X-ray Structure of Human Class IV \(\sigma\sigma\) Alcohol Dehydrogenase

STRUCTURAL BASIS FOR SUBSTRATE SPECIFICITY*

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The structural determinants of substrate recognition in the human class IV, or \(\sigma\sigma\), alcohol dehydrogenase (ADH) isoenzyme were examined through x-ray crystallography and site-directed mutagenesis. The crystal structure of \(\sigma\sigma\sigma\) ADH complexed with NAD\(^+\) and acetate was solved to 3-Å resolution. The human \(\beta_1\beta_1\) and \(\sigma\sigma\) ADH isoenzymes share 69% sequence identity and exhibit dramatically different kinetic properties. Differences in the amino acid positions at 57, 116, 141, 309, and 317 create a different topology within the \(\sigma\sigma\) substrate-binding pocket, relative to the \(\beta_1\beta_1\) isoenzyme. The nicotinamide ring of the NAD(H) molecule, in the \(\sigma\sigma\sigma\) structure, appears to be twisted relative to its position in the \(\beta_1\beta_1\) isoenzyme. In conjunction with movements of Thr-48 and Phe-93, this twist widens the substrate pocket in the vicinity of the catalytic zinc and may contribute to this isoenzyme’s high \(K_m\) for small substrates. The presence of Met-57, Met-141, and Phe-309 narrows the middle region of the \(\sigma\sigma\) substrate pocket and may explain the substantially decreased \(K_m\) values with increased chain length of substrates in \(\sigma\sigma\) ADH. The kinetic properties of a mutant \(\sigma\sigma\) enzyme (\(\sigma\sigma\sigma L317A\)) suggest that widening the middle region of the substrate pocket increases \(K_m\) by weakening the interactions between the enzyme and smaller substrates while not affecting the binding of longer alcohols, such as hexanol and retinol.

Human alcohol dehydrogenase (ADH) isoenzymes are NAD\(^+\)-dependent, zinc metalloenzymes that catalyze the reversible oxidation of alcohols to aldehydes. The ADH system is the major pathway for the metabolism of beverage ethanol as well as biological important alcohols or aldehydes like retinol, 3\(\beta\)-hydroxysteroids, \(\omega\)-hydroxy fatty acids, and 4-hydroxynonenal (1–3). Each isoenzyme in the ADH family is a dimer comprised of two 40-kDa subunits. The individual subunits are comprised of two domains, a catalytic domain and a coenzyme-binding domain (4). Seven human ADH genes (ADH1–ADH7) have been identified (1, 5). The ADH1–ADH5 genes encode the \(\alpha\), \(\beta\), \(\gamma\), \(\pi\), and \(\chi\) subunits, respectively. The protein product of the ADH6 gene has not been identified in vivo. The \(\sigma\) subunit is encoded by ADH7. Polymorphism occurs at both the ADH2 (\(\beta_1\), \(\beta_2\), and \(\beta_3\)) and ADH3 (\(\gamma_1\) and \(\gamma_2\)) loci (6), such that nine distinct human ADH subunits have been identified. ADH isoenzymes have been assigned to five distinct classes based on their amino acid sequences as well as their electrophoretic and enzymatic properties (7). The human \(\alpha\alpha\), \(\beta\beta\), and \(\gamma\gamma\) isoenzymes comprise class I, and the \(\pi\pi\), \(\chi\chi\), \(\sigma\sigma\), and ADH6 comprise classes II, III, IV, and V, respectively. All ADH isoenzymes are expressed in the liver except for \(\sigma\sigma\) ADH, which is primarily localized in epithelial tissue, such as the stomach mucosa (8, 9).

The three-dimensional structures of horse and human class I ADHs have been solved by x-ray crystallography (4, 10–12). Recently, the structure of human class III ADH was reported (13), as well as the structure of a cod liver ADH isoenzyme (14). Thus, an increasingly diverse structural data base exists from which information concerning the determinants of substrate recognition can be obtained by comparing the structures and kinetic properties of ADH isoenzymes. Important amino acids within the substrate-binding site directly affect the substrate specificity of the human ADH isoenzymes (Table I). For instance, \(\gamma_1\gamma_1\) ADH, which has a Ser at residue 48, is the only human isoenzyme able to bind and oxidize 3\(\beta\)-hydroxysteroids (15). Amino acid substitutions within the loops comprised of residues 55–61 and of residues 113–121 in \(\sigma\sigma\) ADH cause these loops to adopt new conformations and contribute to the enzyme’s inability to be saturated with ethanol (13). Mutagenesis studies on the \(\beta_1\beta_1\) isoenzyme indicate that residues 93 and 94 contribute to this isoenzyme’s high \(K_m\) for small substrates. Mutation of Thr-48 and Phe-93, this twist widens the substrate pocket in the vicinity of the catalytic zinc and may contribute to this isoenzyme’s high \(K_m\) for small substrates. The presence of Met-57, Met-141, and Phe-309 narrows the middle region of the \(\sigma\sigma\) substrate pocket and may explain the substantially decreased \(K_m\) values with increased chain length of substrates in \(\sigma\sigma\) ADH. The kinetic properties of a mutant \(\sigma\sigma\) enzyme (\(\sigma\sigma\sigma L317A\)) suggest that widening the middle region of the substrate pocket increases \(K_m\) by weakening the interactions between the enzyme and smaller substrates while not affecting the binding of longer alcohols, such as hexanol and retinol.

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‡The abbreviations used are: ADH, alcohol dehydrogenase; DTT, dithiothreitol; r.m.s.d., root-mean-square deviation; CRBP, cellular retinol-binding protein.

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The three-dimensional structures of horse and human class I ADHs have been solved by x-ray crystallography (4, 10–12). Recently, the structure of human class III ADH was reported (13), as well as the structure of a cod liver ADH isoenzyme (14). Thus, an increasingly diverse structural data base exists from which information concerning the determinants of substrate recognition can be obtained by comparing the structures and kinetic properties of ADH isoenzymes. Important amino acids within the substrate-binding site directly affect the substrate specificity of the human ADH isoenzymes (Table I). For instance, \(\gamma_1\gamma_1\) ADH, which has a Ser at residue 48, is the only human isoenzyme able to bind and oxidize 3\(\beta\)-hydroxysteroids (15). Amino acid substitutions within the loops comprised of residues 55–61 and of residues 113–121 in \(\chi\chi\) ADH cause these loops to adopt new conformations and contribute to the enzyme’s inability to be saturated with ethanol (13). Mutagenesis studies on the \(\beta_1\beta_1\) isoenzyme indicate that residues 93 and 94 contribute to this isoenzyme’s high \(K_m\) for small substrates. Mutation of Thr-48 and Phe-93, this twist widens the substrate pocket in the vicinity of the catalytic zinc and may contribute to this isoenzyme’s high \(K_m\) for small substrates. The presence of Met-57, Met-141, and Phe-309 narrows the middle region of the \(\sigma\sigma\) substrate pocket and may explain the substantially decreased \(K_m\) values with increased chain length of substrates in \(\sigma\sigma\) ADH. The kinetic properties of a mutant \(\sigma\sigma\) enzyme (\(\sigma\sigma\sigma L317A\)) suggest that widening the middle region of the substrate pocket increases \(K_m\) by weakening the interactions between the enzyme and smaller substrates while not affecting the binding of longer alcohols, such as hexanol and retinol.
nonspecific dehydrogenases (22). Retinol, in vivo, is bound to the cellular retinol-binding protein (CRBP). Evidence has been presented showing that holo-CRBP serves as substrate for microsomal dehydrogenases (22) and that CRBP may then transfer retinal to the cytosolic retinal dehydrogenase for oxidation to retinoic acid. Complete dependence on the CRBP pathway for retinoic acid production may deny accessibility of retinol to retinoic acid. However, retinoic acid synthesis during embryogenesis was reported to correlate spatiotemporally with the expression of class IV ADH gene (23). It was proposed that competitive inhibition by ethanol consumed during pregnancy can reduce retinoic acid synthesis and may contribute to the development of fetal alcohol syndrome (24, 25).

In this paper, we examine the structural basis for substrate recognition in σ ADH through x-ray crystallography and site-directed mutagenesis. By comparing the structures of the known human ADH substrate-binding sites, it may be possible to gain a more complete understanding of their roles in the metabolism of endogenous and exogenous alcohols.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Crystallization**—The cDNA for the σ subunit (5) in M13 was subcloned into the vector pK223-3 (Pharmacia Biotech Inc.) by site-directed mutagenesis using a commercial kit (Amersham Corp.) and expressed in Biotech Inc.) by site-directed mutagenesis using a commercial kit (Amersham Corp.). Single-stranded mutagenesis using a commercial kit (Amersham Corp.) and expressed in Biotech Inc.) by site-directed mutagenesis using a commercial kit (Amersham Corp.).

**RESULTS**

**Structure Determination**—The structure of the human class IV, or σ, ADH isozyme was solved to 3.0 Å by molecular replacement using the 2.2-Å structure of the class I human β1,3 isozyme (12) as the starting model. The final refined structure possesses an R_work of 22.5% and an R_free of 30.5% (Table I). The stereospecificity of this model was inspected using the program package PROCHECK (33). The Ramachandran plot showed that 98.6%, or 1471, of the 1492 residues were in the preferred and allowed regions, 1.4%, or 21, of the residues were in the generously allowed region. No nonglycine residues were found in the disallowed region. Due to

**TABLE I**

| Subunit Gene | Sequence identity to β1β4 | Amino acid residues in the substrate-binding site |
|--------------|---------------------------|-----------------------------------|
| α ADH1       | 94                        | T M A I V F M V M L                 |
| β1β4 ADH2    | 100                       | T L F T L F V M M L                 |
| γγ ADH3      | 95                        | S L F T L F V M M L                 |
| ππ ADH4      | 60                        | T F Y A N F V E I                   |
| χχ ADH5      | 62                        | T D Y I V Y M F V                   |
| ADH6         | 63                        | T H F L Q F G V Q F                 |
| α ADH1       | 69                        | T M F L I V Y M F                   |

were determined at a fixed NAD⁺ concentration of 2.5 mM, except those for 1-butanol which were determined both at 2.5 mM NAD⁺ and by co-variation of NAD⁺ and 1-butanol. All kinetic experiments were evaluated using the kinetic programs of Cleland (27). All reported values are expressed as the means of at least three separate experiments with their associated standard deviations.

**X-ray Diffraction Data Collection**—X-ray diffraction data were collected to 3 Å. Higher resolution data were observed initially (2.6 Å), but severe radiation decay and the inability to flash-cool these crystals prevented collection of the higher resolution data. Four crystals (approximate dimensions, 0.3 × 0.15 × 0.07 mm³) were used to collect the native data set at room temperature on a Rigaku 200HB rotating anode generator equipped with an RAXIS IIC image plate area detector with a crystal-to-detector distance of 145 mm. The data collection statistics are listed in Table II. All crystals exhibited radiation decay and were replaced every 12 h. The data were indexed, merged, and scaled using the RAXIS IIC data processing software (38).

**Molecular Replacement and Crystallographic Refinement**—The structure was solved by molecular replacement using the program package AMoRe (28) and the data between 15.0 and 4.0 Å. The β1β4 ADH ternary complex dimer with NAD⁺ and the inhibitor 4-iodopyrazole (Protein Data Bank code 1DEH (13)) served as the search model for these calculations. The correlation coefficients for the top two rotation solutions were 25.7 and 19.7, respectively. After the positions for the two dimers in the asymmetric unit were found, the starting model possessed a correlation coefficient of 54.7 and an R value of 39.9%. All subsequent model refinement was performed using the program package X-PLOR (version 3.1) (29). Rigid-body refinement of the initial model structure with the data between 8.0 and 5.5 Å brought R_work from 40.2 to 37.7% and R_free from 40.5 to 37.3%. The atomic positions were refined to 3 Å using the positional refinement protocol in X-PLOR (30) and a overall temperature factor of 25 A². The resulting structure was inspected using 2Fo − Fo and Fo − Fc maps in CHAIN (31). Amino acid substitutions were introduced as their positions were identified during refinement. Additional solvent z cations and solvent molecule were added when strong positive Fc − Fc electron density indicated their presence. In the last refinement procedure, an overall temperature factor for each subunit was refined, and the non-crystallographic symmetry restraints were removed. The final model possesses an average r.m.s.d. of 0.2 Å for the main chain atoms in the four subunits of the asymmetric unit. Cc alignments between σ ADH and β1,3 isoenzymes were performed using LSQKAB (32) in CCP4 (1994) suite and displayed using QUANTA (Molecular Simulations Inc., Burlington, MA).

**Reagents**—NAD⁺, grade I and DTT were purchased from Boehringer Mannheim, and PEG 6000 was purchased from Hampton Research; ethanol was purchased from Midwest Grain (Pekin, IL). All other reagents were from Sigma and were of the highest grade available.

**RESULTS**

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the presence of zinc acetate in the mother liquor, 8 solvent zinc cations and 10 acetate ions were identified as bound to the enzyme. There was an acetate ion present in the substrate pocket in all four subunits.

Kinetics of the σ ADH Mutant—A σ ADH mutant, α309L317A, was prepared by site-directed mutagenesis. Two residues in the σ isoenzyme, Phe-309 and Cys-317, were mutated to Leu and Ala, respectively. The choice of these two positions for mutagenesis was based on their unique characteristics compared with class I enzymes (Table I). Residue 309 is in the substrate-binding pocket, and residue 317 is behind the nicotinamide ring of NAD⁺. The substrate specificity of this mutant was studied and compared with wild-type σ ADH (Table III). Mutations at these two residues dramatically increase the \( K_m \) values toward small alcohol substrates. For instance, the mutant enzyme exhibits a \( K_m \) for ethanol that is 100-fold higher than the wild-type σ ADH. Interestingly, the \( K_m \) values for substrates with five or more carbons are less affected. The \( K_m \) values for hexanol and retinol are essentially unaffected by these mutations. As the chain length for straight alcohols increases, the \( V_{max} \) for the mutant enzyme increases to a greater extent than does the wild-type enzyme. The \( K_m \) value for NAD⁺ was approximately 2 times higher than the wild-type enzyme, whereas the \( K_m \) (NAD⁺) value obtained from experiments varying both 1-butanol and NAD⁺ concentrations was identical with the wild-type enzyme (0.75 ± 0.03 mM).

Structural Comparison with Other ADH Structures—An alignment of the Ca atoms, excluding residues 115–120 and 244–262, in the dimeric σ and \( \beta_1 \beta_1 \) isoenzymes gives a r.m.s.d. of 0.60 Å. Alignment of the individual domains within each subunit yields similar results, with r.m.s.d. values of 0.49 Å for the catalytic domain and 0.61 Å for the coenzyme-binding domain. Ca alignment of σ ADH with horse and other human ADHs shows that its structure is most similar to the human ADH and horse liver class I ADH isoenzymes. Unlike the recently reported structures of the human class III \( \chi \) isoenzyme (13) and the ADH isoenzyme from cod liver (14), both of which exhibited semi-open domain structures, the human isoenzyme exhibits a fully closed conformation of the catalytic and coenzyme-binding domains when NADH is bound. The alignments reveal that, relative to \( \beta_1 \), there are two major structural differences in each domain of the σ subunit (Fig. 1). In the coenzyme binding domain, the largest difference occurs at the C terminus of an α-helix comprised of residues 251–258 and the following turn. This structural change results from the substitu-
a larger active site in $\chi$ ADH (13).

Our structural comparisons also reveal that there is no evidence that the interaction between coenzyme and residue 223 is weakened in $\sigma$ ADH, as was suggested by a modeling study (34). In fact, the hydrogen-bonding distances between adenosine ribose oxygens and $\gamma$ oxygens of Asp-223 are within the range of 2.6–2.7 Å in both $\sigma$ and $\beta_1\beta_1$ ADHs.

### DISCUSSION

The alcohol binding pocket is an extension of the coenzyme binding site (4) and is fully formed only after coenzyme binding has occurred. In $\sigma$ ADH, the substrate pocket is a cylinder having dimensions of approximately 16 by 7 by 6 Å. The substrate specificity for this enzyme is determined by surface complementarity between the enzyme and the substrates throughout this cylinder. Changes in the $K_m$ values are related to the effective concentration of the ES complex. Mutations can affect $K_m$ by changing the ratio of productive versus non-productive encounters with the enzyme. In ADH, these changes are brought about either through steric exclusion (preventing productive binding), as seen for the binding of secondary alcohols to $\beta_1\beta_1$ ADH (16), or by changing the number of non-productive conformations permitted by altering the accessible volume of the active site (13). The inner part of the alcohol site (near the catalytic zinc) includes residues 48 and 93 and the nicotinamide ring of NAD$^+$. $\sigma$ ADH has a $K_m$ value for ethanol that is 560-fold higher than $\beta_1\beta_1$ ADH (Table III). One possible cause for this difference may be the substitution of Cys for Ala-317 near the nicotinamide ring. To accommodate its longer side chain in $\sigma$ ADH, the main chain atoms of residue 317 move $\sim$1 Å away from Thr-186, toward the carboxamide group relative to $\beta_1\beta_1$ ADH (Fig. 2). To avoid unfavorable contacts with the Cys-317 carbonyl oxygen, the plane of nicotinamide ring appears to twist in $\sigma$ ADH, relative to its position in the $\beta_1\beta_1$ isoenzyme (Figs. 2 and 3). This twist creates more space between the nicotinamide ring and the catalytic zinc (Fig. 3). In addition to these changes, Thr-48 and Phe-93 also shift away from the catalytic zinc. The distance between the Cα atoms of these two residues is 0.9 Å longer in $\sigma$ ADH. Consequently, smaller substrates, such as ethanol, are not as conformationally constrained in this active site as in the $\beta_1\beta_1$ ADH. Thus, a higher concentration of ethanol is required to produce an equivalent number of productively bound conformations.

The structural differences near the catalytic zinc in these two isoenzymes may also explain the weak binding of the inhibitor pyrazole to $\sigma$ ADH ($K_i$ values of 0.60 μM for $\beta_1\beta_1$ and 350 μM for $\sigma$ at pH 7.5 (5, 12)). Pyrazole and its 4-substituted derivatives competitively inhibit the binding of alcohol substrates through the formation of a tight enzyme-NAD$^+$-inhibitor complex (35), in which pyrazole nitrogens interact with both zinc and NAD$^+$. We speculate the bond between the pyrazole nitrogen atom and the C-4 atom on the nicotinamide ring may be distorted due to the twist of the nicotinamide ring in $\sigma$ ADH. In fact, if the active sites of the $\sigma$ and $\beta_1\beta_1$ structures are aligned and the position of 4-iodopyrazole in the $\beta_1\beta_1$ active site structure is used to examine the geometric constraints on pyrazole binding to the $\sigma$ structure, the corresponding angle between the C-4–N-1 and the N-1–N-2 bond is $133^\circ$, while it is close to $120^\circ$ in the $\beta_1\beta_1$ structure, corresponding to a low energy, stable complex. This angular difference would undoubtedly represent a higher energy conformation and could account for up to 2.7 kcal/mol of the observed difference (3.8 kcal/mol) using a harmonic potential with a force constant of 0.27 kcal/(mol°) (36). In addition, the increased distances between the N-1 of pyrazole and the C-4 of the nicotinamide ring (by 0.3 Å) and between residues 48 and 93, where pyrazole is held, could contribute to lowering the affinity for 4-methylpyrazole.

The middle region of the substrate pocket, which plays an important role in the interactions with the aliphatic tail of longer substrates, such as butanol and pentanol, includes residues 57, 141, 294, and 309. Like many other ADH isoenzymes, $\sigma$ ADH exhibits $K_m$ values for primary straight chain alcohols, which decrease with increasing chain length, whereas the $V_{\text{max}}$ values remain relatively constant. Thus, the catalytic efficiency ($V_{\text{max}}/K_m$) increases with increasing chain length. For example, the $V_{\text{max}}/K_m$ for hexanol is 138-fold higher than that for ethanol in $\sigma$ ADH (Table III). In contrast, the $V_{\text{max}}/K_m$ values for $\beta_1\beta_1$ ADH vary only 2- to 3-fold for substrates from ethanol to hexanol. These different characteristics can be explained by differences in the amino acids within the middle region of the substrate pocket. The key amino acids within this region are residues 57, 141, and 309. $\beta_1\beta_1$ ADH possesses Leu residues at all these positions, and their side chains do not appear to create new productive interactions as substrates get longer (Table III). The presence of Phe at position 309, Met at position 141, and Met at position 57, in $\sigma$ ADH, narrows the middle region of the substrate pocket compared with $\beta_1\beta_1$ (Fig. 3). The twist
on the nicotinamide ring also contributes to a shift in Phe-309, to avoid unfavorable contact with NAD(H), which further narrows the channel leading to the catalytic zinc. Although this narrowing does not appear to directly aid the binding of ethanol, it can explain the decreased $K_m$ values for propanol, butanol, and pentanol in the $\sigma$ isoenzyme relative to $\beta_1\beta_1$ ADH.

Our modeling indicated that the side chain of residues 57 and 309 would interact with the substrates at the carbon 4, 5, and 6 positions, whereas the side chain of residue 141 interacts with carbon 3 and 4. In the $\sigma$309L317A mutant, the $K_m$ values for propanol, butanol, and pentanol are increased by 240-, 60-, and 7-fold, confirming the role of residues 309 and 317 in stabilizing the binding of these substrates. Moreover, the increase in $V_{\text{max}}/K_m$ versus the chain length of the substrates is greater for the mutant than for the wild-type enzyme. This behavior in the mutant enzyme is due to a much lower $V_{\text{max}}/K_m$ for small substrates, since the $V_{\text{max}}/K_m$ values for hexanol approach those of the wild-type enzyme. The substitutions in the mutant enzyme thus appear to further widen the substrate-binding site, relative to $\sigma$ ADH, resulting in a greater number of permissible non-productive $E_S$ complexes. These changes in the mutant only affect substrates where binding is dependent on the local topology, such as ethanol, but do not significantly affect the catalytic efficiency toward hexanol or retinol.

The outer part of the substrate-binding pocket is exposed to solvent and includes the loop comprised of residues 114–120 and residue 306 from the other subunit within the dimer. The deletion of residue 117 in $\sigma$ ADH shortens the loop comprised of residues 114–120 (Fig. 3) and widens the entrance to the substrate-binding site. In $\beta_1\beta_1$ ADH, Leu-116 appears to function as a door, opening to allow substrates in or out, but then closing to help keep in bound substrate (17). Consistent with this function, its side chain was found to occupy different conformations in binary and ternary complexes (10–12). In $\sigma$ ADH, the shift in the position of residue 116 due to the deletion of residue 117 does not permit its side chain to function in this manner, leaving an open substrate-binding site (Fig. 3). Widening of the bottleneck at position 116 in $\beta_1\beta_1$ ADH by mutagenesis dramatically increased the apparent $K_m$ values for primary and secondary alcohols (17). Consistent with these observations, $\sigma$ ADH has higher $K_m$ values than the $\beta_1\beta_1$ isoenzyme for straight chain alcohols and very poor efficiency toward all secondary alcohols (5). The $K_m$ for hexanol and retinol are virtually identical in the wild-type and mutant enzymes. With the enlarged entrance to the substrate pocket in the $\sigma$ isoenzyme, the structure of the middle and inner regions of the substrate pocket would seem to be best suited for the oxidation of long chain aliphatic alcohols, such as $\omega$-hydroxy fatty acids, farnesyl alcohols, and retinol. To examine long chain alcohol binding, all-trans-retinol was docked into the active site using program AUTODOCK (37). The results of this simulation confirm our previous results based on modeling studies (5), the $\beta$-ionone ring of retinol binds at the widened entrance of the substrate-binding pocket, such that an ex-
tended conformation of retinol can be adopted. Thus, the ability to bind retinol in a more extended and, presumably, lower energy conformation in $\sigma$ ADH could account for its higher catalytic efficiency.

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