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Crystallization and preliminary X-ray analysis of NADP(H)-dependent alcohol dehydrogenases from *Saccharomyces cerevisiae* and *Rana perezi*

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Different crystal forms diffracting to high resolution have been obtained for two NADP(H)-dependent alcohol dehydrogenases, members of the medium-chain dehydrogenase/reductase superfamily: ScADHVI from *Saccharomyces cerevisiae* and ADH8 from *Rana perezi*. ScADHVI is a broad-specificity enzyme, with a sequence identity lower than 25% with respect to all other ADHs of known structure. The best crystals of ScADHVI diffracted beyond 2.8 Å resolution and belonged to the trigonal space group $P3_121$ (or to its enantiomorph $P3_221$), with unit-cell parameters $a = b = 102.2$, $c = 149.7$ Å, $\gamma = 120°$. These crystals were produced by the hanging-drop vapour-diffusion method using ammonium sulfate as precipitant. Packing considerations together with the self-rotation function and the native Patterson map seem to indicate the presence of only one subunit per asymmetric unit, with a volume solvent content of about 80%. ADH8 from *R. perezi* is the only NADP(H)-dependent ADH from vertebrates characterized to date. Crystals of ADH8 obtained both in the absence and in the presence of NADP$^+$ using polyethylene glycol and lithium sulfate as precipitants diffracted to 2.2 and 1.8 Å, respectively, using synchrotron radiation. These crystals were isomorphous, space group $C2$, with approximate unit-cell parameters $a = 122$, $b = 79$, $c = 91$ Å, $\beta = 113°$ and contain one dimer per asymmetric unit, with a volume solvent content of about 50%.

1. Introduction

Most alcohol dehydrogenases (ADHs) of wide substrate specificity from yeast and vertebrates are members of the medium-chain dehydrogenase/reductase (MDR) superfamily, which has been divided into different enzyme families with regard to structural and functional relationships (Jörnvall *et al.*, 2001).

The classical *S. cerevisiae* ADHs involved in alcohol production and assimilation are tetrameric enzymes structurally related to vertebrate ADHs (Ciriacy, 1997). We have recently reported the characterization of a new yeast ADH, named ADHVI, presumably a dimer of molecular weight 80 kDa, with only 26% sequence identity to the tetrameric enzyme family (Larroy *et al.*, 2002). Primary structure analysis shows that ADHVI belongs to a different family within the MDRs, the cinnamyl alcohol dehydrogenases (CADs; 37% identity). Consistent with this, ADHVI uses NADP(H) as a cofactor, exhibits a broad substrate specificity with aromatic and linear hydrophobic alcohols and shows high activity towards cinnamyl alcohol and cinnamyl aldehyde. In plants, CAD enzymes participate in the synthesis of cinnamyl alcohol derivatives in the last steps of lignin biosynthesis (Luderitz &

Grisebach, 1981). Since *S. cerevisiae* does not synthesize lignin, we hypothesized that the yeast ADHVI could contribute to lignin degradation and the last step of the synthesis of fusel alcohols derived from amino-acid and α-ketoacid metabolism (Larroy *et al.*, 2002). No crystallographic structure has been reported for any member of the CAD family. The closest known structures to ADHVI (25% identity) are of a NADP(H)-dependent ketose reductase from the whitefly *Bemisia argentifolii* (Banfield *et al.*, 2001) and human ADH4 (Xie *et al.*, 1997).

In vertebrates, ADHs constitute a family of eight different classes involved in ethanol metabolism and also in the transformation of a variety of alcohols and aldehydes of physiological importance, such as retinoids, steroids and cytotoxic aldehydes (Duester *et al.*, 1999). All ADHs from vertebrates are dimers of about 80 kDa and contain two Zn atoms per subunit. In mammals, ADH1 and ADH4 have been the most studied classes because of their wide expression level in tissue and their activity towards ethanol and retinol (Boleda *et al.*, 1993). The oxidation of retinol to retinal is the initial step in the synthesis of retinoic acid, an important modulator of gene expression. While ADH1 is present in all vertebrate

groups, ADH4 has only been described in mammals. In our search for an ADH4-type enzyme in amphibians, we found an enzyme with similar kinetic properties to those of ADH4 but, surprisingly, with specificity towards NADP(H) instead of NAD(H), the common coenzyme for all previously described vertebrate ADHs. This enzyme (ADH8) was isolated from the stomach of *R. perezi* and exhibited high activity towards retinal (Peralba *et al.*, 1999). In consequence, we proposed a physiological function in retinal reduction for ADH8, which is also supported by the high cellular NADPH:NADP$^+$ ratio.

Several crystallographic structures of vertebrate ADHs are known: horse ADH1 (Eklund *et al.*, 1976), human ADH1B1, ADH1B2 and ADH1B3 (Hurley *et al.*, 1994), human ADH1A and ADH1C2 (Niederhut *et al.*, 2001), cod ADH1 (Ramaswamy *et al.*, 1996), mouse ADH2 (Svensson *et al.*, 2000), human ADH3 (Yang *et al.*, 1997) and human ADH4 (Xie *et al.*, 1997). All of them correspond to NAD(H)-dependent enzymes and exhibit less than 57% identity with the present amphibian ADH8. Crystallization and structure determination of ADH8 would provide the basis for the unique cofactor specificity of this

Table 1
Data-collection statistics.

Values in parentheses refer to the outer shell.

	ADH8	ADH8 (NADP$^+$)	ScADHVI
Space group	C2	C2	$P3_121$ or $P3_221$
Unit-cell parameters (Å, °)	$a = 122.5$, $b = 79.5$, $c = 91.9$, $\beta = 113.1$	$a = 122.2$, $b = 79.5$, $c = 91.8$, $\beta = 112.8$	$a = b = 102.2$, $c = 149.7$, $\gamma = 120$
No. measured reflections	150436	254342	120598
No. unique reflections	79197	140960	22735
Resolution (Å)	2.20 (2.28–2.20)	1.80 (1.86–1.80)	2.80 (2.90–2.80)
R_{merge}† (%)	11.3 (44.7)	4.8 (33.1)	7.7 (40.0)
Completeness (%)	98.4 (99.0)	96.1 (74.2)	99.8 (99.8)
$\langle I/\sigma(I) \rangle$	11.8 (2.6)	14.4 (1.6)	24.3 (2.2)
Mosaicity (°)	0.4	0.4	0.2

† $R_{merge} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the measured intensity of an individual reflection and $\langle I \rangle$ is the average intensity of the symmetry-related measurements of this reflection.

Figure 1
Crystals of (*a*) ScADHVI and (*b*) ADH8. Despite the size and good morphology of the ScADHVI crystals, diffraction was extremely weak beyond approximately 2.8 Å resolution, which may be related to the high solvent content.

enzyme and for its particularly high activity towards retinal.

In the present report, we describe the crystallization and the crystal properties of two ADHs from the MDR superfamily: *S. cerevisiae* ADHVI, an NADP(H)-dependent ADH and the first member crystallized of the CAD family, and amphibian ADH8, the first NADP(H)-dependent ADH member of the vertebrate ADH family.

2. Experimental results

2.1. Crystallization, data collection and X-ray analysis

2.1.1. NADP(H)-dependent ADHVI from *S. cerevisiae* (ScADHVI). ScADHVI was obtained from yeast cells by a modification of a previously described protocol (Larroy *et al.*, 2002). The modification consisted of performing the DEAE-Sepharose chromatography at pH 8.0 instead of pH 7.0, which resulted in increased purity and allowed bypassing of the hydroxyapatite column. Pure enzyme was used in crystallization screening using the hanging-drop vapour-diffusion method. 1 µl drops of concentrated sample (6 mg ml^{-1}) were mixed with the same volume of a reservoir solution containing 2 *M* ammonium sulfate (AS) and equilibrated at 293 K against a reservoir volume of 1 ml. Well developed bipyramidal hexagonal crystals reaching 0.4 × 0.4 × 0.6 mm in size appeared in 2 d (Fig. 1). These crystals belong to space group $P3_121$ (or to its enantiomorph $P3_221$), with unit-cell parameters $a = b = 102.2$, $c = 149.7$ Å, $\gamma = 120°$). An X-ray diffraction data set was collected using synchrotron radiation and a MAR CCD detector at ESRF (Grenoble, France) at 100 K with 20%(*v/v*) glycerol as cryoprotectant. Data were processed and scaled with the programs *DENZO* and

SCALEPACK (Otwinowski & Minor, 1996), respectively, giving an overall R_{merge} of 7.7% and a completeness of 99% at 2.8 Å (Table 1). An X-ray fluorescence scan of one of the crystals (performed on beamline BM14) showed a clear absorption edge with the peak at 9671.4 eV, strongly supporting the existence of Zn ions in ScADHVI (Kim & Howard, 2002).

Packing considerations indicate that, assuming a protein-subunit molecular weight of about 40 kDa, these crystals could contain one, two or three subunits per asymmetric unit, with corresponding volume solvent contents ranging from 80 to 42% (Matthews, 1968). In turn, the absence of significant peaks in both the native Patterson map and in the self-rotation function (Fig. 2) makes the existence of non-crystallographic symmetry unlikely, although molecular twofold axes oriented almost parallel to the crystallographic twofold axis are still possible. The weakness of the diffraction at high resolution despite the good morphology and size of the ScADHVI crystals could also be related to a high solvent content. Even if these crystals contain only one subunit per asymmetric unit, the ScADHVI molecules could be homodimers as suggested by Larroy *et al.* (2002) if the molecular and crystallographic twofold axes coincide.

No solution has yet been obtained by molecular replacement with the programs *AMoRe* (Navaza, 1994) and *BEAST* (Read, 2001) using search models derived from human ADH1B1 (Hurley *et al.*, 1991), human ADH4 (Xie *et al.*, 1997), horse ADH1 (Adolph *et al.*, 2000) and ketose reductase (Banfield *et al.*, 2001) and from combinations of the N-terminal catalytic and coenzyme-binding domains of these structures. All ADH molecules used as search models exhibit sequence identities with ScADHVI of below 25% which, together
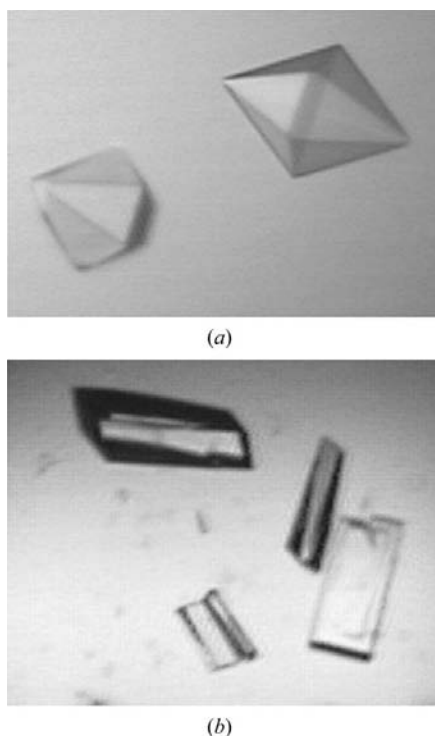
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with the difficulties in finding a molecular-replacement solution, suggests significant structural differences. Searches for heavy-atom derivatives and MAD experiments at the Zn edge are now under way.

2.1.2. NADP(H)-dependent ADH from R. perezi (ADH8). Wild-type ADH8 cDNA was obtained from isolated poly(A)$^+$ RNA and cloned into pBluescript II SK (+) (Peralba *et al.*, 1999). Cloning into pGEX 4T-2 (Amersham Pharmacia Biotech) was performed by amplification of the coding region using the polymerase chain reaction (PCR) with two specific primers, RanaL (5′-TTATA**GGATCC**ATGTGCACTGCGGG-AAAAGAT-3′) and RanaR (5′-CCAC-CT**GAATTC**TTAGTATATCATAATGCT-TCG-3′), including restriction sites (bold) for *Bam*HI and *Eco*RI, respectively. The resulting PCR product was purified by means of the Concert Rapid Gel Extraction System (Life Technologies) and then cloned into the *Bam*HI and *Eco*RI sites of pGEX 4T-2. *Escherichia coli* BL21 competent cells were transformed with the construct.

Expression and batchwise purification were carried out by means of the GST Gene Fusion System (Amersham Pharmacia Biotech). Wild-type cDNA was expressed by inoculating 2×YT liquid medium, containing 75 μg ml^{-1} ampicillin, with an overnight culture (1:100 dilution) of BL21 cells containing the pGEX 4T-2 harbouring the ADH8 cDNA. Cells were grown at 276 K until an absorbance of 0.8–1.0 at 600 nm was reached. Isopropyl β-D-thio-galactoside was then added to a final concentration of 0.1 mM. Bacteria were incubated for 15 h at 296 K and then harvested by centrifugation and stored at

193 K. The pellet was resuspended (100 ml per litre of culture) in cold phosphate-buffered saline (1×PBS; 1.8 mM KH$_2$PO$_4$, 10 mM NaH$_2$PO$_4$, 140 mM NaCl, 2.7 mM KCl pH 7.3) containing 2.5 mM dithio-threitol (DTT). Cells were treated with a 1 mg ml^{-1} final concentration of lysozyme for 30 min at 277 K, sonicated, incubated with bovine DNAse for 30 min at 277 K and finally treated with 1%(v/v) Triton X-100 for 30 min at 277 K to aid in solubilization of the fusion protein. Cell debris was separated by centrifugation at 12 000g for 15 min at 277 K.

A batchwise purification of the fusion protein was carried out by incubation of the cleared cell lysate with glutathione Sepharose 4B (2 ml 50% matrix per litre of culture) at room temperature for 90 min with gentle agitation. The suspension was centrifuged at 500g for 5 min at 277 K and the supernatant was discarded. The matrix containing bound protein was washed three times with 1×PBS by gentle agitation. All centrifugations for washing and elution were performed as described above. Fusion protein bound to matrix was cleaved with thrombin (50 U per litre of culture) by agitation for 15 h at room temperature. Following incubation, the suspension was centrifuged to pellet Sepharose beads. The GST portion of the fusion protein remained bound to the matrix, while the eluate contained ADH8. The purification buffer was exchanged using Centricon Plus-20 (Millipore) to 10 mM sodium phosphate/NaOH pH 6.5 containing 2.5 mM DTT. The protein was then further concentrated and stored at 193 K. The fraction containing ADH activity was subjected to an additional

affinity chromatography step in Red Sepharose CL-6B (Amersham Pharmacia Biotech) to eliminate thrombin. This purifi-cation was performed in batches as described above, but ADH8 was eluted in the presence of 0.6 M NaCl. This fraction was subjected to SDS–PAGE and Coomassie Blue staining to assess the degree of purity.

Samples of ADH8 with an initial protein concentration of 12 mg ml^{-1} (Brad-ford, 1976) were used in crystallization screening with the hanging-drop vapour-diffusion method. Monoclinic crystals belonging to space group $C2$ were obtained in about a week using 20% PEG 4000 and 0.2 M LiSO$_4$ as precipitants in 0.1 M Tris–HCl pH 8 (Fig. 1). The size of the crystals increased, reaching 0.2 × 0.2 × 0.3 mm, when methanol was used as an additive. Crystals with the same morphology and about the same size were also obtained at 277 K under similar crystallization condi-tions from ADH8 samples that had been incubated overnight with 1 M NADP$^+$ (Table 1).

Diffraction data were collected to 2.2 Å using synchrotron radiation at ESRF (Grenoble) from ADH8 crystals grown without nucleotide and flash-cooled to liquid-nitrogen temperature. The cryobuffer was prepared from the crystallization solu-tion supplemented with glycerol to a final concentration of 25%(v/v) in three steps. In a similar way, a diffraction data set to 1.8 Å was collected from ADH8 crystals grown in the presence of NADP$^+$. Both data sets were processed and scaled with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1996), giving overall R_{merge} values of
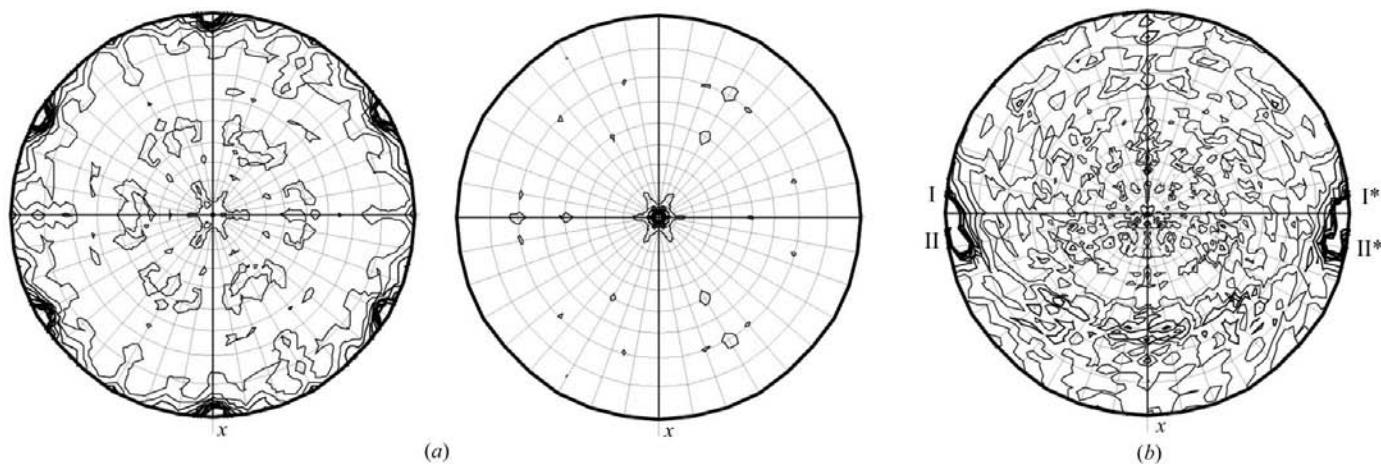

Figure 2
(*a*) Representation of the $\kappa = 180°$ (left) and $\kappa = 120°$ (right) sections containing the only significant peaks found in the self-rotation function of the ScADHVI crystals, which confirm the 3m point-group symmetry. (*b*) Representation of the $\kappa = 180°$ section of the self-rotation function of ADH8 crystals. The largest peak, indicated I in the figure, corresponds to the crystallographic twofold axis. The second peak (II in the figure) corresponds to the molecular twofold axis. All self-rotation calculations were performed in the resolution range 20–3 Å with an integration radius of 30 Å.

11.3 and 4.8 and completenesses of 98 and 96%, respectively (Table 1). These crystals contain two protein subunits, corresponding to one molecule, in the crystal asymmetric unit, with the molecular twofold axis approximately 10° from the crystallographic twofold axis (Fig. 2). A clear molecular-replacement solution, where the first peak in the rotation function was about twice the intensity of the second, has been found using the program *AMoRe* (Navaza, 1994) with a search model derived from the structure of human ADH1B1 (PDB code 1deh; Hurley *et al.*, 1991).

3. Conclusions

Different crystal forms diffracting to high resolution have been obtained for two NADP(H)-dependent alcohol dehydrogenases: ScADHVI from *S. cerevisiae* and ADH8 from *R. perezi*. ScADHVI is a broad-specificity ADH (Larroy *et al.*, 2002), a member of the CAD enzymatic family, with a sequence identity lower than 25% with respect to all other ADHs of known structure. No molecular-replacement solution has yet been found, which suggests important structural differences from the available ADH structures. In turn, a preliminary molecular-replacement solution has already been found for ADH8, the only NADP(H)-dependent ADH from vertebrates characterized to date.

The structure determination at high resolution of both ScADHVI and ADH8 would clarify the molecular peculiarities of the NADP(H)-dependent ADHs, particularly their cofactor preference. Moreover, the ScADHVI structure would provide the first three-dimensional approach to explain the structure–function relationships in the cinnamyl alcohol dehydrogenase family.

References

Adolph, H. W., Zwart, P., Meijers, R., Hubatsch, I., Kiefer, M., Lamzin, V. & Cedergren-Zeppezauer, E. (2000). *Biochemistry*, **39**, 12885–12897.

Banfield, M. J., Salvucci, M. E., Baker, E. N. & Smith, C. A. (2001). *J. Mol. Biol.* **306**, 239–250.

Boleda, M. D., Saubi, N., Farrés, J. & Parés, X. (1993). *Arch. Biochem. Biophys.* **307**, 85–90.

Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.

Ciriacy, M. (1997). *Yeast Sugar Metabolism: Biochemistry, Genetics, Biotechnology and Applications*, edited by F. K. Zimmermann & K. D. Entian, pp. 213–223. Lancaster, Pennsylvania, USA: Technomic Publishing Co. Inc.

Duester, G., Farrés, J., Felder, M. R., Holmes, R. S., Höög, J. O., Parés, X., Plapp, B. V., Yin, S. J. & Jörnvall, H. (1999). *Biochem. Pharmacol.* **58**, 389–395.

Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., Söderberg, B. O., Tapia, O., Brändén, C. I. & Åkeson, Å. (1976). *J. Mol. Biol.* **102**, 27–59.

Hurley, T. D., Bosron, W. F., Hamilton, J. A. & Amzel, L. M. (1991). *Proc. Natl Acad. Sci. USA*, **88**, 8149–8153.

Hurley, T. D., Bosron, W. F., Stone, C. L. & Amzel, L. M. (1994). *J. Mol. Biol.* **239**, 415–429.

Jörnvall, H., Shaqfat, J. & Persson, B. (2001). *Chem. Biol. Interact.* **30**, 491–498.

Kim, K. & Howard, A. J. (2002). *Acta Cryst.* D**58**, 1332–1334.

Larroy, C., Fernandez, M. R., Gonzalez, E., Pares, X. & Biosca, J. A. (2002). *Biochem. J.* **361**, 163–172.

Luderitz, T. & Grisebach, H. (1981). *Eur. J. Biochem.* **119**, 115–124.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.

Niederhut, M. S., Gibbons, B. J., Pérez-Miller, S. & Hurley, T. D. (2001). *Protein Sci.* **10**, 697–706.

Otwinowski, Z. & Minor, W. (1996). *Methods Enzymol.*, **276**, 307–326.

Peralba, J. M., Cederlund, E., Crosas, B., Moreno, A., Julià, P., Martínez, S. E., Persson, B., Farrés, J., Parés, X. & Jörnvall, H. (1999). *J. Biol. Chem.* **274**, 26021–26026.

Ramaswamy, S., El-Ahmad, M., Danielsson, O., Jörnvall, H. & Eklund, H. (1996). *Protein Sci.* **5**, 663–671.

Read, R. J. (2001). *Acta Cryst.* D**57**, 1373–1382.

Svensson, S., Höög, J. O., Schneider, G. & Sandalova, T. (2000). *J. Mol. Biol.* **302**, 441–453.

Xie, P., Parsons, S. H., Speckhard, D. C., Bosron, W. F. & Hurley, T. D. (1997). *J. Biol. Chem.* **272**, 18558–63.

Yang, Z. N., Bosron, W. F. & Hurley, T. D. (1997). *J. Mol. Biol.* **265**, 330–343.