water molecules may also remain in the pocket while ethanol is bound, changing the microenvironment for hydride transfer. Larger substrates, such as hexanol or octanol, fill the pocket better, displacing water and making good interactions so that hydrogen can be transferred.

Such an explanation implies that variations in the size of the pocket should be correlated with the size of the best substrates. In general, the results in Table IV fit this expectation as ethanol is the best substrate with wild-type enzyme, octanol is best with the triple mutant, and pentanol is about equally reactive with most of the enzymes. On the other hand, the activity with branched alcohols (Table V) is considerably better, displacing water and making good interactions so that hydrogen can be transferred.
less than expected from the size of pocket as modeled, and cyclohexanol is at least three orders of magnitude less reactive with the T485S:W93A mutant yeast enzyme than with the liver enzymes. Thus, the shape or accessibility of the pocket appears to be different in the yeast and liver enzymes.

The liver enzyme is a dimer, with amine acid residues from both subunits forming the distal part of the substrate binding pocket. The yeast enzyme is a tetramer, and the arrangement of its subunits is unknown. Furthermore, the yeast enzyme has a deletion of 21 amino acid residues corresponding to residues 119–139 in the horse enzyme. This loop forms part of the bottom of the substrate binding pocket and has been classified as an Ω loop because of its shape (Leszcynski and Rose, 1986). It is easily deleted during model building since residues 118 and 140 are close together. Of course, the modeling does not provide the structure and should be substantiated by experimental evidence.

The results for straight chain primary alcohols (Table IV and Ganzhorn et al., 1987) can be explained with the model by changes in structure close to the catalytic zinc. Ethanol and 1-propanol are readily accommodated in an orientation suitable for hydride transfer, but with longer chain alcohols, steric hindrance could prevent optimal binding. Ganzhorn et al. (1987) showed that the activity with longer chain alcohols could be improved by 10-fold with the M294L substitution, which removed some potential bad contacts and provided apparently more favorable contacts. Changing the position of one methyl group significantly changed activity. In the present study, the activities for the secondary and branched chain alcohols reflect some unknown features of the active site.

The observations that the W57M substitution decreased activities in general and that the triple mutant (T485S:W57M:W93A) enzyme did not have the inverted specificity pattern may indicate that our modeling of the location of Trp-57 is not correct. The yeast enzyme has two residues inserted in this region, and alternative alignments are possible (Sun and Plapp, 1992). We expected the triple mutant enzyme to have specificity patterns resembling that of MmADHa. Nevertheless, the results with the W93A and M294L substitutions support the modeling near the catalytic zinc. Further studies are required to define the pocket, but the kinetic data lead to the suggestion that the substrate binding pocket of the yeast enzyme resembles a bottle, with a narrow opening that restricts access of bulky substrates to the site of catalysis on the zinc.

For the horse liver enzyme, binding of fatty acids to the enzyme-NAD+ complex increased with increasing chain length and reached a maximum at decanoate (Sund and Theorell, 1963). Such an observation is generally attributed to increased hydrophobic interactions of the hydrocarbon chain with the hydrophobic barrel of the substrate binding pocket (Branden et al., 1975). The barrel is open to the solvent and substrates have unrestricted accessibility. Even molecules resembling steroids could fit (Horjales and Brandén, 1985). Nevertheless, the horse EE isoenzyme is not active with steroid substrates, whereas a mutant enzyme where Asp-115 is deleted has good activity (Park and Plapp, 1992). The deletion apparently causes Leu-115 to move so that the steroid can be accommodated. Such a result indicates that both size and shape are important for catalytic activity.

This study illustrates how the pattern of activity of the yeast enzyme can be changed to resemble the pattern of the liver enzymes. Moreover, it shows that it is possible to alter the specificity of an enzyme without sacrificing catalytic power. Similarly, it was shown that the Q102R substitution in lactate dehydrogenase produced an enzyme with equivalent activity on oxaloacetate (Wilks et al., 1988).

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