Biophysical and mutagenic analysis of *Thermoanaerobacter ethanolicus*
secondary-alcohol dehydrogenase activity and specificity

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The *Thermoanaerobacter ethanolicus* 39E adhB gene encoding the secondary-alcohol dehydrogenase (2° ADH) was over-expressed in *Escherichia coli* at more than 10% of total protein. The recombinant enzyme was purified in high yield (67%) by heat-treatment at 85 °C and (NH₄)₂SO₄ precipitation. Site-directed mutants (C37S, H59N, D150N, D150E and D150C were analysed to test the peptide sequence comparison-based predictions of amino acids responsible for putative catalytic Zn binding. X-ray absorption spectroscopy confirmed the presence of a protein-bound Zn atom with ZnS₄(Nimid)(NO₃) co-ordination sphere. Inductively coupled plasma atomic emission spectrometry measured 0.48 Zn atoms per wild-type 2° ADH subunit. The C37S, H59N and D150N mutant enzymes bound only 0.11, 0.13 and 0.33 Zn per subunit respectively, suggesting that these residues are involved in Zn liganding. The D150E and D150C mutants retained 0.47 and 1.2 Zn atoms per subunit, indicating that an anionic side-chain moiety at this position preserves the bound Zn. All five mutant enzymes had ≤ 3% of wild-type catalytic activity, suggesting that the *T. ethanolicus* 2° ADH requires a properly co-ordinated catalytic Zn atom. The His-59 and Asp-150 mutations also altered 2° ADH affinity for propan-2-ol over a 140-fold range, whereas the overall change in affinity for ethanol spanned a range of only 7-fold, supporting the importance of the metal in 2° ADH substrate binding. The lack of significant changes in cofactor affinity as a result of these catalytic Zn ligand mutations suggested that 2° ADH substrate- and cofactor-binding sites are structurally distinct. Altering Gly₁⁹⁸ to Asp reduced the enzyme specific activity 2.7-fold, increased the Kₐ(app) for NAD⁺ 225-fold, and decreased the Kₐ(app) for NAD⁺ 3-fold, supporting the prediction that the enzyme binds nicotinamide cofactor in a Rossmann fold. Our data indicate therefore that, unlike the liver 1° ADH, the Rossmann-fold-containing *T. ethanolicus* 2° ADH binds its catalytic Zn atom using a sorbitol dehydrogenase-like Cys-His-Asp motif and does not bind a structural Zn atom.

INTRODUCTION

Alcohol dehydrogenases (ADHs), central to prokaryotic and eukaryotic metabolism, are also potential biocatalysts for chiral chemical production [1–6]. The functionally and presumably structurally similar ADHs are classified as primary or secondary based on their higher catalytic efficiencies toward primary (1°) or secondary (2°) alcohols. These typically homodimeric or homotetrameric enzymes are almost exclusively Zn-containing metalloenzymes [7] that use NAD(H) (EC 1.1.1.1), NADP(H) (EC 1.1.1.2) or both (EC 1.1.1.71) as cofactor. Fe-linked [8] and ferrodoxin F₄₃0-dependent [9] 1° ADHs have also been reported. The catalytic role of Zn in liver 1° ADH activity has been proposed to involve binding directly to the substrate oxygen atom, facilitating hydride transfer between the adjacent substrate carbon atom and the nicotinamide cofactor [7,10–12]. Although 1° ADHs from diverse sources have been extensively characterized [7], little is known about the molecular basis for their substrate specificity. Far less is known about 2° ADH structure-function, with relatively few reported studies on enzyme purification and characterization [13–17] and a complete lack of site-directed mutagenic information. Recent reports of thermophilic and mesophilic 2° ADH crystallization and X-ray diffraction [18–20] provide the promise of exact structural data; however, the inability of structural characterization alone to explain 1° ADH catalytic properties underscores the importance of a multidisciplinary approach to 2° ADH structure-function research. Because of the central metabolic roles of 2° ADHs [17,21] and their potential biotechnological value [1–6,22,23], structure-function analysis of catalysis and substrate specificity would contribute significantly to the basic understanding of enzyme function and to biocatalyst design.

The exact three-dimensional structure of horse liver 1° ADH has been determined [24], indicating that the catalytic Zn atom is bound by two Cys and one His residue and that the nicotinamide cofactor is bound in a Rossmann fold [7,25]. Research to date has failed to identify the ADH active-site residues specifically responsible for substrate binding. The presumed similarity, based on shared catalytic properties, between 1° and 2° ADH structures provided the rationale for sequence-based comparisons between these enzymes [26–28]. Peptide sequence alignments of thermophilic and mesophilic 1° and 2° ADHs were used to hypothesize (i) that 2° ADHs bind their nicotinamide cofactor in a Rossmann fold, (ii) that they lack a structural Zn-binding loop, and (iii) that they use a unique Cys-His-Asp (Cys⁷²-His⁹⁸-Asp¹³⁸) motif for catalytic metal liganding [28]. Chemical-modification experiments have implicated Zn, Cys and His in *Thermoanaerobium brockii* and *Thermoanaerobacter ethanolicus* 39E 2° ADH ca-

Abbreviations used: ADH, alcohol dehydrogenase; XAS, X-ray absorption spectroscopy; ICP-AES, inductively coupled plasma-atomic emission spectrometry.

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talyis [13,28], but validation of the predicted Zn-liganding motif awaited assessment by biophysical characterization and site-directed mutagenesis. The strong similarities among 2° ADH peptide sequences, kinetic parameters, subunit compositions and molecular masses [28,29] suggest that the results of the T. ethanolicus 2° ADH structure–function analysis will be generally applicable to other 2° ADHs.

The research reported here involves the development of a recombinant overexpression and purification system providing high yields of homogeneous enzyme. The roles of T. ethanolicus 2° ADH amino acids Cys²⁷, His⁵⁹ and Asp³⁵⁹ as catalytic Zn-binding ligands and of Gly¹³⁸ as the single residue responsible for NADP(H) specificity are assessed from site-directed mutation effects on enzyme $K_m$(app), $V_{max}$(app), Zn and cofactor binding. X-ray absorption spectroscopic (XAS) studies support this catalytic Zn-co-ordination sphere. Finally, kinetic data on 2° ADH mutants and exact structural information on 1° ADHs are used to create a hypothetical working model for the 2° ADH active site and to distinguish key differences between 2° and 1° ADH structure–function relationships.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals were reagent/molecular biology grade or purer. Oligonucleotide synthesis and amino acid sequence analysis were performed by the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University. The kanamycin-resistance GenBlock (EcoRI) DNA cartride used in expression vector construction was purchased from Pharmacia (Uppsala, Sweden) [30]. DNA for sequencing was isolated using the Wizard Miniprep kit (Promega, Madison, WI, U.S.A.).

Media and strains

Escherichia coli (DH5α) containing the 2° ADH recombinant plasmids were grown in rich complex medium (20 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) at 37 °C in the presence of 25 µg/ml kanamycin and 100 µg/ml ampicillin.

Mutagenesis

All DNA manipulations were performed using established protocols [31,32]. Point mutations were introduced into the T. ethanolicus 39E adhB gene by PCR [31] using plasmid pADHB25-kan [28] as template. An oligonucleotide primer (KA4N) was synthesized complementary to the non-coding strand that included a KpnI restriction site, the native adhB ribosome assembly site, and the adhB translation initiation codon (5'-CGGGTACCCGGTTAGAGGTGGTTATATAATAGGAAAGGGG-3'). A second oligonucleotide primer (KA4C) that included the complement to the adhB termination codon and an Apal restriction site (5'-CATGCTCAGGCGGCCTTTATGCTAATATTACAACAGGTGGTTG-3') was synthesized complementary to the coding strand. Oligonucleotide 1M1N was identical with primer KA4N except for the substitution of TTA for ATG at positions 31–33. Complementary 30–45-base oligonucleotide primers that contained the mutated bases (C37S: coding, 5'-GGTCTCCGGCC-CTTCCACCTGGAATCATCATACC-3', non-coding, 5'-GGTCTGAAAAGGGAAGGGG-3'); H59N: coding, 5'-CATGATCTCCGGTCAAGCGAAAGTCTCTGACTGTA-3', non-coding, 5'-CATGATCTCCGGTCAAGCGAAAGTCTCTGACTGTA-3'; D150N: coding, 5'-GGAGCTCACTAAAACTGACAGGACTGG-3', non-coding, 5'-GGCTCCGTGAAACCCAGTGGTCATCATAAGAACCATATAAGAACCATATAAG-3'; D150E: coding, 5'-GGGAAGCTGGAATGTCCGAAGTC-3', non-coding, 5'-GGCTCCGTGAAACCCAGTGGTCATCATAAGAACCATATAAGAACCATATAAG-3'; D150E: coding, 5'-GGGAAGCTGGAATGTCCGAAGTC-3', non-coding, 5'-GGCTCCGTGAAACCCAGTGGTCATCATAAGAACCATATAAGAACCATATAAG-3'. The recombinant enzyme was purified from E. coli DH5α by affinity chromatography on a C4-Sepharose column. The recombinant enzyme was purified from E. coli DH5α by affinity chromatography on a C4-Sepharose column. The recombinant enzyme was purified from E. coli DH5α by affinity chromatography on a C4-Sepharose column. The recombinant enzyme was purified from E. coli DH5α by affinity chromatography on a C4-Sepharose column.
of the recombinant 2° ADH protein population expressed from the pADHBKA4-kan plasmid were reported as standard deviations and were calculated on the basis of the relative abundances of the two amino acid species present at each of the first seven peptide positions.

**RESULTS**

**Enzyme overexpression**

*T. ethanolicus* 2° ADH was purified from *E. coli* (DH5α) harbouring plasmid pADHBKA4-kan (Table 1) with a 67% recovery. The recombinant enzyme was expressed at more than 10% of total soluble protein without induction (Figure 1). This construct contained the native ribosome assembly site and translational initiation codons but lacked the native promoter regions, placing the gene under transcriptional control of the pBluescriptII KS(+) lacZ promoter. The recombinant protein population included 56±11% enzyme with a duplicated N-terminal methionine (MMKGFA…) while 44±11% of the protein possessed a single methionine at the N-terminus. Alteration of the ATGATG translational initiation site to TTAATG in the pADHB1M1-kan expression plasmid eliminated the synthesis of 2° ADH with two N-terminal methionines but altered neither the enzyme expression level compared with that seen with pADHBKA4-kan nor the enzyme specific activities toward ethanol and propan-2-ol. The K_m(app) values toward propan-2-ol, ethanol and NADP⁺ were 1.1±0.22 mM, 53±9.0 mM and 17±2.6 µM respectively for the enzyme expressed from pADHBKA4-kan and 0.87±0.32 mM, 37±4.2 mM and 8.5±1.4 µM respectively for the enzyme expressed from pADHB1M1-kan.

**Catalytic Zn ligation**

Chemical-modification experiments have implicated Zn, Cys and His in *T. ethanolicus* 2° ADH activity. Peptide alignments identified Cys37, His99 and Asp130 as potential catalytic Zn ligands [28]. Zn XAS data of native 2° ADH (not shown) were identical with those for the recombinant wild-type 2° ADH (Figure 2). Extraction of the raw XAS data for the recombinant wild-type enzyme by Fourier filtering of the first-shell FT peak, followed by single-shell curve-fitting, suggested a Zn(N,O)₄S₄ first coordination sphere (results not shown). Non-linear least-square fits to unfiltered smoothed EXAFS data (Figure 2b) allow estimation of the number of histidine imidazole ligands. Table 2 summarizes the results obtained assuming either one or two imidazole ligands. The negative Debye–Wall–Waller σ_σ^2 values for the Zn–S ligand and the unnecessarily large Zn–C₉ and Zn–Nᵢ₈ σ_σ^2 values indicate that the co-ordination sphere {Zn(imid)₄(N,O)₄S₄}.

<table>
<thead>
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<th>Table 1 Purification of the recombinant wild-type 2° ADH expressed from plasmid pADHBKA4-kan</th>
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<td>Purification step</td>
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<td>------------------------------------------------------------</td>
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<tr>
<td>Cell extract</td>
</tr>
<tr>
<td>Heat-treatment</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
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</table>

**Figure 1 SDS/PAGE of wild-type recombinant 2° ADH**

Lanes: 3, cell extract of *E. coli* DH5α harbouring plasmid pBluescriptII KS(+) (+); 2, clone KA4 cell extract; 1, clone KA4 dialysed (NH₄)₂SO₄ precipitate. The molecular masses of the standards flanking the sample lanes are indicated on the left. Proteins were stained with Coomassie Brilliant Blue R-250.

![Image](image-url)
tested in fit 1 is physically improbable. The two-sulphur single-imidazole-containing co-ordination sphere modelled in fit 4 \((Zn(\text{imid})(N,O))_3S\) is also an unlikely match to the 2° ADH Zn-liganding environment because of the unreasonably large Zn–S Debye–Waller \(\sigma^2\) values. Although either fit 2 or 3 in Table 2 is reasonable, if two imidazoles are assumed (fit 2) the Debye–Waller \(\sigma^2\) values for outer shell scatterers are 2–3-fold greater than those for the fit including a single imidazole (fit 3). Therefore, on the basis of this analysis, a 5- rather than a 4-co-ordinate Zn site of composition \(Zn(N,O)(\text{imid})_2S\) (fit 3, Table 2) is most consistent with the observed data (Figures 2b and 2c).

Mutant \(adhB\) genes encoding substitutions of residues 37, 59 and 150 were constructed by PCR to test the involvement of these amino acids in catalytic Zn binding. Mutant proteins were expressed at levels similar to, and purified to homogeneity using these amino acids in catalytic Zn binding. Mutant proteins were constructed by PCR to test the involvement of wild-type ADH catalytic Zn-liganding motif, explaining the observed activity. All Zn levels measured by ICP-AES were more than 100-fold higher than the background value. ICP-AES analysis indicated that the enzyme expressed from the wild-type gene (pADHBA4-kan) and the single N-terminal Met mutant enzyme bound 0.47 and 0.50 Zn\(^{2+}\) ions per subunit respectively. The C37S and H59N mutant enzymes contained less than 25\% of the Zn retained by the wild-type enzyme. Altering Asp\(^{150}\) to Glu preserved the wild-type Zn level, whereas that in the D150N mutant was 70\% of wild-type. Changing the putative Asp\(^{150}\) ligand to Cys increased bound Zn 2-fold. Finally, the Gly\(^{198}\) to Asp mutation did not significantly alter 2° ADH Zn content.

Point mutations altering Cys\(^{37}\), His\(^{59}\) and Asp\(^{150}\) significantly reduced enzyme \(V_{\text{max(app)}}\) (Table 3). The conservative Cys\(^{37}\) to Ser mutation replaced the putative Zn ligand residue with one less than 0.4 Å shorter. This mutant retained less than 1\% of the wild-type enzyme \(V_{\text{max(app)}}\) toward both ethanol and propan-2-ol. Replacing His\(^{59}\) with Asn eliminated the ionizable nitrogen atom and reduced the enzyme \(V_{\text{max(app)}}\) to less than 0.5\% of that of the wild-type. The D150C mutant, which presumably mirrored the Cys-His-Cys 1° ADH catalytic Zn-liganding motif, retained approx. 3\% of the wild-type enzyme activity, the highest specific activity among the catalytic Zn mutants. Replacing the Asp carboxy moiety with a thiol group preserved its strong negative ionic character but shortened the amino acid side chain.

Table 2 Curve-fitting results for Zn K-edge smoothed EXAFS of wild-type 2° ADH

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<th>Sample</th>
<th>(k) range</th>
<th>Fit</th>
<th>Group</th>
<th>Shell</th>
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<th>(R_{\text{ref}}(\AA))</th>
<th>(\sigma^2) ((\AA^2))</th>
<th>(\Delta E_0) (eV)</th>
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\[ I' = \frac{\Omega (k^2 \tilde{\chi}^\text{obs} - \chi^\text{theo})^2/M^1/2}{[k^2 \tilde{\chi}^\text{obs} - k^2 \tilde{\chi}^\text{theo}]} \]

The \(N\_f\) numbers in parentheses were not varied during the optimization. Underlined values are chemically or physically unreasonable. Numbers in square brackets were constrained to be a multiple of the value immediately above.

Figure 2 Zn K-edge XAS data for recombinant wild-type \(T.\ ethanolicus\) 2° ADH

(a) Raw XAS data in the Zn K-edge region. (b) plot of Fourier-transformed (FT) data versus distance for raw (- - -) and smoothed (-----) Zn EXAFS data compared with simulation (-----) using parameters from fit no. 3 in Table 4. (c) FT of smoothed EXAFS (-----) compared with FT of the simulation (-----). Both FTs used \(k^2\)-weighted EXAFS data over the range \(k = 1.5–10.5\AA\).
by approx. 0.6 Å. Mutating Asp<sup>150</sup> to Glu also retained the electronic characteristics for metal binding but it lengthened the side chain approx. 1.3 Å. The <i>V</i><sub>max(app)</sub> of this mutant was 0.2% of the wild-type specific activity. Significantly reducing the polarity of this residue while maintaining wild-type geometry, the D150N mutant retained less than 1% of the wild-type <i>V</i><sub>max(app)</sub>.

### Cofactor and substrate specificity

On the basis of the hypothesis of a specific amino acid position responsible for NAD(H) versus NADP(H) cofactor specificity in Rossmann-fold-containing proteins [41], our 1<sup>°</sup> ADH—2<sup>°</sup> ADH peptide alignment [28] predicts that mutating Gly<sup>198</sup> in the <i>T. ethanolicus</i> 2<sup>°</sup> ADH peptide to Asp would exclude NADP(H) from the cofactor-binding site but allow NAD(H) binding. The wild-type enzyme had a 140-fold lower <i>K</i><sub>n(app)</sub> for NADP<sup>+</sup> than for NAD<sup>+</sup> and a catalytic efficiency for propan-2-ol oxidation 2400-fold greater using NADP<sup>+</sup> than using NAD<sup>+</sup> (Table 4). Replacing Gly<sup>198</sup> with Asp increased the <i>K</i><sub>n(app)</sub> for NADP<sup>+</sup> 225-fold and reduced the <i>V</i><sub>max(app)</sub> using NADP<sup>+</sup> 4-fold. The mutant enzyme had a 3-fold lower <i>K</i><sub>n(app)</sub> toward NAD<sup>+</sup> and a 5.5-fold higher propan-2-ol oxidation rate using NAD<sup>+</sup> compared with wild-type, giving the mutant a 6.7-fold lower catalytic efficiency for NADP<sup>+</sup>-linked propan-2-ol oxidation than for the NAD<sup>+</sup>-dependent reaction. In contrast with the G198D mutant, all of the enzymes containing mutations to the putative catalytic Zn-ligand residues had high affinities for NADP<sup>+</sup> (<i>K</i><sub>n(app)</sub> values less than 40 μM (Table 3)). The high <i>K</i><sub>n(app)</sub> and low <i>V</i><sub>max(app)</sub> values using NAD<sup>+</sup> measured for these mutants were similar to those for the wild-type 2<sup>°</sup> ADH (results not shown).

Mutational effects on 2<sup>°</sup> ADH affinity for 1<sup>°</sup> alcohol substrates and on the catalytic efficiencies for oxidizing these substrates indicate a role for the putative catalytic Zn ligands in substrate specificity and in catalytic rate enhancement. Mutating Cys<sup>37</sup> to Ser increased the <i>K</i><sub>n(app)</sub> values for propan-2-ol and ethanol 6-fold and 20-fold respectively (Table 3). Determining the <i>K</i><sub>n(app)</sub> for ethanol of the C37S mutant was complicated by our inability to measure enzyme initial rates using substrate concentrations greater than 700 mM and lower than 300 mM. This <i>K</i><sub>n(app)</sub> value is reported for completeness, but it is an approximate value and is not included in subsequent data analysis. Replacing His<sup>59</sup> with Asn caused a 6-fold increase in the <i>K</i><sub>n(app)</sub> toward propan-2-ol and no significant change in the <i>K</i><sub>n(app)</sub> toward ethanol. The <i>K</i><sub>n(app)</sub> for propan-2-ol of the D150C mutant was similar to the wild-type value and that for ethanol was increased only 2-fold. Mutating Asp<sup>150</sup> to Glu caused a 3-fold increase in the <i>K</i><sub>n(app)</sub> for ethanol but a 29-fold increase in the <i>K</i><sub>n(app)</sub> toward propan-2-ol. Finally, the D150N mutant had a <i>K</i><sub>n(app)</sub> toward propan-2-ol 5-fold lower than the wild-type and a <i>K</i><sub>n(app)</sub> toward ethanol 4-fold greater than the wild-type enzyme. The catalytic efficiencies of all mutants were lower than those of the wild-type enzyme but, for each mutant enzyme, the ratio of the catalytic efficiencies for propan-2-ol versus with ethanol oxidation varied from 4-fold lower (the D150N mutant) to 10-fold higher (the D150N mutant) than that calculated for the wild-type enzyme.

### DISCUSSION

The research described here provides direct evidence for the involvement of specific amino acid residues in <i>T. ethanolicus</i> 39E 2<sup>°</sup> ADH catalysis. The presence of a single specifically bound Zn<sup>2+</sup> ion in the enzyme subunit and the identities of at least two of the three potential liganding protein residues were confirmed by EXAFS analysis. ICP-AES data for the wild-type and mutant enzymes implicated Cys<sup>37</sup>, His<sup>59</sup> and Asp<sup>150</sup> in catalytic Zn binding. Kinetic measurements of mutant enzymes implicated Cys<sup>37</sup>, His<sup>59</sup> and Asp<sup>150</sup> in both catalytic rate enhancement and substrate specificity. Transforming the highly NADP(H)-specific <i>T. ethanolicus</i> 2<sup>°</sup> ADH into an NAD(H)-preferring enzyme with
the Gly\textsuperscript{198} to Asp mutation further validated the sequence comparisons between 1\textdegree{} and 2\textdegree{} ADH, provided experimental evidence that this 2\textdegree{} ADH binds its nicotinamide cofactor in a Rossmann fold, and made the \textit{T. ethanolicus} enzyme more suitable as an industrial biocatalyst. Furthermore construction of the overexpression system provided sufficient yield of purified enzyme for structural analysis and potential industrial-scale biocatalyst production.

The original \textit{T. ethanolicus} 39E 2\textdegree{} ADH gene was expressed in \textit{E. coli} DH5\textalpha{} under the control of its own promoter at approx. 2\% of total protein [28]. In \textit{E. coli} this construct generated a mixed 2\textdegree{} ADH population containing single and double N-terminal methionines, unlike the homogenous single N-terminal methionine-containing population isolated from \textit{T. ethanolicus}. Removing the native promoter region, placing the \textit{adhb} gene under the control of the pBluescriptII KS\textsuperscript{(+)} lacZ promoter (in the pADHBKA4-kan construct), increased the 2\textdegree{} ADH expression to more than 10\% of total protein. High-level expression of a homogeneous 2\textdegree{} ADH population containing a native-enzyme-like single N-terminal methionine was then achieved by modifying the pADHBKA4-kan \textit{adhb} translational initiation ATGATG region to TTAATG (pADHB1M1-kan). A simplified purification procedure using only heat-treatment and (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} precipitation reduced enzyme losses to 33\% while eliminating time-consuming and costly chromatography steps. The similar kinetic results reported here for the homogeneous and mixed recombinant enzyme populations and those reported previously for enzyme isolated from \textit{T. ethanolicus} and the mixed recombinant enzyme populations and those reported previously for enzyme isolated from \textit{T. ethanolicus} and the mixed recombinant protein [28] indicate that neither the additional Met residue nor its codon is needed for expression or processing of binant protein [28,29] indicate that neither the additional Met residue \\

including oxygen ligands plus a \textit{T. ethanolicus} 2\textdegree{} ADH cysteine sulphur and imidazole nitrogen. This is similar to the coordination sphere reported for the 1\textdegree{} ADH catalytic Zn but inconsistent with the four cysteine sulphurs that co-ordinate the 1\textdegree{} ADH structural Zn [7]. The low ICP-AES measured Zn content (\textless{} 1 per subunit) also supports the predicted lack of a 1\textdegree{} ADH-like polycysteine-ligated structural Zn in 2\textdegree{} ADHs [27,28]. These EXAFS and ICP-AES analyses of the wild-type recombinant enzyme identifying a specifically bound Zn provide direct experimental evidence supporting the sequence- and chemical inactivation-based prediction that the \textit{T. ethanolicus} 2\textdegree{} ADH contains a catalytic Zn [28]. However, kinetic analyses of site-directed 2\textdegree{} ADH mutants are required to identify the amino acid ligands and to confirm the catalytic importance of this bound metal.

Peptide alignments of 1\textdegree{} and 2\textdegree{} ADHs identified \textit{T. ethanolicus} 2\textdegree{} ADH residues Cys\textsuperscript{207} and His\textsuperscript{209} as being analogous to two of the liver 1\textdegree{} ADH catalytic Zn ligands [24]. These alignments placed Asp\textsuperscript{150} (conserved in 2\textdegree{} ADHs) in the sequence position corresponding to the second catalytic Zn–Cys ligand of liver 1\textdegree{} ADH. The nearest 2\textdegree{} ADH Cys residue to this position is Cys\textsuperscript{208}, C-terminal to the Rossmann-fold residue Gly\textsuperscript{198}. Although a Cys-His-Asp Zn-binding motif has not been reported for ADHs, it has been described for sorbitol dehydrogenases [42]. Mutating Cys\textsuperscript{207} to Ser, His\textsuperscript{209} to Asn or Asp\textsuperscript{150} to Asn eliminated them as potential Zn ligands and reduced enzyme activity to less than 1\% of wild-type, implicating them in catalysis. The molar ratios of bound Zn per subunit for these mutants were 4-fold, 4-fold and 1.4-fold lower respectively than that for the wild-type enzyme, implicating them in Zn binding. The lack of a substantial change in bound metal as a result of the Gly to Asp mutation at position 198 (predicted to be unrelated to catalytic Zn liganding by sequence alignment and its demonstrated effect on cofactor specificity) further underscores the significance of the reduced Zn content measured for these position-37, -59 and -150 mutants. Altering Asp\textsuperscript{150} to Glu indicated that maintaining the electronic nature of the Asp side chain preserved Zn binding, but the mutant’s dramatically reduced \(V_{\text{max}}\) (app) (0.2\% of wild-type) suggested that the position of the carboxylate was critical to catalysis. The D150C mutant, designed to create the 1\textdegree{} ADH-like Cys-His-Cys catalytic Zn-binding motif, doubled the molar ratio of enzyme-ligated Zn, implicating this residue in Zn binding by enhancing rather than diminishing metal content. The \(K_{\text{m}}\) (app) values resulting from this spatially conservative mutation were also less than those for the D150E mutant, suggesting that the metal position is important to enzyme affinity for substrate. Whether ICP-AES measured 2\textdegree{} ADH Zn-binding stoichiometry (one Zn per dimer) or gauged the enzyme’s Zn affinity relative to that of the Chelex resin under the dialysis conditions does not alter the conclusion that 2\textdegree{} ADH affinity for Zn is specifically affected by non-conservative mutations at positions 37, 59 and 150. The ICP-AES measurements indicating that 10–100\% of the five position-37, -59 and -150 mutant protein subunits bound metal after dialysis and the altered enzyme affinities for substrates also suggest that the low specific activities displayed by these mutants (< 3\% of wild-type \(V_{\text{max}}\) (app) using either substrate) did not result simply from the loss of metal. Although analysis of this Zn-binding motif by EXAFS measurements of the mutant proteins or crystallographic determination of the 2\textdegree{} ADH three-dimensional structure is necessary to elucidate the enzyme–metal stoichiometry and the liganding structural details (both of these studies are underway), the EXAFS, ICP-AES and kinetic data reported here clearly support the hypothesis that \textit{T. ethanolicus} 2\textdegree{} ADH residues Cys\textsuperscript{37}, His\textsuperscript{59} and Asp\textsuperscript{150} ligate a catalytic Zn.
Because horse liver 1° ADH is so well characterized, it is an excellent reference for predicting other ADH structures. 1° and 2° ADH peptide sequence comparisons have suggested that, although similarities exist, these enzyme groups may lack extensive structural similarity in catalytically important regions [27,28]. The data presented here demonstrate that the \textit{T. ethanolicus} 2° ADH is a Zn-dependent Rossmann-fold-containing enzyme that uses a Cys-His-Asp metal-binding motif. Thus, by comparison with the liver 1° ADH (Figure 3a), we propose the hypothetical working model for 2° ADH structure–function studies depicted in Figure 3(b). The kinetic mechanisms proposed for catalysis by both horse liver [7] and \textit{T. brockii} [43] ADHs require bound cofactor before substrate binding. Analysis of the horse liver 1° ADH three-dimensional structure [24] suggests that the cofactor may function to complete the active-site cavity wall. Therefore, as in the liver 1° ADH active site, the nicotinamide cofactor may complete 2° ADH catalytic pocket formation. The His59 and Asp138 mutations altered the 2° ADH $K_v^{\text{app}}$ for propan-2-ol over a 140-fold range, whereas the overall change in affinity for ethanol spanned a range of only 7-fold. The greater sensitivity of 2° ADH secondary-alcohol binding to Zn ligand mutations suggests that the substrate binds the catalytic Zn atom, as in the liver 1° ADH [7]. The physiological acetyl-CoA-linked reductive thioesterase activity for the \textit{T. ethanolicus} 2° ADH also suggested that the long lobe of the previously proposed two-lobe active site [1] may be open-ended, allowing the CoA portion of the substrate to remain outside [17]. Analogously, the active-site channel in horse liver ADH has a closed short segment and a long segment open at the far end, with the catalytic Zn atom at their junction [24]. This may represent a structural similarity between 1° and 2° ADH architectures related to substrate specificity that is also of potential biotechnological importance. Finally, the data presented here indicate that comparative analysis between the two enzyme classes may yield further insights into both 1° and 2° ADH molecular structure–function relationships and obtaining an exact 2° ADH structure will provide the additional information needed to rigorously evaluate these hypotheses.

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REFERENCES

10 Dunn, M. F. and Hutchison, J. S. (1973) Biochemistry 12, 4882–4892

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