Chemico-Biological Interactions 202 (2013) 195–203

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

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

Biocatalytic production of alpha-hydroxy ketones and vicinal diols by yeast and human aldo–keto reductases

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ARTICLE INFO

Article history:
Available online 4 January 2013

Keyword:
Aldo-keto reductases
α-hydroxy ketones and diols
Stereoselectivity of yeast and human AKRs

ABSTRACT

The α-hydroxy ketones are used as building blocks for compounds of pharmaceutical interest (such as antidepressants, HIV-protease inhibitors and antitumorals). They can be obtained by the action of enzymes or whole cells on selected substrates, such as diketones. We have studied the enantiospecificities of several fungal (AKR3C1, AKR5F and AKR5G) and human (AKR1B1 and AKR1B10) aldo–keto reductases in the production of α-hydroxy ketones and diols from vicinal diketones. The reactions have been carried out with pure enzymes and with an NADPH-regenerating system consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase. To ascertain the *regio* and stereoselectivity of the reduction reactions catalyzed by the AKRs, we have separated and characterized the reaction products by means of a gas chromatograph equipped with a chiral column and coupled to a mass spectrometer as a detector. According to the regioselectivity and stereoselectivity, the AKRs studied can be divided in two groups: one of them showed preference for the reduction of the proximal keto group, resulting in the *S*-enantiomer of the corresponding α-hydroxy ketones. The other group favored the reduction of the distal keto group and yielded the corresponding *R*-enantiomer. Three of the AKRs used (AKR1B1, AKR1B10 and AKR3C1) could produce 2,3-butanediol from acetoin. We have explored the structure/function relationships in the reactivity between several yeast and human AKRs and various diketones and acetoin. In addition, we have demonstrated the utility of these AKRs in the synthesis of selected α-hydroxy ketones and diols.

1. Introduction

The Brenda database (<www.brenda-enzymes.org>) lists 560 different oxidoreductase activities that are carried out by more than 300 different proteins in *Saccharomyces cerevisiae* (SGD, <www.yeastgenome.org>). Ten of these enzymes belong to the aldo–keto reductase (AKR, <www.med.upenn.edu/akr>) superfamily, namely AKR2B6 (Gre3p), AKR3A1 (Gcy1p), AKR3A2 (Ypr1p), AKR3C1 (Ara1p), AKR5F (Yjr096p), AKR5G (Ydl124p), AKR9B1 (Aad14p), AKR9B2 (Aad3p), AKR9B3 (Aad4p) and AKR9B4 (Aad10p) [1,2]. The AKRs have been identified in many organisms, prokaryotes and eukaryotes, and are active towards a vast range of substrates, such as aldehydes, ketones, monosaccharides and steroids. Based on their sequence identity, AKRs fall into 15 different families (AKR1-AKR15) where some of them contain multiple subfamilies (e.g., AKR1A-AKR1E) [3]. Most AKRs are NADPH-dependent carbonyl reductases involved in drug detoxification and xenobiotic metabolism. In yeast, they have been implicated in several physiological roles such as oxidative defense and transcriptional regulation [4,5], and in xylose, arabinose and 2,3-butanediol metabolism [6,7]. Moreover, in a recent proteome-wide search, AKR3A1 and AKR5G have been identified as RNA-binding proteins [8]. Although much interest behind the study of AKRs relies on their implication in the development of human diseases, there is also a considerable interest in their use as biocatalysts. Thus, chiral alcohols obtained from the stereoselective reduction of carbonyl compounds are used as building blocks in the synthesis of antitumor agents, such as taxol [9], antidepressants and inhibitors of amyloid-β protein production [10]. Biotechnological applications of AKRs have been described, such as the conversion of xylose to ethanol [11,12] and the enantioselective reduction of prochiral carbonyl groups [13]. Consequently, these enzymes have been screened by several research groups with the aim to find new synthetic routes towards intermediates for the production of fine chemicals and pharmaceuticals [14–16].

In the present work we have studied the biotechnological potential of purified AKRs from human (AKR1B1 and AKR1B10) and yeast (AKR3C1, AKR5F and AKR5G), by characterizing the α-hydroxy ketones and diols produced in the reduction of several vicinal diketones and determining the steady-state kinetic parameters of these reactions.

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http://dx.doi.org/10.1016/j.cbi.2012.12.006

2. Materials and methods

2.1. Materials

Restriction enzymes, T4 DNA ligase, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and the cocktail of protease inhibitors (EDTA-free) were obtained from Roche. KOD DNA polymerase was purchased from Merck. DNA oligomers were synthesized and purified by Sigma–Genosys. Chemicals were purchased from Fluka, Aldrich or Sigma and were of the highest available quality.

2.2. Yeast and bacterial strains, and molecular biology techniques

Escherichia coli XL-1 blue (Stratagene) was used for cloning experiments and was grown at 37 °C in Luria–Bertani medium supplemented with 50 μg/ml ampicillin to select for the desired plasmid constructs. *S. cerevisiae* strains were derived from the Adh deficient strain WV36-405 (*MAT***a,** *ura3-52, trp1, adh1Δ, adh2Δ, adh3, adh4::TRP1*) constructed by Dr. Wolfgang Vogel (Neuherberg, Germany). To avoid interfering reactions in the reduction of diketones, we used WV36-405 *bdh1::kanMX4, ara1::natMX4*, as a recipient strain to over-express the yeast AKRs.

Plasmid pYES2 (Invitrogen) containing the *URA3* gene as a selectable marker was used to overexpress the *AKR* genes in the presence of galactose. All DNA manipulations were performed under standard conditions as described [17]. The oligonucleotides ARA1- Fw: 5′ CGG GAT CCA TGT CTT CTT CAG TAG CC 3′ containing a *Bam*HI site (underlined) and ARA1- Rv: 5′ CTA GTC TAG AAA AGT GAA AAT AAA GTC G 3′ with an *Xba*I site were used to amplify *ARA1* (AKR3C1) by KOD DNA polymerase in a PCR. The amplified fragment and the pYES2 vector were digested with *Bam*HI and *Xba*I and the recombinant construct was introduced into yeast by the lithium acetate method [18], selecting the transformants on SC-Ura medium. The *YDL124W* (AKR5G) gene was amplified with the following oligonucleotides: YDL124-fw: 5′ CGC AAG CTT CAG ATG TCA TTT CAC CAA CAG TTC TTT ACC 3′ and YDL124-his-rv: 5′ GCC GGA TCC TTA ATG ATG ATG ATG ATG ATG TAC TTT TTG AGC AGC GTA GTT GTA TTT ACC G 3′. To amplify *YJR096W* (AKR5F), we used the following oligonucleotides: YJR096-fw: 5′ CGC AAG CTT AAC ATG GTT CCT AAG TTT TAC AAA CTT TCA AAC GG 3′ and YJR096-his-rv: 5′ GCC GGA TCC TTA ATG ATG ATG ATG ATG ATG TGG CGC GTC TGT GCA TTC CC 3′. The oligonucleotides used to hybridize to the 3′ ends of *YDL124W* and *YJR096W* were designed to add six His residues at the carboxyl ends of the corresponding proteins to facilitate their purification. Both genes were cloned in the shuttle vector pYES2 and the resulting constructs were used to transform WV36-405 *bdh1::kanMX4, ara1::natMX4* to uracil prototrophy. The correct constructs were checked by sequencing.

2.3. Purification of yeast AKR3C1, AKR5G(His)$_6$ and AKR5F(His)$_6$. Determination of Mr

AKR3C1 (Ara1p) was purified from a culture of WV36-405 *bdh1::kanMX4, ara1::natMX4* [pYES2-*ARA1*] grown in SC-Ura plus 2% (w/v) galactose and collected at the end of the logarithmic phase. A total yeast extract was obtained by disrupting the cells with glass beads in a Bead Beater from Biospec (Bartlesville, OK) in 50 mM Tris–Cl, 1 mM EDTA and 1 mM DTT, pH 7.4 (buffer A), containing protease inhibitors, followed by centrifugation at 16,000*g*. The supernatant was loaded onto a DEAE-Sepharose (2.5 × 10 cm) column, washed with buffer A and eluted with a 100-mL NaCl gradient (0–0.5 M) in buffer A. The fractions with diacetyl activity were pooled and loaded onto an Affi-Gel Blue (2.5 × 5 cm) column which was washed extensively with buffer A. AKR3C1 was eluted with a 100-mL NaCl gradient (0–0.75 M).

AKR5G(His)$_6$ and AKR5F(His)$_6$ were purified from yeast cultures WV36-405 *bdh1::kanMX4, ara1::natMX4* [pYES2-*YDL124*(His)$_6$] and WV36-405 *bdh1::kanMX4, ara1::natMX4* [pYES2-*YJR096*(His)$_6$] grown until the end of their logarithmic phase in SC-Ura plus 2% galactose. Each yeast pellet was collected and solubilized with detergent Y-Per (Pierce) containing protease inhibitors. The insoluble debris was removed by centrifugation at 10,000*g*. The clear supernatant was loaded into a Ni^{2+}–NTA metal affinity column (GE Healthcare) which was extensively washed with 50 mM sodium phosphate, pH 7.4, 150 mM NaCl and 20 mM imidazole. AKR5G(His)$_6$ and AKR5F(His)$_6$ were eluted with 0.5 M imidazole. The active fractions were pooled and loaded onto a Red-Sepharose (Sigma) column that was washed with 33 mM sodium phosphate pH 7.0. The proteins were eluted with a 100-mL NaCl gradient (0–5 M) in sodium phosphate, pH 7.0. The active fractions were loaded onto a Hi-load (26/60) SuperDex 200 prep grade column and eluted with 33 mM sodium phosphate, pH 7.0. The Mr of the purified yeast enzymes were determined by gel filtration

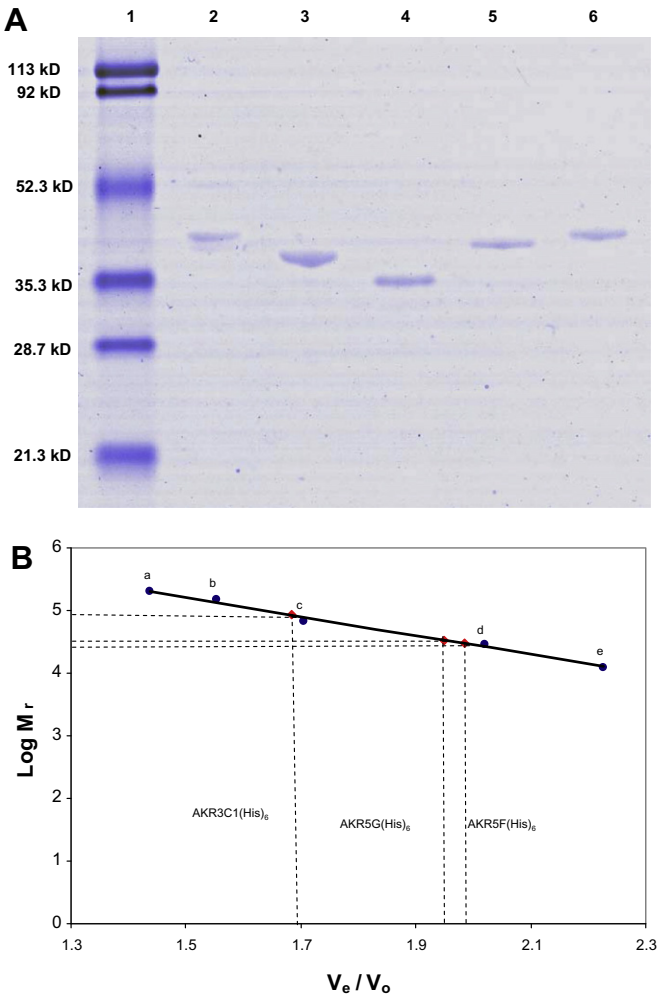

Fig. 1. Analysis of purified yeast and human AKRs. (A) 12% SDS–PAGE of purified AKRs (2 μg). Lane 1: Mr. markers, Lane 2: AKR3C1(His)$_6$, Lane 3: AKR5G(His)$_6$, Lane 4: AKR5F(His)$_6$, Lane 5: (His)$_{10}$AKR1B1 and Lane 6: (His)$_{10}$AKR1B10. (B) Size-exclusion chromatography on a SuperDex 200 column connected to an HPLC system. The Mr. standards used were (a) β-amylase, 200,000, (b) yeast alcohol dehydrogenase, 150,000, (c) bovine serum albumin, 66,000, (d) carbonic anhydrase, 29,000 and (e) cytochrome c, 12,400.