Protein Science (1996), 5:663-671. Cambridge University Press. Printed in the USA.

Crystal structure of cod liver class I alcohol dehydrogenase: Substrate pocket and structurally variable segments

S. RAMASWAMY, MUSTAPHA EL AHMAD, OLLE DANIELSSON, HANS JÖRNVALL, AND HANS EKLUND

[1] Department of Molecular Biology, Swedish University of Agricultural Sciences, S-751 24 Uppsala, Sweden
[2] Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden

(RECEIVED October 31, 1995; ACCEPTED January 30, 1996)

Abstract

The structural framework of cod liver alcohol dehydrogenase is similar to that of horse and human alcohol dehydrogenases. In contrast, the substrate pocket differs significantly, and main differences are located in three loops. Nevertheless, the substrate pocket is hydrophobic like that of the mammalian class I enzymes and has a similar topography in spite of many main-chain and side-chain differences. The structural framework of alcohol dehydrogenase is also present in a number of related enzymes like glucose dehydrogenase and quinone oxidoreductase. These enzymes have completely different substrate specificity, but also for these enzymes, the corresponding loops of the substrate pocket have significantly different structures. The domains of the two subunits in the crystals of the cod enzyme further differ by a rotation of the catalytic domains by about 6°. In one subunit, they close around the coenzyme similarly as in coenzyme complexes of the horse enzyme, but form a more open cleft in the other subunit, similar to the situation in coenzyme-free structures of the horse enzyme. The proton relay system differs from the mammalian class I alcohol dehydrogenases. His 51, which has been implicated in mammalian enzymes to be important for proton transfer from the buried active site to the surface is not present in the cod enzyme. A tyrosine in the corresponding position is turned into the substrate pocket and a water molecule occupies the same position in space as the His side chain, forming a shorter proton relay system.

Keywords: alcohol dehydrogenase; domain rotation; protein structure; proton relay system; refinement; substrate cleft; X-ray crystallography

The catalysis, evolution, NAD interactions, and conformational changes of alcohol dehydrogenases have been investigated thoroughly for several years (Eklund & Brändén, 1987; Danielsson et al., 1994a; Persson et al., 1994). Interpretation of these studies has relied heavily on the structure of the alcohol dehydrogenase from horse liver and on analysis of the differences among separate forms of the enzyme in many vertebrate lines. Crystallographic studies on the highly homologous human alcohol dehydrogenase show that it has practically identical main-chain conformation as the horse enzyme (Hurley et al., 1991).

Alcohol dehydrogenases in vertebrates serve as a group of detoxifying enzymes. Six classes have been recognized (Danielsson et al., 1994a; Jörnvall & Höög, 1995) with at least two forms with fundamental differences in substrate specificity. They are the classical liver alcohol dehydrogenase (class I type) and the ubiquitous, glutathione-dependent formaldehyde dehydrogenase (class III type). The two classes are derived from a gene duplication, and the original class is thought to be like the class III enzyme, because animals of early origin, such as cyclostomes and invertebrates, have this form, but appear to lack the class I form (Kaiser et al., 1993; Danielsson et al., 1994a, 1994b). Significantly, the bony fish appears to be the first vertebrate with both class I and III alcohol dehydrogenases, and its class I enzyme has some mixed-class properties. Thus, the cod class I enzyme has substrate specificities similar to class I enzymes from higher organisms, but sequence relationships closer with class III than class I (Danielsson et al., 1992).

The cod class I enzyme is significantly different from the mammalian class I enzymes, with 53% sequence identity compared to the classical horse enzyme (Fig. 1), but with 64% sequence identity to the human class III enzyme (Danielsson et al., 1992). Residues interacting with the coenzyme that are typical for class III enzymes are found in the class I cod enzyme, most importantly, His 47 and Tyr 51 (mammalian class I numbering). The difference at position 51 is interesting for the mechanism of the enzyme because His 51 in mammalian class I enzymes has been suggested to participate in proton transfer from the buried active site to the surface of the enzyme (Eklund et al., 1976,

Reprint requests to: Hans Eklund, Department of Molecular Biology, Swedish University of Agricultural Sciences, S-751 24 Uppsala, Sweden; e-mail: hasse@xray.bmc.uu.se.

```
                10         20         30         40         50         60         70         80         90         100
Cod      ATVGKVIKCK AAVAWEANKP LVIEEIEVDV PHANEIRIKI IATGVCHTDL YHLFEGKHKDG FPVVLGHEGA GIVESVGPGV TEFQPGEKVI PLFISQCGEC
Horse E  STAGKVIKCK AAVLWEEKKP FSIEEVEVAP PKAHEVRIKM VATGICRSDD HVVSGTLVT-P LPVIAGHEAA GIVESIGEGV TTVRPGDKVI PLFTPQCGKC

                110        120        130        140        150        160        170        180        190        200
Cod      RFCQSPKTNQ CVKGWANESP DVMSPKETRF TCKGRKVLQF LGTSTFSQYT VVNQIAVAKI DPSAPLDTVC LLGCGVCTGF GAAVNTAKVE PGSTCAVFGL
Horse E  RVCKHPEGNF CLKNDLSMPR GTMQDGTSRF TCRGKPIHHF LGTSTFSQYT VVDEISVAKI DAASPLEKVC LIGCGFSTGY GSAVKVAKVT QGSTCAVFGL

                210        220        230        240        250        260        270        280        290        300
Cod      GAVGLAAVMG CHSAGAKRII AVDLNPDKFE KAKVFGATDF VNPNDHSEPI SQVLSKMTNG GVDFSLECVG NVGVMRNALE SCLKGWGVSV LVGWTD-LHD
Horse E  GGVGLSVIMG.CKAAGAARII GVDINKDKFA KAKEVGATEC VNPQDYKKPI QEVLTEMSNG GVDFSFEVIG RLDTMVTALS CCQEAYGVSV IVGVPPDSQN

                310        320        330        340        350        360        370
Cod      VATRPIQLIA GRTWKGSMFG GFKGKDGVPK MVKAYLDKKV KLDEFITHRM PLESVNDAID LMKHGKCIRT VLSL
Horse E  LSMNPMLLLS GRTWKGAIFG GFKSKDSVPK LVADFMAKKF ALDPLITHVL PFEKINEGFD LLRSGESIRT ILTF
```

Fig. 1. Sequence comparison between the cod and horse alcohol dehydrogenases. Identical residues are in bold letters. Notice the lack of identities for residues 50–61.

1982). An enzyme with a tyrosine at this position should give altered catalytic properties. Variations of segments forming parts of the substrate pocket in combination with absence of significant sequence homology make prediction of structural details of the substrate pocket speculative. We have determined the structure of this cod liver enzyme to clarify these important features of the protein.

Results and discussion

The cod liver class I alcohol dehydrogenase crystallizes in a monoclinic space group with one dimer in the asymmetric unit. The general orientation of the dimeric alcohol dehydrogenase molecules in the crystals was obtained from the molecular replacement solution. The correctness of the solution was verified from NAD densities appearing at the expected positions, although NAD was not included in the search model (Ramaswamy et al., 1994b). However, refinement performed at 2.7 Å halted at an intermediary stage and several parts had poor density. When a new data set to 2.05 Å was collected on frozen crystals at the synchrotron in Hamburg, the increase in observations per parameter allowed independent refinement of each of the two subunits in the dimer. Surprisingly, this refinement revealed that they were significantly different. The refinement then proceeded smoothly and, in the final map, all parts of the dimer are in well-defined density, with the usual exception of a few external side chains. The final R-value is 17.7% (free R = 24.8%) for all reflections in the 7–2.05-Å resolution range. The deviation from standard geometry is given in Table 1 and the main-chain torsion angles are plotted in Figure 2. There are no residues in the disallowed regions of the Ramachandran plot and there are only two residues in the generously allowed regions (Cys 175, in both subunits). Cys 175 is a ligand to the catalytic zinc ion and has strained ϕ-ψ angles in the refined structures of the horse alcohol dehydrogenase (Ramaswamy et al., 1994a), where it corresponds to Cys 174. Figure 3 shows a large slice of electron density around the active site, and the insert shows electron density for the structural zinc and its ligands.

The overall structure

The liver alcohol dehydrogenases are dimeric molecules throughout the vertebrate system (Danielsson et al., 1992). The horse class I alcohol dehydrogenase molecule has been investigated extensively in two major conformations dependent on whether NAD is present or not (Eklund et al., 1976, 1981, 1984; Al-Karadaghi et al., 1994). With NAD, the cleft between the two domains of each subunit closes around the coenzyme, whereas in the absence of NAD, the cleft is open. The conformational change of the protein is essentially a rigid body rotation of the catalytic domains by 10°, but also a smaller rotation of the coenzyme binding domains. To allow the rotation of the catalytic domain, a loop in the coenzyme binding domain has to move away. This movement is probably triggered by the binding of the coenzyme.

One interesting result from the structure determination of the cod class I enzyme is that the two subunits in the crystals have different conformation; one subunit has roughly the expected closed conformation, whereas the other has a conformation very

Table 1. *Crystallographic refinement statistics*

Resolution range (Å)	7.0–2.05
No: of reflections	
Observed	83,875
Unique	39,749
Completeness	
Overall	86.9%
2.08–2.05 Å resolution shell	73.7%
R-merge	12%
R-factor	
Free	24.8%
Conventional	17.7%
Number of atoms	
Total	6,346
Protein	5,606
NAD	88
Zn	4
Solvent	648
Average B-factor (Å^2) subunit A/subunit B	
Main chain A/B	8.0/7.7
Side chain A/B	10.0/10.0
Zn A/B	9.5/13.3
NAD A/B	6.0/16.5
Solvent	18.7
RMSDs from geometry	
Bonds	0.01 Å
Angles	1.6°
Dihedrals	25.1°
Impropers	1.3
G-factor (overall) from Procheck	0.28

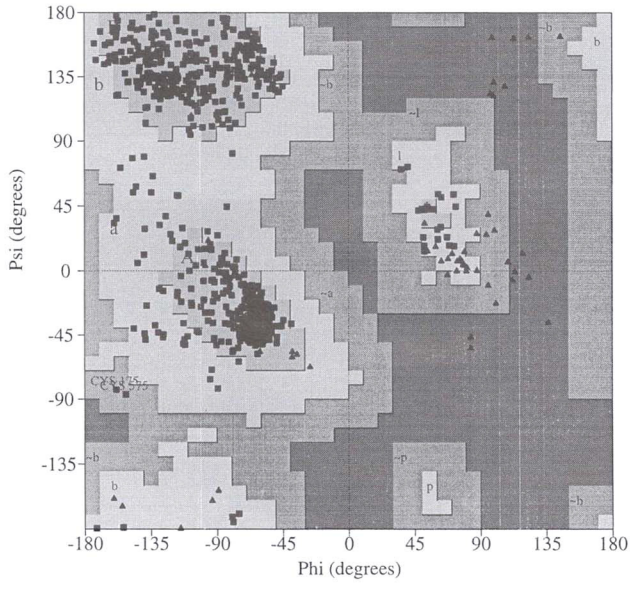

Fig. 2. Ramachandran plot. The only outliers are the Zn-ligand Cys 175 in both subunits.

similar to the open conformation. A superposition of the cod enzyme on top of the horse enzyme places the catalytic domains in a position between the open and closed form, which was the preliminary result reported in conference reports (Ramaswamy et al., 1994b; El-Ahmad et al., 1995). However, when the coenzyme binding domain of each subunit is superimposed on top of the other, it is obvious that one exists essentially as the closed form, whereas the other is in the open form (Fig. 4 and Kinemage 1; Table 2).

Independent superposition of the coenzyme and catalytic domains of the holo and apo form of the horse enzyme on the cod enzyme subunits demonstrates that the structures of the domains are very similar, except in a few loop regions. The RMS deviations (RMSDs) of such superpositions are 0.5–0.9 Å (Table 2). One of the measures of the open/closed conformations can be determined by superimposing the coenzyme binding domains and then calculating the angle of rotation required to superpose the catalytic domains. The rotation axis is very similarly located, close to the 3_{10}-helix preceding the zinc ligand Cys 174 (175), in both the horse and cod enzymes. The angle between the two axes is 15°. Results of these calculations (Table 3) suggest that subunit 2 of the cod enzyme is slightly less open than the apo form of the horse enzyme. Whether or not this is the completely open form of the enzyme can be ascertained only when the structure of the apo form of the cod enzyme also has been determined. The rotation of the catalytic domain of the apo form is highly dependent on interactions of residues 51–61 with the loop 293–298 in the coenzyme binding domain. This loop moves significantly in the conformational transition between the open and closed form of the horse enzyme. The loop in the cod enzyme has a different conformation than that in either of those conformations found in the horse enzyme.

Crystal packing in one direction is caused by the formation of anti-parallel β-sheet interactions between the β-strands around residue 348 in one molecule with the same region of its crystallographic neighbor. These interactions extend the pleated sheet of the catalytic domain into a larger sheet between molecules. This tight packing might explain the very small change in cell dimensions, contrary to what is observed normally for cryo-cooled crystals. The small energy obtained by these crystal packing interactions may be sufficient to open the cleft between the coenzyme and catalytic domain. A small energy difference between the open and closed forms has also been estimated by molecular mechanics calculations for the horse enzyme (Colonna-Cesari et al., 1986).

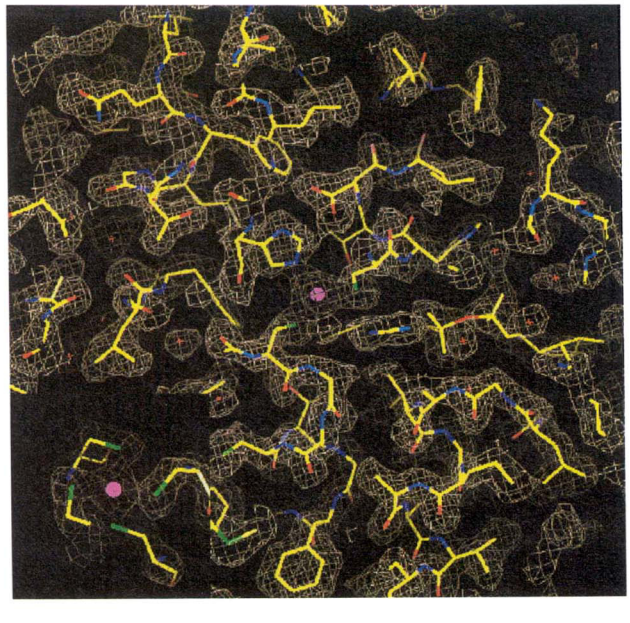

Fig. 3. Electron density maps. Final electron density map ($3F_o - 2F_c$ map contoured at 1σ) with the active site Zn ion in the center (magenta-colored sphere) surrounded by its ligands, Cys 175 to the left, His 67 at the top and Cys 46 to the right. Below Cys 46 is the NAD going toward the right. Insert in the lower left corner shows the structural Zn ion (magenta-colored sphere) surrounded by its four cysteine ligands.

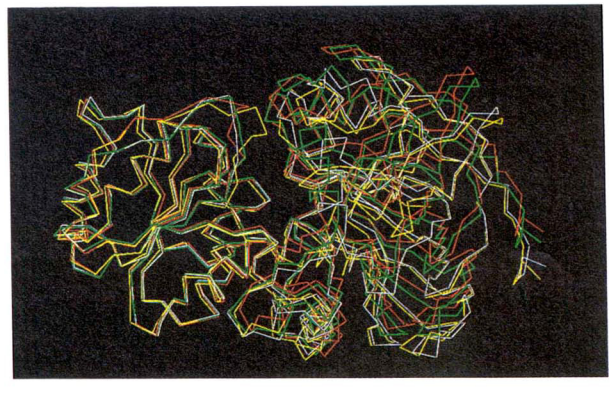

Fig. 4. Comparison with horse class I alcohol dehydrogenase. Superposition of the Cα chains of the cod enzyme, subunit A in green and subunit B in white, and the NAD complex of the horse enzyme with penta-fluoro-benzyl alcohol in red and the apo form of the horse enzyme in yellow. Coenzyme binding domains of each subunit were superimposed. One subunit of the cod enzyme is essentially as in the closed form of the horse enzyme, whereas the other is as in the open form.

Table 2. *Comparisons with horse class I alcohol dehydrogenase*[a]

A	B	Domain	RMSDs (Å)
Cod subunit 1	Cod subunit 2	Catalytic	0.3
Cod subunit 1	Holo horse	Catalytic	0.9
Cod subunit 2	Holo horse	Catalytic	0.9
Cod subunit 1	Apo horse	Catalytic	0.9
Cod subunit 2	Apo horse	Catalytic	0.9
Cod subunit 1	Cod subunit 2	Coenzyme	0.3
Cod subunit 1	Holo horse	Coenzyme	0.5
Cod subunit 2	Holo horse	Coenzyme	0.5
Cod subunit 1	Apo horse	Coenzyme	0.5
Cod subunit 2	Apo horse	Coenzyme	0.5

[a] Results of superposition of the two cod subunits on the holo and the apo forms of the horse enzyme. Superposition was done using the *lsq* option in "O." In all cases, the loop regions were omitted and hence the RMS does not reflect the differences in the loop regions.

A few residues in the coenzyme binding domain of subunit 2 are disordered, which might be derived from the fact that the more open conformation of this subunit allows higher flexibility in the domain–domain interactions. The nicotinamide of the coenzyme is disordered in subunit 2 (the one with the open conformation), and its atoms have high temperature factors. The movement of the nicotinamide ring is possible because it is not tightly locked by the interaction between the two domains. The open conformation of the enzyme is similar to the one that has been observed for ADP-ribose and other coenzyme analogues bound to the horse enzyme (Samama et al., 1977; Cedergren-Zeppezauer et al., 1982; Eklund et al., 1984).

Domain conformation

When the coenzyme binding domains of the cod and horse enzymes are superimposed separately, they are remarkably similar in spite of the many sequence differences, and only one region has significantly different conformation, that of residues 295–303. The Cα positions for these residues differ by 1.5–3.5 Å from each other. This is the loop in the coenzyme binding domain that undergoes a large conformational change when the coenzyme binds (Eklund et al., 1981, 1984). Although the two subunits are different, this loop has essentially the same conformation in both, but is shifted slightly due to the differences in domain interactions. The only other difference in Cα positions of more than 1.5 Å in the coenzyme binding domain is for loop residues 284–285. Both these differences are related to sequence differences between the cod and horse enzymes.

For the catalytic domain, there are two parts with very different conformation, residues 55–59 and 117–126. The former difference may depend on one extra residue in the cod enzyme. A key difference in the 117–126 loop is that some of the residues that stabilize the conformation of the loop by side-chain hydrogen bonds in the horse enzyme are different in the cod enzyme. The whole structural zinc ion loop, residues 95–113, is shifted by up to 2.5 Å compared to that of the horse ADH structure, as a result of the different subunit interactions. Such differences are also responsible for the shift of residues 343–344.

Table 3. *Angle of rotation needed to superimpose the catalytic domains after superpositioning of the coenzyme domains*

A	B	Angle (°)
Cod subunit 1	Cod subunit 2	6.7
Cod subunit 1	Halo horse	3.2
Cod subunit 2	Holo horse	9.7
Cod subunit 1	Apo horse	7.5
Cod subunit 2	Apo horse	1.0

[a] The *lsq* option in "O" (Jones et al., 1991) was used to determine the superposition matrices, and the program "cello" (Gerard Kleywegt, Uppsala) to determine the angles. The superpositions were done without the use of the loop regions, with their large differences.

Differences between the two subunits

The structures of the domains are very similar in the two subunits when compared separately. Most of the side chains also have very similar conformations, except a few charged side chains at the surface of the molecule. Two exceptions exist at the coenzyme binding site between the domains, His 47 and Trp 294. These differences were verified using simulated annealing omit maps. In subunit 1, His 47 is hydrogen bonded to Glu 55 and connects via a water molecule to an oxygen atom of a NAD-phosphate. In subunit 2, His 47 has turned but still binds to NAD via a water molecule. Trp 294 is in contact with the NMN-ribose and is exposed to the substrate pocket. In subunit 2, where the substrate pocket is more open, the side chain has turned around and points out more into the pocket. In both subunits, it is in van der Waals contact to Tyr 51, but, because the domain rotation is different in the two subunits, the Cα atoms of the two residues are 2.6 Å more apart in subunit 2, which allows the rotation of the Trp 294 side chain.

The role of histidine in the proton relay system

It was recognized early that Ser 48–His 51 could be a possible proton relay system to shuttle protons from the internal active site to the surface (Eklund et al., 1976). Further studies also involved the 2′-oxygen of the nicotinamide linked ribose of NAD in the proton relay system: substrate oxygen-Ser 48–O2′–His 51 from the active site in the center of the molecule to the surface (Eklund et al., 1982). Mutagenesis studies supported this view and the mutant yeast and human $\beta_1\beta_2$ enzymes, where the histidine was substituted by a Gln or Glu, had significantly lower activity (Plapp et al., 1990; Ehrig et al., 1991; Plapp, 1995). Sequence studies and homology modeling of the class II and III alcohol dehydrogenases indicated, however, that these enzymes do not have a similar system, because the residue corresponding to His 51 was Ser and Tyr, respectively (Jörnvall et al., 1987; Eklund et al., 1990). In class II ADH, it was suggested that the O2′ was hydrogen bonded to Ser 51 via a water molecule, whereas in class III, Tyr 51 might form a hydrogen bond to O3′ (Eklund et al., 1990). In both cases, the proton transfer should be affected and the pH-profile of the catalysis should be influenced. These differences were also suggested to affect pyrazole inhibition. In the cod enzyme, the corresponding residue is Tyr as in class III, but, because there is an insertion in the cod se-

quence in the region 51–61, this Tyr could be the inserted residue (Danielsson et al., 1992) and it was possible that the adjacent His 52 might have occupied a position similar to that of His 51 in the horse enzyme.

The structure of this cod ADH shows that neither Tyr 51 nor His 52 forms a hydrogen bond to the ribose. Tyr 51 is in a similar position as His 51 (horse), but the side chain points away from the ribose and instead lines the substrate pocket. His 52 is buried and forms a hydrogen bond to the carbonyl oxygen of residue 61. However, a water molecule is bound to O2′ of the ribose in a water network linked to His 47 (Fig. 5 and Kinemage 1). This demonstrates that the His 51 interactions are not essential and a water molecule can form a substitution without significant alterations of catalytic efficiency. However, this water molecule is close to His 47, and this suggests that there may be a necessity for a histidine at either position 47 or 51 for efficient proton transfer. Inspection reveals that there is a histidine either at position 47 or 51 in all alcohol dehydrogenases except human ADH V and deer-mouse ADH VI, and for some species there are histidines at both positions. However, the water molecule substituting for His 51 is not hydrogen bonded to His 47, so the proton relay system cannot be considered to involve this residue.

The substrate binding pocket

The substrate binding pocket in cod liver class I alcohol dehydrogenase is as hydrophobic as the one in the horse enzyme. The inner parts of the substrate pockets are very similar, with Phe 93, Phe 140, and Leu 141 in both cases, but the latter two have slightly different positions (Fig. 6 and Kinemage 1). The main difference is Thr at position 48 instead of Ser, which restricts the space close to the active site zinc atom. Thr at this position is common among alcohol dehydrogenases, and is generally related to lower activity for secondary alcohols (Höög et al., 1992). The activity of cod ADH with cyclohexanol is more similar to the human class I $\beta_1\beta_1$ isozyme, which has Thr 48, than to the $\gamma\gamma$ isozyme, which has Ser 48 (Danielsson et al., 1992). K_m and k_{cat} are 4.3 mM and 8 min^{-1} for the cod enzyme, 0.042 mM and 130 min^{-1} for the $\gamma\gamma$ isozyme, and 14.5 and 14 for the $\beta\beta$ isozyme. Ethanol and pentanol activities are very similar for the cod and the $\beta\beta$ isozyme.

In the middle of the substrate pocket there are significant changes. Val 294 is substituted by Trp and the larger side chain alters the topology of the cleft on one side. Close to this Trp, Met 306 (from subunit 2) and Ile 318 in horse ADH are compensated for by substitutions in cod ADH to Ile and Met, respectively. On the opposite side of the pocket, there are even more significant differences, mainly due to the differences in the loop regions 57–60 and 120–130. The side chain of Tyr 51, which has a completely different conformation than that of His 51 in the horse enzyme, and Met 124 cover the space that Thr 56 and Leu 57 occupy in the horse enzyme. Furthermore, Trp 115 and Ala 116 substitute for Leu 116–Ser 117 in the horse form. The substrate pocket has the same hydrophobic character as in the other class I enzymes (there is no His 57 in the center of this substrate cleft anticipated from earlier considerations), but the geometry of the substrate pocket differs between the horse and cod enzymes. One side of the cleft is more narrow in the horse enzyme due to residues 110, 114, and 116, whereas the cod enzyme is more narrow on the opposite side by residues 48, 124, 294, and 306 (from subunit 2).

Coenzyme binding

In the dimer molecule, NAD is bound differently to the two subunits (Tables 4 and 5). In the subunit with closed conformation, the interactions are essentially as those in NAD complexes with the horse enzyme, whereas in the subunit with open conformation, the interactions are more like those observed in the ADP-ribose complex, where more water molecules bridge the interactions between the coenzyme and the catalytic domain. For example, there is a water molecule between the phosphate oxygen in subunit 2 and the main-chain nitrogen atom of residue

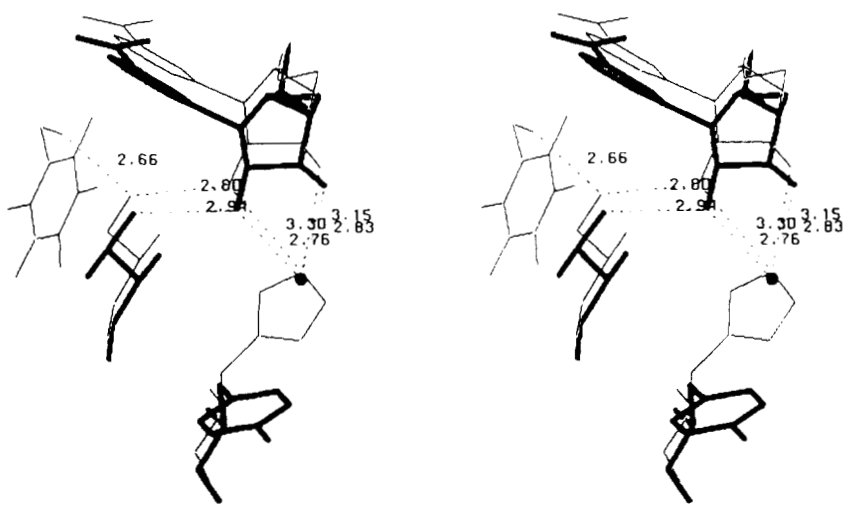

Fig. 5. The proton relay systems in cod ADH (thick lines) and horse ADH (thin lines) superimposed. Hydrogen bonds are from the substrate oxygen of penta-fluorobenzyl alcohol bound to Zn (to the left) to the side-chain oxygen of Thr/Ser 48, to O2′ of the ribose, and to a water molecule in the cod enzyme and His 51 in the horse enzyme. Tyr 51 in the cod enzyme has a very different side-chain conformation and is not involved at all in the proton relay system.

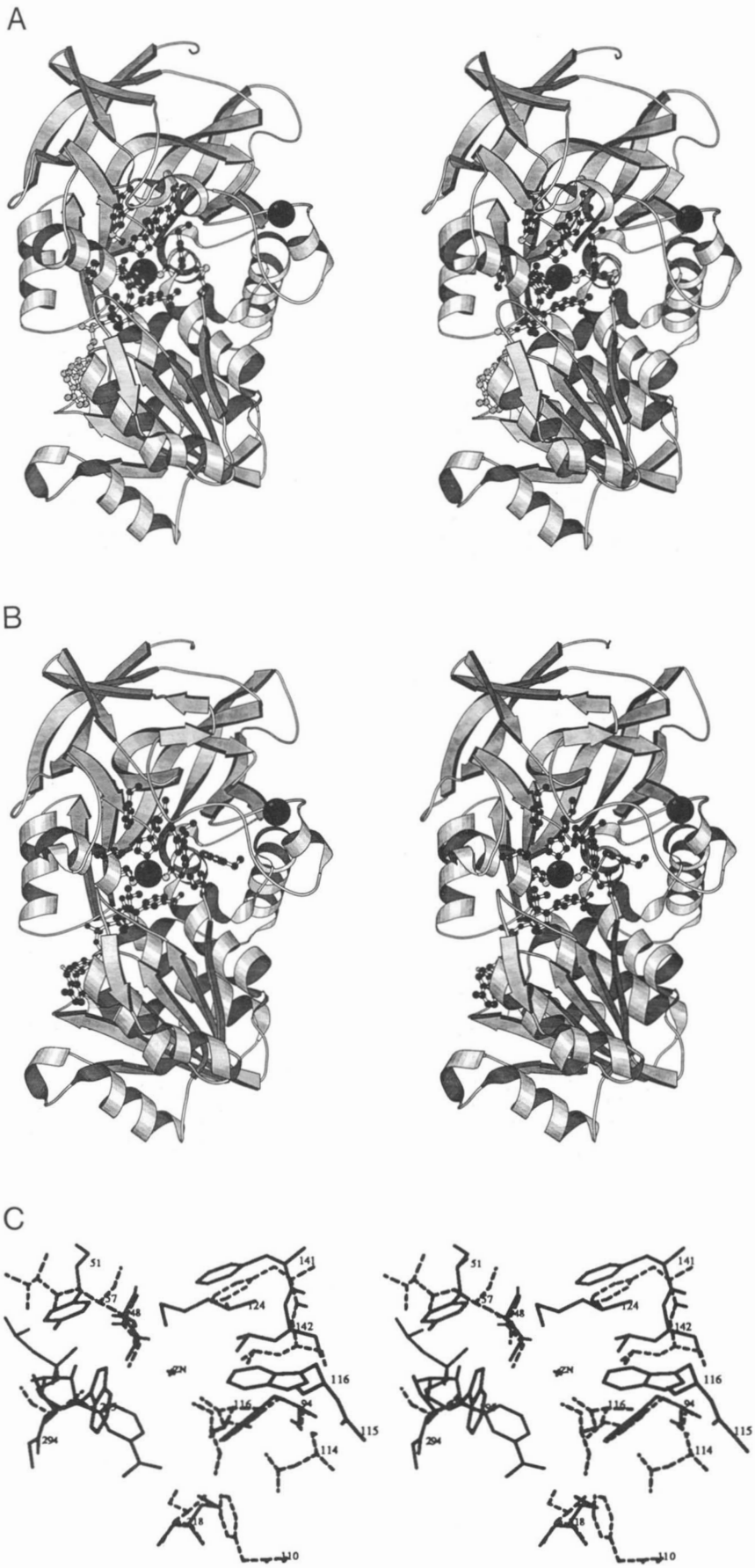

Fig. 6. The substrate pocket in the cod and horse class I alcohol dehydrogenases. **A:** Stereo diagram of the cod subunit showing residues at the substrate pocket. The top of the substrate pocket is formed by residues Met 124, Phe 141, Leu 142, and Trp 116. The right of the pocket is formed by Phe 94 and Met 318. The bottom of the pocket is formed by the nicotinamide ring. The left of the pocket is formed by Trp 294, Thr 48, and Tyr 51. The left side of the substrate pocket is formed by variable segments. In the inner parts of the substrate pocket, the zinc ion is represented as a black sphere and the zinc ligands are shown. **B:** Stereo diagram of the horse subunit showing residues at the substrate pocket. The top of the substrate pocket is formed by residues Phe 140 and Leu 141. The right of the pocket is formed by Leu 116, Phe 93, Phe 110, and Ile 318. The bottom of the pocket is formed by the nicotinamide ring. The left of the pocket is formed by Val 293, Ser 48, and Leu 57. The zinc ion is represented as a black sphere and the zinc ligands are shown. **C:** Stereo diagram of the residues lining the substrate pocket in cod and horse alcohol dehydrogenases superimposed. The cod residues are drawn in solid lines and the horse residues in dotted lines. The active site zinc atom is at the center of the figure at the bottom of the substrate pocket. The nicotinamide ring of NAD covers the left side of the pocket.

47 at the N-terminus of a helix. Thr 48 forms a hydrogen bond to the O2′ oxygen in subunit 1, but is 5.7 Å away in subunit 2.

The interactions of coenzyme with the subunits of the enzyme are listed in Table 4, and for the coenzyme in subunit 1, they are very similar to that in the closed horse conformation. The adenine ring is sandwiched between two hydrophobic Leu and Val residues. In the horse enzyme, similar interactions occur with two Ile. The differences in side chains result in a slight rotation of the adenine plane by about 10° along its longest direction. The adenosine ribose has the same hydrogen bonding interactions as in the horse enzyme. One difference between the interaction of the coenzyme in the cod and horse enzymes is at position 47, where an Arg in the horse enzyme forms a hydrogen bond to OPA1, whereas the cod enzyme has a His. His 47 binds to the phosphate oxygen via a water molecule in both subunits. Other interactions with the phosphates are very similar to those for the horse enzyme. The nicotinamide-linked ribose has main-chain interactions, but only one side-chain hydrogen bond to Thr 48. Instead of the His 51 interactions, there is a water molecule. The nicotinamide ring has the same hydrogen bonds as in the horse enzyme. The proximity of the nicotinamide ring with O 292, Thr 48 OG1, and Thr 178 OG1 is similar in the horse enzyme.

Loop variations

The mixed-class cod alcohol dehydrogenase of class I represents an interesting enzyme in the evolution of alcohol dehydrogenases because it is more related in sequence to the class III enzymes. The conclusion from the three-dimensional structure of this cod enzyme is that the narrow deep hydrophobic substrate cleft, characteristic of the class I enzymes, has diverged structurally from that of the mammalian class I enzymes. Whereas most of the structure serves as a framework that is very similar for the different alcohol dehydrogenases, three loops forming part of the substrate binding site are significantly different. As a consequence, the relative location of the substrate pocket is different in the cod and horse/human enzymes (Fig. 6 and Kinemage 1). The substrate pocket is still hydrophobic and similar in size to that of horse class I alcohol dehydrogenase, in spite of many main-chain and side-chain differences.

Alcohol dehydrogenases belong to a larger family of similar structures that include, among others, sorbitol dehydrogenase (Eklund et al., 1985), glucose dehydrogenase (John et al., 1994), and quinone oxidoreductase (Thorn et al., 1995). The general structures of both glucose dehydrogenase and quinone oxidoreductase are very similar to alcohol dehydrogenase. The largest differences occur also in these cases around the substrate pocket, and the loop corresponding to residues 50–60 in the cod alcohol dehydrogenase is very different in both these structures. Glucose dehydrogenase has a structural zinc ion like alcohol dehydrogenases, but the next loop (corresponding to residues 115–125 in the cod alcohol dehydrogenase) has a very different conformation. Quinone oxidoreductase lacks the structural zinc ion and the corresponding loop is also different. The third loop of the alcohol dehydrogenases that shows structural variation, residues 293–300, is also somewhat different in the other enzymes.

The differences observed between the cod and horse alcohol dehydrogenase structures are amplified in the comparisons of the members of the larger structural family, and these members have clearly more distinct substrate specificities. This theme has been found in several other structural families that have a common framework where differences in loops determine substrate specificity, as for example, the TIM-barrel structures (Brändén, 1991).

Table 4. *Coenzyme hydrogen bonding interactions in Cod class I and horse class I alcohol dehydrogenases*[a]

	Cod subunit 1	Cod subunit 2	Horse NADH
Adenine			
N1		H_2O	H_2O
N6		H_2O	H_2O
N7	H_2O	H_2O	H_2O
Ribose			
O2′	Asp 224 OD1, H_2O	Asp 224 OD1, H_2O	Asp 223 OD, H_2O
O3′	Asp 224 OD2	Asp 224 OD2	Asp 223 OD
	Lys 229 NZ	Lys 229 NZ	Lys 228 NZ
Phosphates			
OP1A	H_2O, H_2O	H_2O, H_2O	Arg 47 NH1, NH2
OP1B	H_2O, H_2O	H_2O	H_2O, H_2O
OP2A	Arg 369, N 47	Arg 369, H_2O	Arg 369, N 47
OP2B	N203, N204, H_2O	N204, H_2O	N203, H_2O
Ribose			
O2′	Thr 48, H_2O	H_2O	Ser 48, His 51
O3′	O 270, N295, H_2O	O 270	O 269, His 51
Nicotinamide			
O7	N319	N319	N319
N7	O 292, O 317	O 292, O 317	O 292, O 317

[a] Horse ADH–NADH interactions are from Ramaswamy et al. (1994a) and ADP-ribose interactions from Eklund et al. (1984).

Table 5. *Torsion angles of NAD bound to alcohol dehydrogenases*[a]

Torsion angle	Cod		Horse		Human	
	Subunit 1	Subunit 2	Subunit 1	Subunit 2	Subunit 1	Subunit 2
χ_a	−101	−103	−102	−99	−111	−107
γ_a	−78	−68	−70	−75	−82	−78
β_a	157	155	151	152	133	144
α_a	79	74	79	86	105	90
ζ_a	96	85	92	88	48	75
ζ_n	−168	−166	−164	−160	−117	−137
α_n	42	40	62	58	69	42
β_n	−167	−177	−168	−166	176	−162
γ_n	67	86	50	51	59	70
χ_n	−115	−107	−97	−103	−106	−98
θ_n	26	−10	22	22	−1	9

[a] Torsion angles are defined in Eklund et al. (1984). The values for horse are from PDB-file 1HDX and the values for the human from PDB-file 3HUD.

Methods

Class I cod alcohol dehydrogenase was crystallized in hanging drops over a reservoir that contained 1 mL of 50 mM TES buffer, pH 6.9, 10 mM NAD, 1 mM pyrazole, and 15.2% PEG 2000. The class I crystals grow as very thin plates and the average size of the crystals used for data collection was 0.5×0.1 mm, with a very small dimension along the longest crystallographic cell axis (Ramaswamy et al., 1994b). The crystals belong to the monoclinic space group $P2_1$, with cell dimensions $a = 102.9$ Å, $b = 47.4$ Å, $c = 80.7$ Å, with $\beta = 104.6°$ and $V_M = 2.4$ Å^3/Da, and one dimer per asymmetric unit.

Data collection and data processing

Data were collected from three cryo-cooled crystals to 2.05 Å resolution at the X11 station at Hamburg. A total of 195 frames were obtained and the data were processed using Denzo (Otwinowski, 1993). All the individual frames were merged together using the program SCALEPACK (Otwinowski, 1993) with an R-merge for all reflections of 12.1% (for 39,749 unique reflections and 83,875 observed reflections). The amplitudes were computed from intensities using the program TRUNCATE in the CCP4 suite (1994). The total completeness for reflections in the range 7–2.05 Å is 86.9%, with 73.7% at highest resolution between 2.08 and 2.05 Å.

Structure solution and refinement

Prior to collection of the cryo-cooled data, a room-temperature data set of 2.8 Å resolution was first collected and an initial structure was solved using X-PLOR (Ramaswamy et al., 1994b). The cell dimensions of the cryo-cooled crystals changed only noticeable along the a-axis, which was 0.4 Å shorter. Because this change is very small, the room-temperature, partially refined model was used to start the refinement. Because only one monomer was used in the earlier refinement calculations, a dimer was generated. This was followed by a rigid body refinement. The data were bifurcated to measure R-free (Brünger, 1993). All refinement was done using X-PLOR (version 3.1, Brünger et al., 1987) and all model building was done using the program "O" (Jones et al., 1991).

The two catalytic domains and the two coenzyme domains were considered independent in the initial rigid body refinement. This was followed by various cycles of simulated annealing refinement, temperature-factor refinement, and model building. Rounds of refinement were done omitting the regions of the structure that were poorly defined and those that were expected to have the largest differences (residues 50–60, 115–130, and 290–300). The lack of low-resolution data (<10 Å resolution) made the rebuilding of these loops difficult. At this stage, the R-factor was 22.3 and the R-free, 31.2. A few cycles of unrestrained refinement using the program ARP, after omitting the bad regions (Lamzin & Wilson, 1993), decreased the R-factor to 16.6, whereas the R-free remained the same. ARP added a total of 888 water molecules. Most of these (500 molecules), were uncertain and were deleted before refinement was continued using X-PLOR and "O." However, inspection of the maps at this stage revealed that most of the loop regions and the loops could be rebuilt. Progress in refinement was monitored using improvement in R-factor and R-free and real space correlation (Jones et al., 1991) of the maps to the model. Good stereochemistry was achieved, which was analyzed using Procheck (Laskowski et al., 1993) and Prosa (Sippl, 1993). It was clear from the electron density maps that several residues had multiple conformations, but these were not modeled because the resolution does not permit refinement of multiple conformations. The final model has 648 water molecules and has been deposited in the Protein Data Bank, code 1CDO.PDB.

Acknowledgments

We thank Maria Paula Sebastiao, EMBL, Desy, Hamburg for help with cryo-cooling and Pete Dunten for linguistic corrections of the manuscript. This work was supported by grants from the Swedish Natural Science and Medical Research Councils.

References

Al-Karadaghi S, Cedergren-Zeppezauer ES, Hovmöller S, Petratos K, Terry H, Wilson KS. 1994. Refined crystal structures of liver alcohol dehydrogenase-NADH complex at 1.8 Å resolution. *Acta Crystallogr D 50*:793–807.

Brünger AT. 1993. Assessment of phase accuracy by cross validation: The free *R* value, methods and applications. *Acta Crystallogr D 49*:24–36.

Brünger AT, Kuriyan J, Karplus M. 1987. Crystallographic *R* factor refinement by molecular dynamics. *Science 235*:458–460.

CCP4 suite. 1994. CCP4 suite: Programs for protein crystallography by collaborative computational project number 4. *Acta Crystallogr D 50*: 760–763.

Cedergren-Zeppezauer E, Samama JP, Eklund H. 1982. Crystal structure determinations of coenzyme analogue and substrate complexes of liver alcohol dehydrogenase: Binding of 1,4,5,6-tetrahydronicotinamide adenine dinucleotide and trans-4-(*N*,*N*-dimethylamino)cinnamaldehyde to the enzyme. *Biochemistry 21*:4895–4908.

Colonna-Cesari F, Perahia D, Karplus M, Eklund H, Brändén CI, Tapia O. 1986. Interdomain motion in liver alcohol dehydrogenase. *J Biol Chem 261*:15273–15280.

Danielsson O, Atrian S, Luque T, Hjelmqvist L, Gonzàlez-Duarte R, Jörnvall H. 1994a. Fundamental molecular differences between alcohol dehydrogenase classes. *Proc Natl Acad Sci USA 91*:4980–4984.

Danielsson O, Eklund H, Jörnvall H. 1992. The major piscine liver alcohol dehydrogenase has class-mixed properties in relation to mammalian alcohol dehydrogenase of class I and III. *Biochemistry 31*:3751–3759.

Danielsson O, Shafqat J, Estonius M, Jörnvall H. 1994b. Alcohol dehydrogenase class III contrasted to class I. Characterization of the cyclostome enzyme, the existence of multiple forms as for the human enzyme, and distant cross-species hybridization. *Eur J Biochem 225*:1081–1088.

Ehrig T, Hurley TD, Edenberg HJ, Bosron WF. 1991. General base catalysis in a glutamine for histidine mutant at position 51 of human liver alcohol dehydrogenase. *Biochemistry 30*:1062–1068.

Eklund H, Brändén CI. 1987. Alcohol dehydrogenase. *Biological Molecules and Assemblies 3*:73–142. In: Jurnak F, McPherson A, eds. *Active sites of enzymes*. New York: Wiley and Sons.

Eklund H, Horjales E, Jörnvall H,Brändén CI, Jeffery J. 1985. Molecular aspects of functional differences between alcohol and sorbitol dehydrogenases. *Biochemistry 24*:8005–8012.

Eklund H, Müller-Wille P, Horjales E, Levina O, Holmquist B, Vallee BL, Höög JO, Kaiser R, Jörnvall H. 1990. Comparison of three classes of human liver alcohol dehydrogenase. *Eur J Biochem 193*:303–310.

Eklund H, Nordström B, Zeppezauer E, Söderlund G, Ohlsson I, Boiwe T, Söderberg BO, Tapia O, Brändén CI, Åkeson Å. 1976. Three-dimensional structure of horse liver alcohol dehydrogenase at 2.4 Å resolution. *J Mol Biol 102*:27–59.

Eklund H, Plapp BV, Samama JP, Brändén CI. 1982. Binding of substrate in a ternary complex of horse liver alcohol dehydrogenase. *J Biol Chem 257*:14349–14358.

Eklund H, Samama JP, Jones TA. 1984. Crystallographic investigations of nicotinamide adenine dinucleotide binding to horse liver alcohol dehydrogenase. *Biochemistry 23*:5982–5996.

Eklund H, Samama JP, Wallén L, Brändén CI, Åkeson Å, Jones TA. 1981. Structure of a triclinic ternary complex of horse liver alcohol dehydrogenase at 2.9 Å resolution. *J Mol Biol 146*:561–587.

El-Ahmad M, Ramaswamy S, Danielsson O, Karlsson C, Höög JO, Eklund H, Jörnvall H. 1995. Crystallizations of novel forms of alcohol dehydrogenase. In: Weiner H, Holmes RS, Wermuth B, eds. *Enzymology and molecular biology of carbonyl metabolism 5*. New York: Plenum. pp 365–371.

Höög JO, Eklund H, Jörnvall H. 1992. A single-residue exchange gives human recombinant $\beta\beta$ alcohol dehydrogenase $\gamma\gamma$ isoenzyme properties. *Eur J Biochem 205*:519–526.

Hurley TD, Bosron WF, Hamilton JA, Amzel LM. 1991. Structure of human $\beta 1\beta 1$ alcohol dehydrogenase catalytic effects of non-active site substitutions. *Proc Natl Acad Sci USA 88*:8149–8153.

John J, Crennel SJ, Hough DW, Danson MJ, Taylor GL. 1994. The crystal structure of glucose dehydrogenase from thermoplasma acidophilum. *Structure 2*:385–393.

Jones TA, Zou JY, Cowan SW, Kjeldgaard M. 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr 47*:110–119.

Jörnvall H, Höög JO, von Bahr-Lindström H, Vallee BL. 1987. Mammalian alcohol dehydrogenases of separate classes: Intermediates between different enzymes and intraclass isozymes. *Proc Natl Acad Sci USA 84*:2580–2584.

Jörnvall H, Höög JO. 1995. Nomenclature of alcohol dehydrogenases. *Alcohol and Alcoholism 30*:153–161.

Kaiser R, Fernández MR, Parés X, Jörnvall H. 1993. Origin of the human alcohol dehydrogenase system. Implications from the structure and properties of the octopus protein. *Proc Natl Acad Sci USA*:11222–11226.

Lamzin VS, Wilson KS. 1993. Automated refinement of proteins models. *Acta Crystallogr D 49*:129–147.

Laskowski RA, McArthur MW, Moss DS, Thornton JM. 1993. PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Crystallogr 26*:282–291.

Otwinowski Z. 1993. Data collection and processing. In: Sawyer L, Issacs N, Bailey S, eds. *Proceedings of the CCP4 study weekend*. Warrington, UK: SERC Daresbury Laboratory. pp 56–62.

Plapp BV. 1995. Site-directed mutagenesis: A tool for studying enzyme catalysis. *Methods Enzymol 249*:91–119.

Plapp BV, Ganzhorn AJ, Gould RM, Green DW, Jacobi T, Warth E, Kratzer DA. 1990. Catalysis by yeast alcohol dehydrogenase. In: Weiner H et al., eds. *Molecular biology of carbonyl metabolism 3*. New York: Plenum Press. pp 241–251.

Persson B, Zigler JS Jr, Jörnvall H. 1994. A super-family of medium-chain dehydrogenases/reductases (MDR). Sub-lines including z-crystallin, alcohol and polyol dehydrogenases, quinone oxidoreductases, enoyl reductases, VAT-1 and other proteins. *Eur J Biochem 226*:15–22.

Ramaswamy S, Eklund H, Plapp BV. 1994a. Structures of horse liver alcohol dehydrogenase complexed with NAD+ and substituted benzyl alcohols determined in two crystal forms. *Biochemistry 33*:5230–5237.

Ramaswamy S, El-Ahmad M, Danielsson O, Jörnvall H, Eklund H. 1994b. Crystallisation and crystallographic investigations of cod alcohol dehydrogenase class I and class III enzymes. *FEBS Lett 350*:122–124.

Samama JP, Zeppezauer E, Biellmann JF, Brändén CI. 1977. The crystal structure of complexes between horse liver alcohol dehydrogenase and the coenzyme analogues 3-iodopyridine-adenine dinucleotide and pyridine-adenine dinucleotide. *Eur J Biochem 81*:403–409.

Sippl MJ. 1993. Recognition of errors in three-dimensional structures of proteins. *Proteins Struct Funct Genet 17*:355–362.

Thorn JM, Barton JD, Dixon NE, Ollis DL, Edwards KJ. 1995. Crystal structure of *Escherichia coli* QOR quinone oxidoreductase complexed with NADPH. *J Mol Biol 249*:785–799.