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Molecular characterization of an aldo-keto reductase from *Marivirga tractuosa* that converts retinal to retinol

Seung-Hye Hong[a,1], Hyun-Koo Nam[a,1], Kyoung-Rok Kim[a], Seon-Won Kim[b], Deok-Kun Oh[a,*]

[a] Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea
[b] Division of Applied Life Science, EB-NCRC and PMBBRC, Gyeongsang National University, Jinju 660-701, Republic of Korea

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ABSTRACT

A recombinant aldo-keto reductase (AKR) from *Marivirga tractuosa* was purified with a specific activity of 0.32 unit ml^{-1} for all-*trans*-retinal with a 72 kDa dimer. The enzyme had substrate specificity for aldehydes but not for alcohols, carbonyls, or monosaccharides. The enzyme turnover was the highest for benzaldehyde (k_{cat} = 446 min^{-1}), whereas the affinity and catalytic efficiency were the highest for all-*trans*-retinal (K_m = 48 μM, k_{cat}/K_m = 427 mM^{-1} min^{-1}) among the tested substrates. The optimal reaction conditions for the production of all-*trans*-retinol from all-*trans*-retinal by *M. tractuosa* AKR were pH 7.5, 30 °C, 5% (v/v) methanol, 1% (w/v) hydroquinone, 10 mM NADPH, 1710 mg l^{-1} all-*trans*-retinal, and 3 unit ml^{-1} enzyme. Under these optimized conditions, the enzyme produced 1090 mg ml^{-1} all-*trans*-retinol, with a conversion yield of 64% (w/w) and a volumetric productivity of 818 mg l^{-1} h^{-1}. AKR from *M. tractuosa* showed no activity for all-*trans*-retinol using $NADP^+$ as a cofactor, whereas human AKR exhibited activity. When the cofactor-binding residues (Ala158, Lys212, and Gln270) of *M. tractuosa* AKR were changed to the corresponding residues of human AKR (Ser160, Pro212, and Glu272), the A158S and Q270E variants exhibited activity for all-*trans*-retinol. Thus, amino acids at positions 158 and 270 of *M. tractuosa* AKR are determinant residues of the activity for all-*trans*-retinol.

1. Introduction

Retinoids have been extensively studied over the last decades (Blomhoff and Blomhoff, 2006). Retinoids include retinal, retinol, retinoic acid, and retinyl ester, which are the aldehyde, alcohol, oxidized, and ester forms of vitamin A, respectively, and they participate in growth (Raifen et al., 1996), immune system (Mora et al., 2008), vision (von Lintig, 2012), and gene signaling (Pino-Lagos et al., 2010).

Commercial retinol production is currently conducted *via* chemical synthesis through the acid or base reduction of a pentadiene derivative followed by the acidification/hydrolysis of the isomeric mixture to generate retinol (Mercier and Chabardes, 1994). Although this chemical process is economic, the method has some disadvantages, including complex purification steps, the formation of chemical wastes, and the formation of undesired by-products. Moreover, the product of the chemical process is not retinol but retinol acetate. Thus, the biological manufacture of retinol has been focused in recent years (Kim and Oh, 2010).

The enzymes catalyzing the oxidation and reduction of retinal are classified into 4 distinct types: alcohol dehydrogenases (ADHs) of the medium-chain dehydrogenase (MDR) family, short-chain dehydrogenase (SDR), the aldehyde dehydrogenase (ALDH), and aldo-keto reductase (AKR) families (Duester et al., 2003). ADHs, SDRs and AKRs oxidize retinol to retinal and reduce retinal to retinol, whereas ALDHs oxidize retinal to retinoic acid. SDRs and AKRs prefer $NADP^+$/NADPH over NAD^+/NADH and catalyze the reverse reaction, reduction of retinal to retinol which can then be esterified and stored as retinyl esters (Ruiz et al., 2012). ADHs also catalyze the reversible reaction but prefer NAD^+/NADH to $NADP^+$/NADPH (Duester, 1996). In contrast, ALDHs catalyze the irreversible oxidation of retinal to retinoic acid.

AKR family (EC 1.1.1.21) includes a number of NADPH-dependent oxidoreductases, such as aldehyde reductase, aldose reductase, prostaglandin F synthase, xylose reductase, rho crystallin, and many other enzymes (Bohren et al., 1989). The activity of AKR for retinal as a substrate has been reported in the subfamily 1B (aldose reductase) and 1C (prostaglandin F synthase), including AKR1B1, AKR1B10, and AKR1B12 from human, AKR1C7 from chicken, and AKR1C15 from rat (Crosas et al., 2001; Duester, 2000;

* Corresponding author. Tel.: +82 2 454 3118; fax: +82 2 444 5518.
E-mail address: deokkun@konkuk.ac.kr (D.-K. Oh).
[1] These authors equally contributed to this paper.

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Endo et al., 2007; Jez et al., 1997; Peralba et al., 1999; Ruiz et al., 2012). These AKRs have been reported not only to oxidize retinol and but also to reduce retinal. However, bacterial AKRs for retinal have not yet been characterized.

In this study, the *AKR* gene from *Marivirga tractuosa* was cloned and expressed in *Escherichia coli* for retinal reduction. The reaction conditions of the enzyme, including organic solvent, anti-oxidant, pH, temperature, and the concentrations of substrate and enzyme, were optimized. Under the optimized conditions, the production of all-*trans*-retinol from all-*trans*-retinal was performed. Moreover, the elucidation on the activity of *M. tractuosa* AKR for all-*trans*-retinol was tried.

2. Materials and methods

2.1. Bacterial strains, plasmid, and culture conditions

The expression vectors pET-22b(+) and pET-15b(+) were purchased from Novagen (San Diego, CA, USA). The expression host, *E. coli* ER 2566, and all restriction enzymes were purchased from New England Biolabs (Hertfordshire, UK). Luria-Bertani (LB) medium was purchased from BD Biosciences (San Jose, CA, USA). The substrates retinal and retinol and the cofactors NADH, NADPH, NAD^+, and $NADP^+$ (purity > 99%) were purchased from USB (Santa Clara, CA, USA). Pre-stained protein makers for SDS-PAGE and gel filtration calibration kit were purchased from MBI Fermentas (Hanover, MD, USA) and GE Healthcare (Piscataway, NJ, USA), respectively.

2.2. Gene cloning and site-directed mutagenesis

The gene encoding a putative AKR was amplified by PCR using the genomic DNA of *M. tractuosa* KCTC 2958 as a template. Sequences of primers used for gene cloning were based on DNA sequence of AKR from *M. tractuosa* DSM4126 (GenBank accession number NC_017459). Forward (5′-GCGGGATCCGATGAGAAAATT AACTTTCAGAAAC-3′) and reverse (5′-ATACTCGAGAGTTTCTCCCAAAGGCCAGCCATG-3′) primers for the insertion of pET-22b(+) plasmid were designed to introduce the underlined *Bam*HI and *Xho*I restriction sites, respectively, and forward (5′-GGCCATATGAGAAAATTAACTTTCAGAAAC-3′) and reverse (5′-ATACTCGAGTTCTCCCAAAGGCCAGCCATG-3′) primers for the insertion of pET-15b(+) plasmid were designed to introduce the underlined *Nde*I and *Xho*I restriction sites, respectively. The primers were synthesized by Bioneer (Daejon, Korea). The amplified DNA fragment was purified using a PCR purification kit (Promega, Fitchburg, WI, USA) and ligated into the *Bam*HI and *Xho*I sites of pET-22b(+) and the *Nde*I and *Xho*I sites of pET-15b(+). Each plasmid was transformed into *E. coli* ER2566 as an expression host, the transformed host was grown on LB agar (1.0% tryptone, 0.5% yeast extract, 1.0% sodium chloride, and 1.5% agar), and an ampicillin-resistant colony was selected. Gene expression was evaluated by both sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme activity assay. Mutations of the NADPH-binding residues in *M. tractuosa* AKR were generated by site-directed mutagenesis using a QuickChange Kit (Stratagene, Beverly, MA). DNA sequencing was performed at the Macrogen facility (Seoul, Korea).

2.3. Culture conditions and enzyme expression

M. tractuosa was grown in 50 ml of growth medium containing 0.5% glucose, 0.5% yeast extract, 1.0% casein peptone, and 0.5% sodium chloride in a 250 ml flask at 37 °C with shaking at 200 rpm. For protein expression, the recombinant *E. coli* cells were grown in LB medium in a 2000 ml flask containing 20 μg ml^{-1} ampicillin at 37 °C with shaking at 200 rpm. At the culture optical density of 0.6 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM. For the enzyme expression, the culture was then incubated for additional 16 h at 16 °C with shaking at 150 rpm. The expression of *M. tractuosa* AKR was determined by SDS-PAGE.

2.4. Enzyme purification

The cells were harvested from the culture broth by centrifugation at 6000 × *g* for 30 min at 4 °C, washed twice with 0.85% NaCl, resuspended in 50 mM phosphate buffer (pH 8.0) containing 300 mM KCl and 10 mM imidazole, and disrupted on ice using a sonicator. The unbroken cells and cell debris were removed by centrifugation at 13,000 × *g* for 20 min at 4 °C and the supernatant was filtered through a 0.45-μm filter. The recombinant protein, which was expressed from pET-22b(+), was applied to an immobilized metal ion affinity chromatography cartridge (Bio-Rad, Hercules, CA, USA) equilibrated with 50 mM phosphate buffer (pH 8.0). The cartridge was washed extensively with the same buffer, and the bound protein was eluted with a linear gradient of 10–500 mM imidazole at a flow rate of 1 ml min^{-1}. The eluate was collected and loaded onto a Bio-Gel P-6 desalting cartridge (Bio-Rad) equilibrated with 50 mM *N*-(2-hyroxyethyl)piperazine-*N*′-(3-propanesulfonic acid) (HEPPS) buffer (pH 7.5). The protein was eluted with 50 mM HEPPS buffer (pH 7.5) at a flow rate of 1 ml min^{-1} to obtain a solution of purified enzyme. The hexahistidine tag of the recombinant protein, which was expressed from pET-15b(+), was cut with a thrombin capture kit (Novagen, Madison, WI, USA). The thrombin-treated enzyme was applied to a His-trap HP affinity chromatography column (GE Healthcare, Piscataway, NJ, USA) equilibrated with 50 mM phosphate buffer (pH 7.0) for removing the histidine tag. The active factions were pooled and subsequently loaded onto a Benzamidine Sepharose column (Novagen, Madison, WI, USA) equilibrated with 50 mM phosphate buffer (pH 8.0) for removing thrombin. The columns were eluted in 50 mM HEPPS buffer (pH 7.5) at a flow rate of 1 ml min^{-1}. The resulting solution was used as a purified untagged protein, which was confirmed by SDS-PAGE. All purification steps using cartridges were carried out in a cold room at 4 °C with a Profinia protein purification system (Bio-Rad).

2.5. Determination of molecular mass

The subunit molecular mass of AKR from *M. tractuosa* was examined by SDS-PAGE (12% gels) under denaturing conditions, using the prestained protein makers as reference proteins. All protein bands were stained with Coomassie blue for visualization. The molecular mass of the native enzyme from *M. tractuosa* was determined by gel filtration chromatography using Sephacryl S-300 preparative-grade column HR 16/60 (GE Healthcare). The purified enzyme solution was applied to the gel filtration column and eluted at a flow rate of 0.3 ml min^{-1} with 50 mM HEPPS buffer (pH 7.5) containing 150 mM NaCl. The column was calibrated with ferritin (453 kDa), catalase (206 kDa), ovalbumin (44 kDa), and carbonic anhydrase (29 kDa) as reference proteins (GE Healthcare). The molecular mass of the native AKR was calculated by comparing with the migration time of the enzyme to those of the reference proteins.

2.6. Enzyme assay

Unless otherwise stated, the reaction was performed at 30 °C in 50 mM HEPPS buffer (pH 7.5) containing 0.2 mM all-*trans*-retinal, 0.016 unit ml^{-1} (0.05 mg ml^{-1}) enzyme, 5% (v/v) methanol, 1% (w/v) hydroquinone, and 1 mM NADPH for 5 min. After incubation, the reaction was stopped by adding the same volume of absolute