Methionine-141 directly influences the binding of 4-methylpyrazole in human $\sigma\sigma$ alcohol dehydrogenase

PEIGUANG T. XIE AND THOMAS D. HURLEY

Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5122

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Abstract

Pyrazole and its 4-alkyl substituted derivatives are potent inhibitors for many alcohol dehydrogenases. However, the human $\sigma\sigma$ isoenzyme exhibits a 580-fold lower affinity for 4-methylpyrazole than does the human $\beta_1\beta_1$ isoenzyme, with which it shares 69% sequence identity. In this study, structural and kinetic studies were utilized in an effort to identify key structural features that affect the binding of 4-methylpyrazole in human alcohol dehydrogenase isoenzymes. We have extended the resolution of the human $\sigma\sigma$ alcohol dehydrogenase (ADH) isoenzyme to 2.5 Å resolution. Comparison of this structure to the human $\beta_1\beta_1$ isoenzyme structure indicated that the side-chain position for Met141 in $\sigma\sigma$ ADH might interfere with 4-methylpyrazole binding. Mutation of Met141 in $\sigma\sigma$ ADH to Leu (σ 141L) lowers the K_i for 4-methylpyrazole from 350 to 10 μ M, while having a much smaller effect on the K_i for pyrazole. Thus, the mutagenesis results show that the residue at position 141, which lines the substrate-binding pocket at a position close to the methyl group of 4-methylpyrazole, directly affects the binding of the inhibitor. To rule out nonspecific structural changes due to the mutation, the X-ray structure of the σ 141L mutant enzyme was determined to 2.4 Å resolution. The three-dimensional structure of the mutant enzyme is identical to the wild-type enzyme, with the exception of the residue at position 141. Thus, the differences in 4-methylpyrazole binding between the mutant and wild-type $\sigma\sigma$ ADH isoenzymes can be completely ascribed to the local changes in the topology of the substrate binding site, and provides an explanation for the class-specific differences in 4-methylpyrazole binding to the human ADH isoenzymes.

Keywords: alcohol dehydrogenase; enzyme kinetics; pyrazole; X-ray crystallography

Human alcohol dehydrogenases (EC 1.1.1.1, ADH) isoenzymes are dimeric, zinc-dependent oxidoreductases with subunit molecular masses of 40,000 Da. Seven ADH genes (*ADH1–ADH7*) have thus far been identified in humans (Edenberg & Bosron, 1997). The *ADH1–ADH5* genes encode the α , β , γ , π , and χ subunits, respectively, and the *ADH7* gene encodes the σ subunit. A protein product associated with the *ADH6* gene has not been identified in vivo (Yasunami et al., 1991). Polymorphism occurs at both the *ADH2* (β_1 , β_2 , β_3) and *ADH3* (γ_1 , γ_2) loci, such that nine distinct human ADH subunits have been identified (Ehrig et al., 1990). The human ADH isoenzymes have been assigned to five distinct classes based on their amino acid sequences, electrophoretic and enzymatic properties, and their sensitivity to inhibition by pyrazole and its four-substituted derivatives (Vallee & Bazzone, 1983; Edenberg & Bosron, 1997). The dimeric human $\alpha\alpha$, $\beta\beta$, and $\gamma\gamma$ isoenzymes and their polymorphic variants comprise the class I forms, and are the most sensitive to inhibition by 4-methylpyrazole and exhibit K_i

values in the low μ M range. In contrast, the $\pi\pi$, $\chi\chi$, $\sigma\sigma$, and *ADH6* isoenzymes comprise the class II, III, IV, and V forms, respectively, and are less sensitive to 4-methylpyrazole inhibition, with K_i values generally two to three orders of magnitude higher. However, it is not clear whether specific amino acid substitutions within the active sites of these other isoenzymes are responsible for the differences in affinity for 4-methylpyrazole or whether more generalized structural perturbations due to the overall 30–40% sequence variation are responsible.

The substrate-binding pockets of $\beta_1\beta_1$ and $\sigma\sigma$ ADH isoenzymes exhibit different topological features due to a number of amino acid substitutions. These different characteristics lead to different affinities for 4-methylpyrazole. The K_i for 4-methylpyrazole exhibited by $\sigma\sigma$ ADH is 580-fold greater than that of $\beta_1\beta_1$ ADH, reflecting a free energy of binding difference ($\Delta\Delta G$) of 3.8 kcal/mol (350 μ M vs. 0.6 μ M at pH 7.5; Kedishvili et al., 1995; Davis et al., 1996). We previously attributed the lower affinity for 4-methylpyrazole in $\sigma\sigma$ ADH to the distorted covalent bond angles between the pyrazole ring and the enzyme due to different positioning of the nicotinamide ring in the $\sigma\sigma$ ADH isoenzyme (Xie et al., 1997). In this paper, we report the kinetic and structural characterization of a mutant of $\sigma\sigma$ ADH (σ 141L) where residue 141 is mutated to Leu, the corresponding residue in $\beta_1\beta_1$ ADH.

Reprint requests to: Thomas D. Hurley, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5122; e-mail: thurley@iupui.edu.

Abbreviations: ADH, alcohol dehydrogenase; DTT, dithiothreitol; NCS, noncrystallographic symmetry; RMSD, root-mean-square deviation.

Materials and methods

Site-directed mutagenesis, protein purification, and crystallization

The mutant $\sigma\sigma$ ADH enzyme containing the Leu141 substitution was generated by oligonucleotide site-directed mutagenesis (Sculptor, Amersham Life Sciences Inc., Arlington Heights, Illinois). Single-stranded σ subunit cDNA in the M13HinEco1 vector (Edenberg et al., 1987) was used as the template to generate the mutant. The mutated cDNA was subcloned into the expression plasmid pKK223-3 and completely sequenced prior to expression to ensure no unwanted mutations were introduced by the mutagenesis procedure. The expression and purification of the protein were performed as reported previously (Xie et al., 1997). The protein was crystallized under conditions similar to those previously reported except that the crystals were grown at 4 °C rather than at room temperature. The components of the crystallization solution were 100 mM cacodylate, pH 6.5, 100 mM zinc acetate, 7.5 mM NAD^+ , 18% (w/v) polyethylene glycol 6000, and 8 mg/mL of the recombinant human $\sigma\sigma$ isoenzyme.

Steady-state kinetics

The steady-state kinetic constants (K_m and $V_{\max}$) were determined by single and covariations experiments at pH 7.5 in 100 mM sodium phosphate buffer at 25 °C. The inhibition constants (K_i) were determined at a fixed 2.5 mM NAD^+ concentration and by covariation of five different concentrations of ethanol and five different concentrations of inhibitor. The concentrations of ethanol and inhibitor ranged by at least eightfold, and spanned the calculated values of K_m and K_i . All individual rate measurements were run in duplicate, and each overall experiment was performed at least three times. All kinetic experiments were evaluated using the kinetic programs of Cleland (1979), modified for a personal computer with an input and output interface (T.D. Hurley, unpubl. results). The K_i values were calculated using the equation,

$$V = \frac{V_{\max}[S]}{K_m(1 + [I]/K_i) + [S]}$$

assuming that the inhibitor binds to a single enzyme species in a competitive manner. Fits to the noncompetitive and uncompetitive equations were also evaluated; in all cases the best fits, as determined by an analysis of variance, were to the competitive inhibition equation.

Diffraction data collection and processing

Two data sets, a wild-type $\sigma\sigma$ (2.5 Å) set and a σ 141L mutant (2.4 Å) set, were collected at −165 °C using a liquid nitrogen crystal cooling system (Molecular Structure Corp., The Woodlands, Texas). To stabilize the protein crystals for flash freezing, 30% (v/v) ethylene glycol was added to the crystallization solution as a cryoprotectant. The crystals were transferred to this cryoprotectant solution in a two-step procedure and immediately mounted in a loop of appropriate size for flash freezing by the cold nitrogen stream. Each data set was collected on a single crystal. All X-ray diffraction data were collected on a Rigaku 200HB rotating anode generator equipped with an RAXIS IIC image plate area detector using a crystal-to-detector distance of 145 mm and the appropriate 2θ offset for the resolution. The generator was operated at 100 kV

and 50 mA. All diffraction data were processed using the processing program Denzo/HKL (Otwinowski & Minor, 1997).

Molecular replacement and crystallographic refinement

The 2.5 Å $\sigma\sigma$ ADH structure was solved by the rigid-body refinement in X-PLOR 3.1 (Brünger, 1992) with the 3.0 Å room temperature structure (1AGN; Xie et al., 1997), followed by iterative cycles of refinement using X-PLOR 3.8 and manual inspection in the program CHAIN (Sack, 1988). The diffraction data between 45 and 8 Å were included in refinement by applying a bulk solvent correction (Jiang & Brünger, 1994) as implemented in X-PLOR 3.8. Fourfold NCS restraints were applied to the main-chain atoms throughout the refinement with a weight of 10.0 kcal/mol Å^{−2}. These restraints excluded those loops that make unique contacts within the crystal lattice; residues 13–21, 97–105, 133–141, 190–193, 209–218, 232–239, 338–342, and 354–364. The 2.4 Å σ 141L structure was solved using a partially refined 2.5 Å $\sigma\sigma$ ADH structure as the model. Refinement of this structure was performed following the protocol used for the 2.5 Å $\sigma\sigma$ ADH structure. Refinement of the catalytic and structural zinc atoms and their respective ligands were accomplished using a set of stereochemical restraints developed in our laboratory that maintain the geometry observed in the higher resolution structures of horse alcohol dehydrogenase. A total of 408 ordered water molecules, 13 bound acetate molecules (including one bound to the catalytic zinc atom in each subunit), and 11 surface bound zinc ions were added to the wild-type enzyme structure during refinement. A total of 371 ordered water molecules and an identical number of solvent acetate and zinc ions as added to the wild-type structure were added to the 141L mutant structure during refinement.

Reagents

NAD^+ , grade I, and DTT were purchased from Roche Molecular Biochemicals (Indianapolis, Indiana), PEG 6000 was purchased from Hampton Research (Laguna Niguel, California), ethanol was purchased from Midwest Grain (Pekin, Illinois). *trans*-2-Hexen-1-ol and all-*trans*-retinol were from Aldrich (Milwaukee, Wisconsin). All other reagents were from Sigma (St. Louis, Missouri) and were of the highest grades available and used without further purification.

Results

Kinetic constants of σ 141L

Residue 141 is located in the substrate-binding site, with its C_α 9.7 Å away from the catalytic zinc in $\sigma\sigma$ ADH. Met141 was mutated to Leu, as occurs in $\beta_1\beta_1$, in the σ 141L mutant. The kinetic constants for this mutant were determined (Table 1). Its K_m for ethanol does not change within the experimental error. The catalytic efficiency for substrates from ethanol to hexanol resembles those of wild-type $\sigma\sigma$ ADH. The largest change in substrate characteristics occurs with 1-propanol, where the $V_{\max}/K_m$ decreased approximately fivefold in the σ 141L mutant. The most striking difference between the wild-type and mutant enzymes is in the K_i for 4-methylpyrazole, a known potent inhibitor for many ADH isoenzymes. The K_i value decreased by 35-fold to 10 μM in the σ 141L mutant enzyme, compared to a value of 350 μM for wild-type $\sigma\sigma$ ADH (Table 2). However, the K_i for pyrazole was

Table 1. Kinetic constants for $\sigma\sigma$ and the $\sigma141L$ mutant ADH (pH 7.5)^a

Alcohol substrate	K_m (mM)		V_{max} (min ⁻¹)		V_{max}/K_m (mM ⁻¹ min ⁻¹)	
	$\sigma\sigma$	$\sigma141L$	$\sigma\sigma$	$\sigma141L$	$\sigma\sigma$	$\sigma141L$
Ethanol	28	33 ± 6	1,840	1,300 ± 160	65	42 ± 7
1-Propanol	1.4	6.0 ± 1.4	1,030	590 ± 40	570	100 ± 30
1-Butanol	0.79	0.90 ± 0.08	2,090	1,050 ± 20	2,640	1,170 ± 80
1-Pentanol	0.28	0.11 ± 0.03	960	710 ± 70	3,400	6,500 ± 800
1-Hexanol	0.13	0.035 ± 0.002	1,170	290 ± 10	9,000	8,500 ± 700
<i>trans</i> -2-Hexen-1-ol	0.019 ± 0.002	0.018 ± 0.002	2,200 ± 100	1,030 ± 10	120,000 ± 12,000	57,000 ± 5,600
All- <i>trans</i> -retinol	0.042 ± 0.002	0.014 ± 0.003	67 ± 3	31 ± 6	1,600 ± 100	2,300 ± 160

^aKinetic constants are expressed as the mean ± SD for the experimental data in this manuscript. The remaining values are from Kedishvili et al. (1995).

much less affected by the same mutation with a K_i value of 700 ± 100 μ M for the wild-type enzyme and a K_i value of 140 ± 30 μ M for the $\sigma141L$ mutant enzyme (Table 2).

Data collection and structure refinement

The structure of the human $\sigma\sigma$ ADH isoenzyme to 2.5 Å and the structure of a site-directed mutant of $\sigma\sigma$ ADH, $\sigma141L$, to 2.4 Å are presented in this paper. Each data set was collected at −165 °C using a single crystal. When compared to the room temperature data, the ability to eliminate scaling problems associated with radiation decay and with the use of multiple crystals for data collection dramatically improved the collection statistics and resolution of the data (Table 3).

Rigid-body refinement against the 3 Å $\sigma\sigma$ ADH structure (Xie et al., 1997) using data between 8.0–3.5 Å brought R_{free}/R_{work} from 44.7/44.8% to 34.6/33.5%. Simulating annealing brought R_{free}/R_{work} to 35.1/28.7%. The inclusion of the bulk-solvent correction without further refinement decreased both the R_{free} and R_{work} by 1%. The final structure of $\sigma\sigma$ ADH possesses a R_{work} value of 21.7% and R_{free} of 26.8%. The 2.4 Å $\sigma141L$ structure was solved by rigid body refinement using a partially refined 2.5 Å $\sigma\sigma$ structure and data between 8.0–3.5 Å. This brought R_{free} from 36.8 to 28.3% and R_{work} from 36.9 to 28.7%. The final structure has an R_{free} of 27.4% and an R_{work} of 21.6% (Table 4).

Structures of wild-type $\sigma\sigma$ and $\sigma141L$ mutant

A Ramachandran plot for the wild-type $\sigma\sigma$ ADH structure and for the $\sigma141L$ mutant showed that 99.2% of the 1,256 nonglycine and

nonproline residues were in the preferred and allowed regions, and 0.6% of the residues were in the generously allowed region (data not shown). Two residues, Cys174 in two subunits, were found in the disallowed region. Cys174 is one of the catalytic zinc ligands and its unusual peptide angles have been noted in other ADH enzymes (Eklund et al., 1976; Al-Karadaghi et al., 1994; Davis et al., 1996). Each of the four subunits in the wild-type and $\sigma141L$ mutant enzyme structures has a bound NAD⁺ molecule and an acetate ion bound in the active site. The acetate ion in the active site is directly liganded to the catalytic zinc ion and is within hydrogen bonding distance to the side-chain hydroxyl of Thr48, thus, roughly occupying the position of a substrate during catalysis. The wild-type and $\sigma141L$ mutant structures are essentially identical within the limits of the current resolution and exhibit RMSDs for all their C $_{\alpha}$ atoms in the range of 0.3 Å.

No difference in the main-chain conformation was observed between the new structures and the original 3 Å structure, and an alignment of the new 2.5 Å $\sigma\sigma$ subunit with the $\beta_1\beta_1$ structure showed no difference in relative domain orientation. As was observed in our 3 Å structure, the nicotinamide ring of the bound cofactor differs in its glycosidic dihedral angle by ~5° compared to its conformation in the class I human and horse ADH structures. The conformation of the nicotinamide ring appears to be independent of the zinc-bound ligand in the human $\beta\beta$ structures we have

Table 2. Inhibition constants for pyrazole derivatives in human $\beta_1\beta_1$ and $\sigma\sigma$ ADH isoenzymes (pH 7.)^a

Human ADH isoenzyme	K_{IS} for 4-methylpyrazole	K_{IS} for pyrazole
$\beta_1\beta_1$	0.6	4.1 ± 0.6
$\sigma\sigma$	350	700 ± 100
$\sigma141L$	10 ± 1	140 ± 30

^aThe competitive inhibition constants are expressed as the mean ± SD. The values for the inhibition of $\beta_1\beta_1$ and $\sigma\sigma$ by 4-methylpyrazole are from Davis et al. (1996), and Kedishvili et al. (1995), respectively.

Table 3. Crystal and data collection statistics^a

	Wild-type $\sigma\sigma$ ADH	$\sigma141L$ ADH
Resolution range	50–2.5 Å	50–2.4 Å
Subunits per asymmetric unit	4	4
Space group	P2 ₁	P2 ₁
Cell constants; <i>a</i> , <i>b</i> , <i>c</i> (Å), β (°)	86.4, 90.9, 121.1, 99.6	85.9, 90.5, 119.8, 99.3
Total observations	254,379	171,372
Unique reflections	58,678	64,234
<i>I</i> / σ ₁	17.1 (2.6)	16.5 (2.1)
R_{merge}	8.5% (36.8%)	7.7% (40.3%)
Completeness	91.4% (76%)	91.0% (77%)

^a $R_{merge} = \sum_{hkl} |I_i - I_n| / \sum I_n$, where I_i is an observed intensity, and I_n is the average of the observed equivalents. Values in parentheses are those for the highest resolution shell in each data set (2.6–2.5 Å for wild-type $\sigma\sigma$ and 2.5–2.4 Å for $\sigma141L$).

Table 4. Refinement statistics for $\sigma\sigma$ ADH structures^a

	Wild-type $\sigma\sigma$ ADH	σ 141L ADH
Resolution range included	50–2.5 Å	50–2.4 Å
Total number of reflections	58,662	64,216
Completeness for range (%)	91	91
$R_{\text{work}}/R_{\text{free}}$ (%)	21.7/26.8	21.6/27.4
Average B value (all atoms)	36.4	39.4
Luzzatti estimate of error (Å)	0.3	0.3
RMSD from ideal bond lengths (Å)	0.01	0.009
RMSD from ideal bond angles (°)	1.51	1.50
RMSD from ideal torsion angles (°)	24.8	24.6
RMSD from ideal improper angles (°)	1.39	1.42

^a $R_{\text{work}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{cal}}|| / \sum_{hkl} |F_{\text{obs}}|$, where $|F_{\text{obs}}|$ and $|F_{\text{cal}}|$ are the observed and calculated structure factor amplitudes of a reflection hkl , respectively. R_{free} is the same as R_{work} except that the summation is over the portion of data (7%) that is not included in the refinement.

solved; thus, we feel that the difference in the nicotinamide ring positioning observed between the $\beta_1\beta_1$ and the $\sigma\sigma$ isoenzymes is not due to the different zinc bound ligands.

Discussion

The improved resolution of the $\sigma\sigma$ ADH structure provides us with new information about the structure and function of $\sigma\sigma$ ADH. In general, the higher resolution structures are very similar to the original 3 Å structure (Xie et al., 1997). Thus, the unique main-chain path taken by residues 258 to 261 is unchanged in these structures, as is the conformation of residues 114–120 that contains the single amino acid deletion at position 117 relative to the class I enzymes (Xie et al., 1997). In addition, the difference in glycosidic dihedral angle of the nicotinamide ring, relative to the class I structures, is also observed in both higher resolution structures presented here. We now believe that this rotation of the nicotinamide ring is not due to the substitution of Ala317 in the class I structures by Cys317 in the class IV structure. It is more likely that the increased accessible volume of the active site in the $\sigma\sigma$ isoenzyme leads to a different ground state binary complex structure.

Aside from confirming many of our original observations, the higher resolution structures allow a more accurate fitting of several amino acid side chains. In fact, a new side-chain conformation for Met141 identified in the substrate binding pocket of these higher resolution structures led us to consider whether this residue was responsible for the class IV isoenzyme's weak interaction with pyrazole-based inhibitors.

It was discovered over 30 years ago that pyrazole and its derivatives with substituents the 4-position are good inhibitors for many ADH isoenzymes (Theorell et al., 1969). The inhibition constant for 4-methylpyrazole is often used to compare the kinetic characteristics of different ADH isoenzymes. In conjunction with the K_m for ethanol, the behavior of different ADH isoenzymes toward pyrazole formed the early basis for the distinction between the different classes of isoenzymes. For instance, the human class IV $\sigma\sigma$ ADH isoenzyme is inhibited by 4-methylpyrazole to a much lesser extent (580-fold) than is the class I $\beta_1\beta_1$ ADH isoenzyme

(Kedishvili et al., 1995). Likewise, the class II and class III isoenzymes are greater than 1,000-fold less sensitive to inhibition by 4-methylpyrazole than are the class I isoenzymes. Thus, the different classes of ADH isoenzyme bind this inhibitor with free energies that vary by >4.0 kcal/mol. Pyrazole is thought to mimic the transition state for alcohol dehydrogenases by donating a pair of electrons to the catalytic zinc atom and another pair of electrons to the C4 position of the nicotinamide ring through the adjacent nitrogen atoms on the pyrazole ring (Theorell et al., 1969; Eklund et al., 1982). Therefore, the variation in 4-methylpyrazole binding among the distinct classes of ADH isoenzymes might reflect differences in the nature of the transition-state in these isoenzymes (i.e., the extent of electrostatic stabilization of the alcoholate anion by the zinc atom). Alternatively, the differences in binding might be more simply due to amino acid substitutions at or near the catalytic zinc atom that alter the van der Waals interactions that help to stabilize the position of pyrazole in the active site.

We previously suggested that this weakened binding of 4-methylpyrazole to the $\sigma\sigma$ isoenzyme might be due to distorted bond angles and longer bond distances between the nitrogen atoms of the pyrazole ring and the catalytic zinc and the C4 of the nicotinamide ring (Xie et al., 1997). This suggestion was primarily based on the differences in the angular orientation of the nicotinamide ring within the active site of the $\sigma\sigma$ isoenzyme that we thought might be due to the larger amino acids at positions 309 and 317. However, mutation of Phe309 and Cys317 in $\sigma\sigma$ ADH to the corresponding residues in the $\beta_1\beta_1$ enzyme did not significantly affect the K_i for 4-methylpyrazole, but did affect substrate specificity (Xie et al., 1997). New positions for amino acid side chains in the substrate binding site of our higher resolution structure suggests an alternative explanation for the poor binding of 4-methylpyrazole. A structural alignment between this new $\sigma\sigma$ structure and class I $\beta_1\beta_1$ isoenzyme ternary complex with 4-iodopyrazole allows us to examine the relative positioning of the active site residues in $\sigma\sigma$ ADH compared to the positioning of 4-iodopyrazole in the $\beta_1\beta_1$ structure. This alignment indicates that the substitution of Met in the $\sigma\sigma$ structure for Leu in the $\beta_1\beta_1$ structure at position 141 sterically interferes with the binding of pyrazole compounds with substituents at the 4-position (Fig. 1). This is especially true of the iodine atom at the 4-position in the actual $\beta_1\beta_1$ structure, but is also true if a methyl group, with the appropriate changes in the bond distances, is substituted for the iodo group on the inhibitor (Fig. 1A). The distances between a methyl group at the 4-position on the pyrazole ring and two delta methyl groups of Leu141 in $\beta_1\beta_1$ are 3.8 and 4.5 Å, respectively. However, the distance between the epsilon methyl group of Met141 in $\sigma\sigma$ ADH and the methyl group at the 4-position of the pyrazole ring is only 2.2 Å, which is clearly too close for optimal van der Waals interactions. In addition, the delta sulfur atom of Met141 is also only 3.2 Å from the 4-methyl group of pyrazole; thus, simple rotation of the epsilon methyl group of Met141 still leaves a relatively close contact between the inhibitor and residue 141.

When Met141 is mutated to Leu in $\sigma\sigma$ ADH, the K_i for 4-methylpyrazole decreases 35-fold, from 350 to 10 μM (Table 2). The increased affinity for the inhibitor in the mutant enzyme seems to be attributable to the removal of the steric hindrance arising from Met141. This argument is further enhanced by the similarity of the inhibition constants of pyrazole toward wild-type $\sigma\sigma$ ADH and the σ 141L mutant. With the methyl group removed at the 4-position, the interaction between pyrazole and the side chain of residue 141 would be expected to diminish and, as a consequence,

Fig. 1. Binding of 4-methylpyrazole in the $\beta_1\beta_1$ isoenzyme and alignment to the active sites of the $\sigma\sigma$ and $\sigma141L$ enzymes. The $\sigma\sigma$ and $\sigma141L$ structures were aligned to the $\beta_1\beta_1$ structure using the C_α atoms of their respective coenzyme binding domains, and the resulting aligned set of coordinates were used to generate this figure using the program MOLSCRIPT (Kraulis, 1991). The binding of 4-methylpyrazole was modeled based on the position of 4-iodopyrazole in the $\beta_1\beta_1$ structure by replacing the iodo group with a methyl group. **A:** The modeled position of 4-methylpyrazole (green) in $\beta_1\beta_1$ ADH (PDB code; 1DEH, blue) and within the aligned structure of the wild-type $\sigma\sigma$ isoenzyme (red). **B:** The modeled position of 4-methylpyrazole in $\beta_1\beta_1$ (blue) compared to the aligned structure of the $\sigma141L$ enzyme (red).

we would expect the affinity for pyrazole to be similar in the wild-type and $\sigma141L$ mutant isoenzymes. Indeed, the difference in the inhibition constants for pyrazole exhibited by these two isoenzymes is only fivefold compared to the 35-fold difference when 4-methylpyrazole is used. The small difference in the pyrazole inhibition constant between the wild-type and $\sigma141L$ enzymes might indicate that Met141 still affects the binding of pyrazole by limiting access to the binding site.

To examine the structural consequences of the substitution at position 141 in $\sigma\sigma$ ADH, we solved the structure of the $\sigma141L$ mutant to 2.4 Å resolution. The structure is essentially identical to the wild-type structure except at position 141. The C_α alignments between the wild-type and mutant subunits yield RMSDs in the range of 0.3 Å, which is identical to the RMSD between individual subunits within each of the two $\sigma\sigma$ ADH structures. The similarity of the overall structures is reflected in their similar kinetic behavior toward alcohol substrates (Table 1). This contrasts to the large changes in Michaelis constants we observed with the $\sigma309L317A$ mutant (Xie et al., 1997). To compare the active site topologies and their possible influence on pyrazole binding, the $\sigma141L$ structure

was aligned to the $\beta_1\beta_1$ ADH structure with 4-methylpyrazole bound in the substrate binding site. The distances between the methyl group carbon atom and the terminal carbon atoms in $\sigma141L$ are 3.3 and 4.7 Å, respectively, which are similar to those observed in the $\beta_1\beta_1$ structure (Fig. 1B). Indeed, this model can also be used to explain the fivefold decrease in the $V_{\max}/K_m$ for 1-propanol observed for the $\sigma141L$ isoenzyme. The increased accessible volume in the substrate binding site resulting from the substitution of Leu for Met occurs exactly where the C3 position of 1-propanol would be located when productively bound within the active site.

Therefore, the kinetic and structural data show that the side chain of Met141 interferes with the binding of 4-methylpyrazole to $\sigma\sigma$ ADH. That additional as of yet unidentified factors also influence the binding of 4-methylpyrazole is indicated by the remaining 16-fold difference in 4-methylpyrazole binding observed between human $\beta_1\beta_1$ and the $\sigma141L$ mutant enzyme. The remainder of the difference in 4-methylpyrazole binding could be influenced by a number of structural features unique to the class IV isoenzyme. These features include the shortened loop created by the deletion of residue 117 that may influence solvent access to the active site

or the difference in the angular orientation of the nicotinamide ring. Alternatively, additional amino acid substitutions in the substrate binding site, such as Met57, could also influence access to the pyrazole binding site in $\sigma\sigma$ ADH.

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