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Expression, crystallization and activities of the two family 11 aldo–keto reductases from *Bacillus subtilis*

Andreas Ehrensberger and David K. Wilson*

Section of Molecular and Cellular Biology, University of California, Davis, CA 95616, USA

Correspondence e-mail: dave@alanine.ucdavis.edu

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Two members of the aldo–keto reductase family 11 from *Bacillus subtilis* have been crystallized and their oxidoreductase activity confirmed. AKR11A is a protein induced by inositol and repressed by glucose. AKR11B is induced when the cell is stressed by heat, acid, ethanol, starvation or osmotic shock and is therefore classified as a general stress protein. The expected NADPH-dependent sugar reductase activities for both proteins have been confirmed kinetically with several substrates. AKR11B exhibited typical aldo–keto reductase kinetics. However, only trace activity was found in AKR11A. To examine the effects of differences in sequence on the structures and functions of these enzymes, a crystallographic study has been initiated. AKR11A has been crystallized in its apo form and AKR11B crystals were obtained in complex with $NADP^+$.

1. Introduction

The aldo–keto reductase (AKR) superfamily includes approximately 120 different proteins that have been found throughout the eukaryotes and prokaryotes. They have been categorized into 12 families through amino-acid homology (Jez & Penning, 2001). Many of these enzymes play important roles in various metabolic pathways including steroid (family 1) and sugar (families 1 and 2) catabolism and vitamin C biosynthesis (family 5). Most of the other AKR families have not yet been well characterized. Structurally, aldo–keto reductases are composed of a single-domain $(\beta/\alpha)_8$ barrel. They catalyze the reversible reduction of carbonyl-containing compounds to the corresponding alcohols using NADPH and sometimes also NADH as the hydride donor. Most aldo–keto reductases can reduce a large variety of sugars but usually prefer hydrophobic substrates. Catalytic activity has been demonstrated for many of these, but only a subset have been studied structurally. These include members from families 1 (El-Kabbani *et al.*, 1995; Hoog *et al.*, 1994; Rondeau *et al.*, 1992; Wilson *et al.*, 1992; Ye *et al.*, 2000), 2 (Kavanagh *et al.*, 2002), 3 (Hur & Wilson, 2001), 5 (Khurana *et al.*, 1998), 6 (Gulbis *et al.*, 1999) and 7 (Kozma *et al.*, 2002).

We have crystallographically and kinetically characterized AKR11A and AKR11B, the only known members of family 11, which are both present in the prokaryote *Bacillus subtilis*. These have been found to show some similarity in their primary sequence to other aldo–keto reductases, especially in the consensus-sequence regions corresponding to catalytic amino acids and cosubstrate-binding residues. Significant differences are noted in putative substrate-binding residues.

AKR11A, also known as the *iol*S gene product (Yoshida *et al.*, 1997), YxbF and vegetative protein 147 (Antelmann *et al.*, 1997), is one of the proteins whose gene is located adjacent to the operon coding for the enzymes responsible for *myo*-inositol catabolism, including inositol dehydrogenase (Ramaley *et al.*, 1979; Yoshida *et al.*, 1997). Although AKR11A is transcribed in the opposite direction from the other genes in this operon, it is also induced by inositol. Its disruption does not affect *myo*-inositol metabolism (Yoshida *et al.*, 1997).

AKR11B, also called GSP69 (Antelmann *et al.*, 1997) or YhdN (Petersohn *et al.*, 1999), belongs to a larger group of general stress proteins in *B. subtilis* induced by the σ^B transcription factor (Petersohn *et al.*, 1999). σ^B is in turn synthesized when the cell is under general stress, such as heat, acid, ethanol, starvation or osmotic stress (Hecker *et al.*, 1996). No study has been performed on the specific function of AKR11B, but it may play some role in protecting or healing the cell under stressful conditions, a role that has also been assigned to other eukaryotic AKRs.

The genes for AKR11A and AKR11B are present at different loci on the chromosome and are regulated by separate promoters and induced under distinct conditions. Despite these differences, both are encoded by genes under glucose repression (Yoshida *et al.*, 2001) and are the members of the superfamily most closely related to each other, sharing 37% sequence identity.

In order to gain more insight into the structures and functions of these two proteins, they have been crystallized. Their activities as aldo–keto reductases have also been examined and characterized.

2. Materials and methods

The DNA sequences of both genes were obtained (GENBANK accession Nos. P46336 and P80874 for AKR11A and AKR11B, respectively) and the genes amplified *via* PCR. The primers used are 5′-GCTAGTGCATATGAAAAAAGCGAAG-CTCGGA and 5′-ACTGCCCGGGTGCG-AACAGCTTATCAATAAA for AKR11A, and 5′-GCTAGTGCATATGGAATATAC-CAGTATAGC and 5′-ACTGCCCGGG-TATTTCCTCTCTGGTCGGCGG for AKR11B. *B. subtilis* genomic DNA was used to amplify the insert using VENT polymerase (New England Biolabs). The genes were then inserted into the *Nde*I and *Sma*I sites of the *Escherichia coli* expression plasmid pTYB2 (New England Biolabs). Positive clones were identified and sequenced to assure that no mutations were introduced from the PCR amplification. The *E. coli* expression strain BL21* was transformed with the resulting plasmid.

Each of the transformed strains was grown in 6 l LB medium until an OD_{600} of 0.6 was reached and then induced with 500 µ*M* isopropylthiogalactoside for 3 h (AKR11A) and 8 h (AKR11B) at 288 K. The cells were resuspended in buffer containing 0.5 *M* NaCl, 0.1 m*M* EDTA, 20 m*M* Tris pH 8.0 with 0.1% Triton X-100 (buffer *A*), then microfluidized and sonicated at 277 K and clarified by centrifugation at 39 000*g*. The supernatant containing the soluble protein was filtered and loaded onto 15 ml chitin beads (New England Biolabs). The protein of interest was fused to an intein/chitin-binding domain coded in the pTYB2 vector and thus remained bound to the beads. The column was extensively washed with buffer *A* and the Triton X-100 was then removed by washing with the same buffer without detergent until the OD_{280} had decreased to a minimum. Self-cleavage of the intein tag was achieved by adding 0.3% β-mercaptoethanol. After elution, the protein product was concentrated and desalted in 10 m*M* HEPES pH 7.3. In view of the predicted isoelectric points of 5.5 for AKR11A and 5.0 for AKR11B, each protein was loaded on a Poros HQ anion-exchange column (Perseptive Biosystems). A peak was observed from approximately 250–350 m*M* NaCl upon elution with a 0–450 m*M* NaCl gradient at pH 7.6. Fractions were collected, desalted and concentrated. After the final purification step, the total yield of AKR11A was of 0.54 mg per litre of culture, while AKR11B was expressed at 0.31 mg per litre of culture.

In order to establish the kinetic parameters of both proteins, their initial velocities were measured by observing the rates of decrease in absorbance at 340 nm as NAD(P)H was oxidized to $NAD(P)^+$. Assays were taken in a buffer containing 50 m*M* K_2HPO_4, 10 m*M* KCl and 0.5 m*M* EDTA at pH 7.0 and 298 K using a Hewlett–Packard 8453 spectrophotometer. Measurements at five to eight different substrate concentrations were taken for the substrates that showed activity. The concentration of NAD(P)H was kept at 250 µ*M* when measuring activity for other substrates. Kinetic constants for NAD(P)H with AKR11B were determined by keeping DL-glyceraldehyde at 30 m*M*. K_M and k_{cat} values were determined by curve fitting the resulting values to the Michaelis–Menten equation with the program *DeltaGraph* (SPSS Inc.).

In order to determine whether AKR11A and AKR11B formed oligomers in solution, dynamic light-scattering measurements were taken. The experiments were performed on a Protein Solutions Dynapro-99 using 0.75 mg ml^{-1} protein in 10 m*M* HEPES pH 7.3.

Crystallization conditions were screened by the hanging-drop vapor-diffusion method using the Wizard crystallization screen (Emerald Biostructures). AKR11A was used at a concentration of 12.5 mg ml^{-1} and AKR11B at 12.2 mg ml^{-1}. Both proteins were kept in 10 m*M* HEPES pH 7.3. They were crystallized by combining 1 µl protein with 1 µl well solution and suspending this droplet over 500 µl well solution. Crystal quality was optimized by varying the temperature, pH and concentration of precipitants and salts. AKR11B crystals were grown at 295 K with 25% PEG 8000, 140 m*M* NaCl and 100 m*M* imidazole pH 7.0. $NADP^+$ was added to the drop to a final concentration of 5 m*M*. AKR11A crystals were obtained using 32% PEG 3000 and 100 m*M* CHES pH 9.9 at 277 K. The best crystals appeared in a period of 1–5 d for both proteins and were of a size suitable for diffraction.

For cryocooling, the crystals were harvested in 20% glycerol, 80% well solution using a nylon-fiber loop and flash-cooled to 100 K. Native data for AKR11A were collected at beamline 9-2 at Stanford Synchrotron Radiation Laboratory on an ADSC Quantum 4 detector. The unit-cell parameters and space group were determined and the data were reduced and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Data for AKR11B were collected on a Bruker SMART 6000 CCD area detector mounted on a rotating-anode generator producing Cu $K\alpha$ radiation. Crystallographic parameters for this data set were determined using the *Proteum* software package (Bruker Inc.).

Table 1
Kinetic constants for AKR11B.

Substrate	K_m (m*M*)	k_{cat} (s^{-1})
DL-Glyceraldehyde	2.2	1.37
p-Nitrobenzaldehyde	0.67	1.09
NADPH	0.0122	2.08

3. Results and discussion

Kinetic results show that both AKR11A and AKR11B have aldo–keto reductase activity and can use NADPH as a cosubstrate.

AKR11B was shown to reduce the standard AKR substrates DL-glyceraldehyde and *p*-nitrobenzaldehyde in the presence of NADPH (Table 1). Some AKRs have been shown to utilize NADH, but no activity was observed in either enzyme when assayed with this potential cosubstrate. Furthermore, glucose does not function as a substrate for AKR11B.

AKR11A was able to reduce DL-glyceraldehyde at a barely detectable level with NADPH as a cosubstrate. The rate constant for DL-glyceraldehyde in this reaction was 0.014 s^{-1} at a substrate concentration of 255 m*M* and an NADPH concentration of 250 µ*M*. Owing to the low activity of the enzyme, K_M could not be obtained accurately. AKR11A is unable to oxidize *myo*-inositol with either $NADP^+$ or NAD^+ as a cosubstrate and also does not use glucose as its substrate. The structure of AKR11A may aid in the identification of better substrates.

In view of the fact that several families of AKRs are functional as dimers (families 2 and 7) and tetramers (family 6), we investigated the oligomeric structure of the enzymes. From the dynamic light-scattering experiments, the molecular weights of AKR11A and AKR11B were individually estimated to be 36 and 37 kDa, respectively, clearly indicating their monomeric nature. To examine the possibility of heterodimerization, the proteins were mixed in equimolar quantities, but the resulting molecular weight of the proteins was still approximately 40 kDa, suggesting that there

Table 2
Crystallographic parameters and statistics.

Values given in parentheses are for the highest resolution shell.

	AKR11A (apo)	AKR11B (holo)
Space group	$P2_12_12_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 45.9, b = 87.7, c = 94.3	a = 54.8, b = 113.0, c = 57.5, β = 90.8
Resolution (Å)	30–1.9 (1.93–1.90)	30–2.8 (2.92–2.8)
Unique reflections/ observations	30304/159075	17299/76628
Completeness (%)	98.5 (92.5)	99.4 (99.4)
$I/\sigma(I)$	26.1 (5.0)	8.2 (3.1)
$R_{\rm merge}$ (%)	5.2 (31.9)	8.5 (20.6)

is no heterodimerization between these two aldo–keto reductases. The molecular weights based upon sequence data are 35 kDa for AKR11A and 37 kDa for AKR11B.

AKR11A crystallized in space group $P2_12_12_1$, with unit-cell parameters a = 45.9, b = 87.7, c = 94.3 Å and a $V_{\rm M}$ of 2.7 Å^3 Da^{-1} for one molecule per asymmetric unit. AKR11B bound to NADP$^+$ formed crystals in space group $P2_1$, with unit-cell parameters a = 54.8, b = 113.0, c = 57.5 Å, β = 90.8°, giving a $V_{\rm M}$ of 2.4 Å^3 Da^{-1} for two molecules per asymmetric unit. Crystallographic parameters and data-collection statistics for both enzymes are summarized in Table 2.

Attempts to obtain the phases for both structures are under way using various aldo–keto reductases as models for molecular replacement. The proteins whose structures have been determined that are most promising as suitable models because of sequence homology are the rat K$^+$ channel β-subunit (PDB code 1qrq) and aflatoxin aldehyde reductase (PDB code 1gve). Each of these shares between 24 and 29% identity with AKR11A and AKR11B.

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