Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary characterization of crystals of *R*-alcohol dehydrogenase from *Lactobacillus brevis*

Karsten Niefind,[a]† Bettina Riebel,[b]† Jörg Müller,[a] Werner Hummel[b] and Dietmar Schomburg[a]*

[a]Universität Köln, Institut für Biochemie, Zülpicher Strasse 47, D-50674 Köln, Germany, and [b]Forschungszentrum Jülich, Postfach 2050, D-52404 Jülich, Germany

† BR and KN should be considered as first authors.

Correspondence e-mail: d.schomburg@uni-koeln.de

Received 30 May 2000
Accepted 29 August 2000

The *R*-specific alcohol dehydrogenase (RADH) from *Lactobacillus brevis* is a valuable catalyst for the production of chiral alcohols that can be used as synthons in asymmetric syntheses. RADH is a homotetramer with 222 symmetry and a molecular mass of 107 kDa. The recombinant enzyme has been expressed in *Escherichia coli*, purified to homogeneity and crystallized. The crystals belong to the orthorhombic space group *I*222, with unit-cell parameters a = 56.5, b = 85.1, c = 115.4 Å, and diffract X-rays to at least 1.8 Å resolution. The calculated crystal packing parameter V_M = 2.59 Å^3 Da^{-1}, corresponding to a solvent content of 52.5% and suggesting that one RADH monomer is contained in the asymmetric unit. The RADH tetramer lies on a special position with its molecular dyads coinciding with the crystallographic twofold axes and with its centre of mass on the origin of the unit cell.

1. Introduction

Chiral alcohols are valuable synthons in organic and pharmaceutical chemistry. Therefore, enzymes that catalyze the reduction of prochiral ketones to chiral alcohols are biotechnologically important (Hummel, 1997). This is especially true for oxidoreductases of low substrate specificity, as they can be used for the production of a large spectrum of alcohols, and those of *R* stereospecificity at the reaction centre, as the first alcohol dehydrogenases of biotechnological interest discovered were restricted to *S* alcohols (Hummel, 1997).

An *R*-alcohol dehydrogenase (RADH) promising in this respect was found in *L. brevis* [Deutsche Sammlung für Mikroorganismen (DSM) 20054] during a screen for oxidoreductases with low substrate specificity (Hummel, 1990), together with a very similar enzyme from *L. kefir* (DSM 20587). RADH is a homotetramer with a molecular mass of 26 758 g mol^{-1} as calculated from its amino-acid sequence and catalyzes reactions of the type

$$R_1\mathrm{CO}R_2\mathrm{1} + \mathrm{NADPH} + \mathrm{H}^+ \overset{\mathrm{RADH}}{\rightleftharpoons} R_1\mathrm{CHOH}R_2 + \mathrm{NADP}^+.$$

R_1 is typically a methyl group, while for R_2 a variety of chemical groups are acceptable (Riebel, 1996). The standard reference substrate in the literature is acetophenone. RADH from *L. brevis* is of special value because it is significantly more stable than the homologous enzyme from *L. kefir* (Riebel, 1996).

RADH was purified, characterized, sequenced and cloned by Riebel (1996). A crystal structure determination of RADH is especially interesting with respect to rational protein design, by which it may be possible to improve the stability of the enzyme in organic solvents. Furthermore, it would be valuable to identify the important residues for cosubstrate specificity with the aim of producing RADH mutants which utilize NADH instead of the expensive NADPH.

2. Experimental procedure

2.1. Expression, purification and crystallization

Recombinant RADH was expressed in *E. coli* and purified as described by Riebel

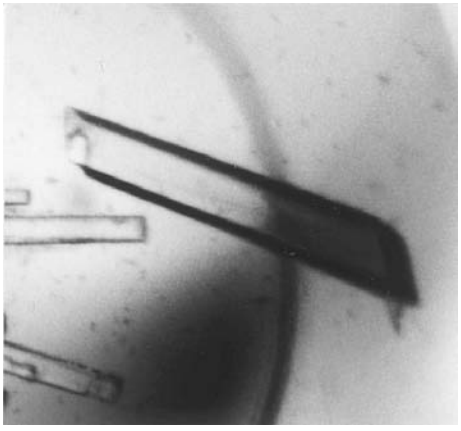

Figure 1
Orthorhombic crystals of recombinant RADH from *L. brevis*.

(1996). Purified RADH was concentrated and rebuffered by ultrafiltration against a solution of 1 m*M* $MgCl_2$, 20 m*M* triethanolamine pH 7.0. The final protein concentration in the RADH stock solution for crystallization setups was 15 mg ml^{-1}.

All crystallization experiments were performed according to the sitting-drop variant of the vapour-diffusion method (Ducruix & Giegé, 1992) using Cryschem crystallization plates. For each setup, temperatures of 289 and 277 K were tested in parallel. Generally, a 4 µl droplet of RADH stock solution was mixed with 4 µl of the reservoir solution. Each well contained 500 µl of reservoir solution. Preliminary crystallization conditions were established using Crystal Screens I and II (Hampton Research, USA).

These initial crystallization conditions were refined by application of the grid-screen approach (Bergfors, 1999). The pH value, the concentration of the precipitating agent and the protein concentration were varied systematically. Three different temperatures (289, 293 and 298 K) were used.

2.2. X-ray diffraction experiment

RADH crystals were characterized by X-ray diffraction. Diffraction data were collected with a MacScience DIP-2030H imaging-plate detector and Cu $K\alpha$ radiation. The X-rays were generated by a Nonius FR591L rotating-anode generator operating at 45 kV and 100 mA. They were monochromated by a graphite crystal and parallelized using a 0.5 mm collimator.

For diffraction experiments RADH crystals were mounted in glass capillaries. The crystal-to-detector distance was 120 mm. The width of each oscillation frame was 0.5° in φ, with an exposure time of 10 min. All measurements were carried out at 283 K.

The data were processed using *XDISP*, *DENZO* and *SCALEPACK* from the *HKL* package (Otwinowski & Minor, 1997) and *SCALEPACK2MTZ*, *TRUNCATE*, *CAD* and *MTZ2VARIOUS* from the *CCP4* program suite (Collaborative Computational Project, Number 4, 1994). As two possible space groups remained after scaling, molecular-replacement searches were performed to complete the space-group determination. For this purpose, the program system *X-PLOR* (Brünger, 1992) was applied using the tutorial scripts of version 3.851 for rotation and translation searches.

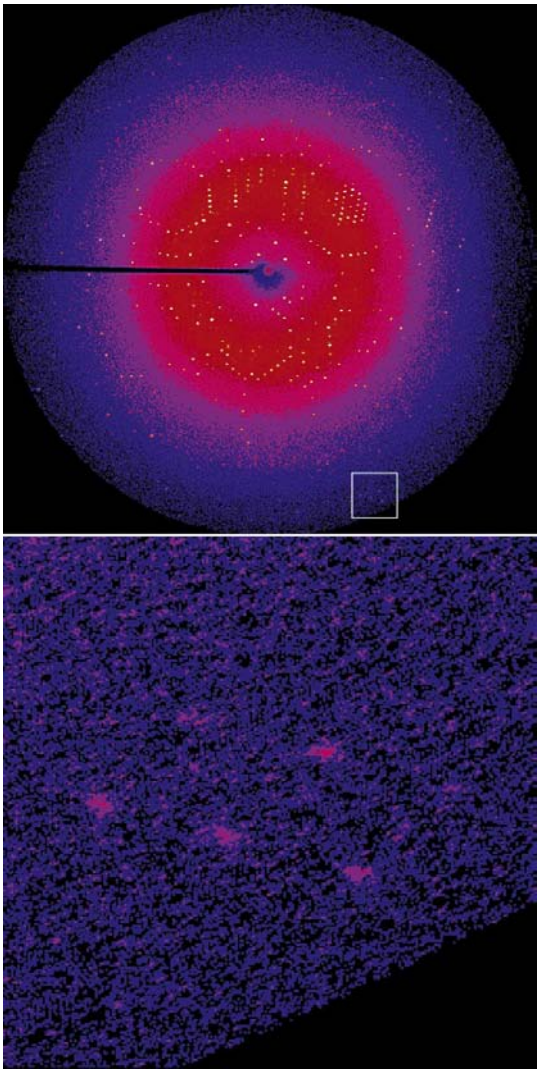

Figure 2
A 0.5° rotation diffraction frame of an RADH crystal. The white square marks the region which is enlarged in the lower part of the figure. The diffraction angles of the outermost reflections correspond to a resolution of 1.8 Å.

3. Results and discussion

RADH crystals with good diffraction qualities could be found (Fig. 1). They grew at 289 K within 10 d with the following optimal reservoir composition: 40%(*v*/*v*) 2-methyl-2,4-pentanediol (MPD), 50 m*M* calcium chloride, 100 m*M* *N*-[2-hydroxyethyl]-piperazine-*N*′-[4-butanesulfonic acid] (HEPES buffer) pH 7.0. These crystals had typical dimensions of 0.4 × 0.2 × 0.2 mm.

Table 1
Native data set statistics of RADH crystals.

Total number of observations, 247 529; number of outlier rejections, 1289.

High-resolution limit (Å)	No. of unique reflections	$I/\sigma(I)$	Completeness (%)	Multiplicity	R_{sym} (%)
3.86	2798	80.5	99.8	9.5	3.2
3.07	2685	71.2	100	9.7	4.3
2.68	2650	47.7	100	9.7	6.0
2.43	2644	36.7	100	9.9	7.4
2.26	2623	31.2	99.9	9.3	8.6
2.13	2621	25.4	99.8	9.3	10.4
2.02	2601	18.7	99.7	9.7	13.6
1.93	2604	14.7	99.2	9.5	18.2
1.86	2607	12.8	99.2	8.5	22.1
1.79	2349	11.2	91.3	8.3	23.5
Total	26182	46.4	98.9	9.5	5.9

The crystals were stable in the X-ray beam for more than 3 d during the diffraction experiments and diffracted X-rays to at least 1.8 Å resolution (Fig. 2). A body-centred orthorhombic crystal lattice with unit-cell parameters a = 56.5, b = 85.1, c = 115.4 Å could be found with *DENZO*, leaving $I222$ or $I2_12_12_1$ as possible space groups. Assuming one RADH monomer per asymmetric unit, this lattice leads to a V_M value of 2.59 $Å^3$ Da^{-1}, corresponding to a solvent content of 52.5% of the crystallographic unit cell (Matthews, 1968). No other V_M values and crystal packings are plausible. Hence, if RADH crystallized as an intact tetramer with 222 symmetry, its local twofold axes tetramer must coincide completely with the crystallographic dyads and its centre of mass must lie on the origin of the unit cell. As a consequence, $I222$ should be the correct space group. A verification of this conclusion from the diffraction data alone is not possible because $I222$ and $I2_12_12_1$ possess identical extinction rules.

A native data set consisting of 494 single frames was collected from one RADH crystal (Table 1). The data extend to 1.8 Å resolution. However, considering the fact that in the last resolution shell (1.86–1.79 Å) the signal-to-noise ratio [$I/\sigma(I)$] is still more than 11 (Table 1) and the portion of reflections with $I/\sigma(I)$ lower than 1 is negligibly small, it should be possible to collect data to even higher resolution.

To confirm the space-group determination Patterson search calculations (molecular replacement) were performed using all reflections between 15 and 5 Å resolution from the collected data set and one subunit

Table 2
Translation peaks.

(*a*) Space group *I*222.

Peak No.	Components of translation vector t_x	t_y	t_z	Relative peak height $[T(t_x, t_y, t_z) - T_{\text{average}}]/\sigma_T$
1	0.059	0.186	0.115	4.51
2	0.176	0.100	0.154	3.43
3	0.353	0.429	0.423	3.35

(*b*) Space group $I2_12_12_1$.

Peak No.	Components of translation vector t_x	t_y	t_z	Relative peak height $[T(t_x, t_y, t_z) - T_{\text{average}}]/\sigma_T$
1	0.176	0.343	0.212	3.49
2	0.059	0.429	0.173	3.48
3	0.353	0.186	0.481	3.32

of hydroxy steroid dehydrogenase from *Streptomyces hydrogenans* [PDB (Berman *et al.*, 2000) code 2hsd; Ghosh *et al.*, 1994] reduced to a polyalanine chain as a search model. The amino-acid sequence of hydroxy steroid dehydrogenase is 39.6% identical to that of RADH, so the Patterson searches were expected to be successful. In fact, the correct orientation of the search model could easily be found: the highest rotation peak, which characterized the correct solution, was 5.1 standard deviations above the average of the rotation function with a difference of 0.8 standard deviations to the next peak.

Translation searches were performed with both space groups *I*222 and $I2_12_12_1$. As expected from the crystal packing considerations, a significant solution with a relative peak height of 4.51σ above the average was only found with space group *I*222, while with $I2_12_12_1$ the highest translation peak was only 3.49σ above the average (Table 2). Hence, the space group *I*222 was confirmed. Simultaneously, the solution found defines the starting point for a refinement of the RADH structure, which is in progress. The resulting structure will be described in detail in a subsequent paper.

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