Biochemical and Biophysical Research Communications 474 (2016) 104–110

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Structural characterization of an aldo-keto reductase (AKR2E5) from the silkworm *Bombyx mori*

Kohji Yamamoto [a, *], Akifumi Higashiura [b], Mamoru Suzuki [b], Takahiro Shiotsuki [c], Ryohei Sugahara [c], Takeshi Fujii [d, e], Atsushi Nakagawa [b]

[a] Kyushu University Graduate School, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
[b] Institute for Protein Research, Osaka University, Suita 565-0871, Japan
[c] Institute of Agrobiological Sciences, NARO, Tsukuba, Ibaraki 305-8634, Japan
[d] Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
[e] Department of Science of Biological Production, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan

ARTICLE INFO

Article history:
Received 15 April 2016
Accepted 16 April 2016
Available online 19 April 2016

Keywords:
Protein crystal structure
Enzyme specificity
NADPH
Lepidoptera

ABSTRACT

We report a new member of the aldo-keto reductase (AKR) superfamily in the silkworm *Bombyx mori*. Based on its amino acid sequence, the new enzyme belongs to the AKR2 family and was previously assigned the systematic name AKR2E5. In the present study, recombinant AKR2E5 was expressed, purified to homogeneity, and characterized. The X-ray crystal structures were determined at 2.2 Å for the apoenzyme and at 2.3 Å resolution for the NADPH-AKR2E5 complex. Our results demonstrate that AKR2E5 is a 40-kDa monomer and includes the TIM- or $(\beta/\alpha)_8$-barrel typical for other AKRs. We found that AKR2E5 uses NADPH as a cosubstrate to reduce carbonyl compounds such as DL-glyceraldehyde, xylose, 3-hydroxy benzaldehyde, 17α-hydroxy progesterone, 11-hexadecenal, and bombykal. No NADH-dependent activity was detected. Site-directed mutagenesis of AKR2E5 indicates that amino acid residues Asp70, Tyr75, Lys104, and His137 contribute to catalytic activity, which is consistent with the data on other AKRs. To the best of our knowledge, AKR2E5 is only the second AKR characterized in silkworm. Our data should contribute to further understanding of the functional activity of insect AKRs.

1. Introduction

The aldo-keto reductase (AKR) superfamily comprises ubiquitously expressed enzymes responsible for the reduction of aldehydes, ketones, steroid hormones, and monosaccharides [1], mainly via NADPH-dependent reversible reactions. Multiple AKR families and subfamilies have been defined based on the differences in amino acid sequences [2], which influence substrate specificity. For example, the AKR family 1 (AKR1) comprises hydroxysteroid dehydrogenases, whereas the AKR2 family contains aldose reductases. Other AKR families are involved in prostaglandin synthesis, xenobiotic detoxification, reduction of phospholipid aldehydes, metabolic assimilation of carbohydrates, and other secondary metabolic pathways [2]. In all characterized members, AKR structures contain the triose phosphate isomerase $(\alpha/\beta)_8$-barrel (TIM barrel) motif with a cylindrical core of eight parallel β-strands surrounded by eight α-helices running antiparallel to the strands. AKR binding sites are formed when the cofactor NADPH binds to the pocket generated by the C-terminal face of the TIM barrel. Residues involved in cofactor bindings, such as the catalytic tetrad Asp, Tyr, Lys, and His, are highly conserved [3].

In our previous study, we identified mRNA coding for a hitherto unknown AKR in the silkworm (*Bombyx mori*), a model Lepidoptera [4]. Here, we biochemically characterized this enzyme designated AKR2E5. Our kinetic studies demonstrated that AKR2E5 recognized aldehydes, steroid hormones, sugars, and insect pheromones. We also determined AKR2E5 X-ray crystal structure and investigated structure–function relationships in enzyme catalysis using the recombinant AKR2E5 overexpressed in *Escherichia coli*. Our results suggest that conserved amino acid residues in the NADPH-binding region of AKR2E5 are critical for catalysis.

Abbreviations: AKR, aldo-keto reductase; DLG, DL-glyceraldehyde; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TIM, triose phosphate isomerase.

* Corresponding author.
E-mail address: yamamok@agr.kyushu-u.ac.jp (K. Yamamoto).

http://dx.doi.org/10.1016/j.bbrc.2016.04.079
0006-291X/

2. Materials and methods

2.1. Insects and tissue dissection

Silkworm larvae were reared on mulberry leaves in the Institute of Genetic Resources at Kyushu University Graduate School (Fukuoka, Japan). Day-3 fifth-instar larvae were dissected on ice, and the resulting tissues were stored at −80 °C until use. Total RNA was extracted from the dissected tissues using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol, and then used for RT-PCR.

2.2. Cloning and sequencing of AKR2E5-encoding cDNA (akr2e5)

Total RNA was analyzed using RT-PCR. First-strand cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and an oligo-dT primer and used as a template for PCR with the following oligonucleotide primers: 5′-AGAACACATATGAATAAGGTGAATGTACCC-3′ (sense) and 5′-AAGGATCCTTAATCGATGTCACCGTTGGCA-3′ (antisense). Primers were designed based on the sequence obtained from the SilkBase EST database [5]; the underlined and double-underlined regions are *Nde*I and *Bam*HI restriction enzyme sites, respectively, used to insert the PCR product into the plasmid. The thermocycling conditions were as follows: 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min, with a final extension step at 72 °C for 10 min AKR2E5 cDNA (*akr2e5*) was ligated into the pGEM-T Easy Vector (Promega) and used to transform *E. coli* TOP10. The clone was sequenced using the GenomeLab DTCS Quick Start Kit (Beckman) and an automated DNA Sequencer (CEQ 8000; Beckman). We used GENETYX-MAC software (ver. 14.0.12) to obtain the complete *akr2e5* gene sequence and to deduce the amino acid sequence.

Sequence identity alignments were performed with ClustalW (ver. 1.83); the gap creation penalty and gap extension were set to 10 and 0.2, respectively.

2.3. Overexpression and purification of the recombinant protein

The pGEM-T Easy Vector carrying *akr2e5* was digested with *Nde*I and *Bam*HI, subcloned into the expression vector pET-11b (Novagen), and transformed into competent *E. coli* Rosetta-gami™ 2 (DE3) cells (Novagen). Cells were grown at 37 °C in Luria–Bertani (LB) medium containing 100 μg/mL ampicillin until the optical density (OD_{600}) reached 0.7, and induced with 1 mM isopropyl 1-thio-β-D-galactoside (PMSF) for the production of recombinant protein. After further incubation for 3 h, cells were centrifuged, resuspended in 20 mM Tris–HCl containing 0.5 M NaCl (pH 8.0), 4 mg/mL lysozyme, and 1 mM PMSF, and disrupted with sonication.

Unless otherwise noted, all subsequent operations were conducted at 4 °C. The disrupted cells were centrifuged at 10,000 × *g* for 15 min, and the resulting supernatant was subjected to ammonium sulfate fractionation (30%–70% saturation). The precipitate was resuspended in 10 mM Tris–HCl buffer (pH 8.0), dialyzed against the same buffer, and subjected to anion-exchange chromatography on DEAE-Sepharose (GE Healthcare); the bound protein was eluted by a linear gradient (0–0.3 M) of NaCl. Enzyme-containing fractions were pooled, concentrated using a centrifugal filter (Millipore), applied to a Superdex 200 column (GE Healthcare), and equilibrated in Tris–HCl containing 0.2 M NaCl. SDS-PAGE was performed in a 15% polyacrylamide slab gel containing 0.1% SDS, as previously described [6]. Protein bands were visualized by staining with Coomassie Brilliant Blue R250.

2.4. Crystallization, data collection, and structure determination

Purified AKR2E5 protein was concentrated to approximately 10 mg/mL in 10 mM HEPES (pH 7.4). Crystallization was performed using a sitting-drop vapor diffusion method at 20 °C. Each drop was formed by mixing equal volumes (1 μL) of protein and reservoir solutions. Crystals used for X-ray analysis were grown at 20 °C for 1 month in 0.1 M Tris–HCl (pH 8.5) containing 4 M ammonium acetate. X-ray diffraction data sets were collected with beamline

Table 1
Data collection and refinement statistics for AKR2E5 (Values in parentheses indicate the highest-resolution shell).

Parameter	apo-AKR2E5	AKR2E5 complexed with NADPH
Space group	$P6_122$	$P6_122$
Unit cell parameters (Å)	$a = b = 112.18$, $c = 147.63$	$a = b = 111.95$, $c = 147.65$
Beam line	SPring-8, BL44XU	SPring-8, BL44XU
Wavelength (Å)	0.9	0.9
Resolution range (Å)	37.0–2.20 (2.24–2.20)	46.1–2.30 (2.34–2.30)
Total number of reflections	343,695	383,297
Number of unique reflections	28,491 (1398)	24,781 (1215)
Multiplicity	12.1 (12.3)	15.5 (15.4)
R_{merge}[a] (%)	7.7 (48.8)	7.3 (46.3)
$\langle I\rangle/\langle\sigma(I)\rangle$	35.6 (6.97)	30.0 (5.04)
Completeness (%)	99.8 (99.7)	99.7 (99.3)
Refinement statistics		
Resolution range (Å)	37.0–2.20	46.1–2.30
Number of reflections	27,609	23,541
R_{work}[b] (%)/R_{free}[c] (%)	15.7/20.4	15.9/20.6
Root-mean-square deviations		
Bond lengths (Å)/Bond angles (°)	0.021/1.909	0.019/1.935
Average B-factors (Å^2)/Number of atoms		
Protein	34.8/2614	35.4/2637
Small molecules[d]	42.8/37	44.7/77
Water	41.3/252	41.0/241
Ramachandran plot		
Favored region (%)	96.9	96.3
Allowed regions (%)	3.1	3.7

[a] $R_{merge} = \sum(I - \langle I\rangle)/\sum\langle I\rangle$, where I is the intensity measurement for a given reflection and $\langle I\rangle$ is the average intensity for multiple measurements of this reflection.
[b] $R_{work} = \sum|F_{obs} - F_{cal}|/\sum F_{obs}$, where F_{obs} and F_{cal} are the observed and calculated structure-factor amplitudes.
[c] The R_{free} value was calculated using only an unrefined, randomly chosen subset of reflection data (5%) that were excluded from refinement.
[d] Small molecules include NADPH, ethylene glycol, acetate ion and calcium ion.