

Stereoselective Reduction of 4-Benzoylpyridine in the Heart of Vertebrates

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Abstract

The stereoselectivity in the reduction of 4-benzoylpyridine (4-BP) was examined in the cytosolic fractions from the heart of 9 vertebrates (pig, rabbit, guinea pig, rat, mouse, chicken, soft-shelled turtle, frog and flounder). 4-BP was stereoselectively reduced to *S*(-)- α -phenyl-4-pyridylmethanol [*S*(-)-PPOL] in the cytosolic fractions from the heart of pig, rabbit and guinea pig. However, of mammalian heart cytosol tested, only rat heart cytosol had little ability to reduce stereoselectively 4-BP. In an attempt to elucidate this reason, amino acid sequence of rat heart carbonyl reductase (RatHCR) was deduced from the cloned cDNA and compared with that of pig heart carbonyl reductase (PigHCR), which shows a high stereoselectivity in the reduction of 4-BP to *S*(-)-PPOL. RatHCR showed a high identity with PigHCR in amino acid sequence. Furthermore, recombinant RatHCR was confirmed to reduce stereoselectively 4-BP to *S*(-)-PPOL with a high optical purity comparable to recombinant PigHCR. It is possible that in the cytosolic fraction from the heart of rat, constitutive reductase other than RatHCR counteracts the stereoselective reduction of 4-BP to *S*(-)-PPOL, by catalyzing the reduction of 4-BP to the *R*(+)-enantiomer.

Keywords: Stereoselective reduction; Carbonyl reductase; 4-Benzoylpyridine; Species difference; Heart

Introduction

Carbonyl reductase (EC 1.1.1.184) catalyzes the reduction of endogenous and exogenous carbonyl compounds to their corresponding alcohols (Forrest and Gonzalez, 2000; Oppermann and Maser, 2000; Rosemond and Walsh, 2004). Many carbonyl reductases have been purified from various tissues such as the brain, liver and kidney in humans and animal species (Wermuth, 1981; Ikeda et al., 1984; Usui et al., 1984; Imamura et al., 1993). In general, carbonyl reductases are monomeric and cytosolic enzymes with molecular weight of around 34 KDa. The enzymes have been considered as a member of the aldo-keto reductase family based on their functional properties (Maser, 1995). However, recent structural investigations including amino acid sequences have demonstrated that most of carbonyl reductases belong to the short chain dehydrogenase/reductase (SDR) family (Forrest and Gonzalez, 2000; Oppermann et al., 2001).

We have purified a tetrameric form of carbonyl reductase from cytosolic fractions of rabbit and pig heart, using 4-benzoylpyridine (4-BP) as a substrate (Imamura et al., 1999; Usami et al., 2003). The enzyme purified from pig heart (pig heart carbonyl reductase, PigHCR) belongs to the SDR family, and has the ability to reduce efficiently alkyl phenyl ketones, α -dicarbonyl compounds and all-*trans* retinal. Furthermore, recombinant PigHCR isolated from the cell extract of *Escherichia coli* (*E. coli*) expressing its cDNA, like native PigHCR, is demonstrated to catalyze the stereoselective reduction of 4-BP to *S*(-)- α -phenyl-4-pyridylmethanol [*S*(-)-PPOL] (Fig.

1) (Shimada et al., 2003). As evident from the chemical structure of 4-BP, the phenyl group shows a structural resemblance to the pyridyl group in size and stereochemical characteristics. Thus, it is interesting that PigHCR has the ability to reduce stereoselectively 4-BP.

Recently, the reduction of 4-BP in the cytosolic fraction of pig heart has been reported to exhibit a stereoselectivity comparable to that in recombinant PigHCR (Shimada et al., 2004). In our preliminary experiment, however, such a high stereoselectivity was not observed in the reduction of 4-BP in the cytosolic fractions from the heart of several vertebrates. One of the most important aspects of drug metabolism, particularly in relation to preclinical safety evaluation, is species difference in metabolic pathways (Fournel and Caldwell, 1986; Suckow et al., 1986; Takasaki et al., 1999). However, information about species difference in the stereoselective reduction of drugs containing a ketone group within their chemical structures has been very limited.

The present study was designed to evaluate the stereoselectivity in the reduction of 4-BP in cytosolic fractions from the heart of 9 vertebrates (pig, rabbit, guinea pig, rat, mouse, chicken, soft-shelled turtle, frog and flounder). Furthermore, amino acid sequence of rat heart carbonyl reductase (RatHCR) was deduced from the cloned cDNA and the stereospecificity of recombinant RatHCR for 4-BP reduction was compared with that of recombinant PigHCR.

Materials and methods

Materials

4-Benzoylpyridine (4-BP) was purchased from Wako Pure Chemicals (Osaka, Japan). *S*(-)- and *R*(+)- α -phenyl-4-pyridylmethanol [*S*(-)-PPOL and *R*(+)-PPOL] were synthesized from 4-BP as reported previously (Shimada et al., 2003). NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). All other chemicals were of reagent grade.

Animals

Pig hearts were supplied from a slaughterhouse and stored at -20°C . Male rabbits at 15 weeks of age (Japanese White) and guinea pigs at 10 weeks of age (Hartley) were purchased from KBT Oriental (Saga, Japan). Male rats at 8 weeks of age were obtained from the following sources: Fischer 344, Wistar, Sprague-Dawley (SD) and WKY (Japan SLC, Shizuoka, Japan); Long-Evans (LE) [KBT Oriental (Saga, Japan)]; Wistar-Imamichi (WI) [Imamichi Institute for Animal Reproduction (Ibaraki, Japan)]. Male mice (ddY) at 10 weeks of age were purchased from Japan SLC. Male chickens were supplied from Kumamoto Prefectural Agricultural Research Center (Kumamoto, Japan). Male frog (*Rana catesbeiana*) was purchased from Kyudo (Saga, Japan). Male soft-shelled turtle (*Pelodiscus sinensis*) and flounder (*Paralichthys olivaceus*) were supplied from Hattori SST (Kumamoto, Japan) and Kumamoto Prefectural Fisheries Research Center (Kumamoto, Japan), respectively. All animal experiments

were performed in accordance with the Guidelines for Animal Experiments of Kumamoto University.

Preparation of cytosolic fraction

The hearts were excised from animals anesthetized. The tissues were homogenized in three volumes of 10 mM sodium potassium phosphate buffer containing 1.15% KCl (pH 6.0). The homogenates were centrifuged at 105,000g for 60 min to obtain the cytosolic fraction.

Stereoselective reduction of 4-BP

The stereoselective reduction of 4-BP was estimated by measuring *S*(-)- and *R*(+)-PPOL formed from 4-BP in the reaction mixture (Shimada et al., 2003). The reaction mixture consisted of substrate (0.5 mM 4-BP), NADPH-generating system (50 μ M NADP, 1.25 mM glucose-6-phosphate, 50 munits glucose-6-phosphate dehydrogenase and 1.25 mM MgCl₂), the cytosolic fraction (or recombinant RatHCR) and 100 mM sodium potassium phosphate buffer (pH 6.0) in a final volume of 0.5 ml. The reaction mixture was incubated at 37 °C for 10 min and boiled for 2 min to stop the reaction. After centrifugation at 5,000 rpm, the supernatant (20 μ l) was subjected to high-performance liquid chromatography (HPLC) for the determination of the reduction products, *S*(-)- and *R*(+)-PPOL, of 4-BP. HPLC was carried out using a Waters 600E HPLC apparatus (Japan Waters, Tokyo, Japan) equipped with a Daicel Chiralpak AD-RH column (Daicel, Tokyo, Japan) and a Waters 484 UV monitor (254 nm).

Mixture of 20 mM borate buffer (pH 9.0)-acetonitrile (6:4, v/v) was used as a mobile phase at a flow rate of 0.5 ml/min. The amount of the reduction products formed from 4-BP was estimated as nmol/mg protein. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Isolation of cDNA for RatHCR

To isolate the cDNA for RatHCR, we searched rat Expressed Sequence Tag (EST) sequences and found a rat EST (accession number: AW141472), which shows 82% sequence identity with the cDNA for rabbit and pig HCRs, but lacks its 5'-sequence (486 base pairs) of the putative open reading frame. The 5'-end of the sequence of the putative open reading frame of the rat EST was generated from a total RNA preparation of a male Wister rat heart by using a 5'-rapid amplification of cDNA end (RACE) kit (Gibco-BRL, Rockvill, MD, USA). The sequences of three primers used in the RACE were 5'-cggaaagcaggaactaac (ra-1r), 5'-ctgggcagagctgattttgtg (ra-2r), and 5'-ccctgggtaggctgtctagg (ra-3r), which correspond to positions 901-919, 879-899 and 785-804, respectively, of the full-length cDNA. The DNA sequencing was performed using an ABI 373A DNA sequencing system (Applied Biosystems Japan, Tokyo, Japan). The amplification of the full-length cDNA (804 base pairs, accession number: AB062758) was achieved by PCR using a set of forward and reverse primers, 5'-atggccagttccgggct (that anneals to position 1-17) and ra-3r, and its sequence was confirmed by repeating RT-PCR and sequencing of the amplified products.

Expression and purification of recombinant RatHCR

The bacterial expression plasmid harboring the cDNA for RatHCR was constructed using pCR T7 TOPO vectors (Invitrogen, Carlsbad, CA, USA), and transformed into *E. coli* BL21(DE3)pLysS cells (Invitrogen) as described previously (Usami et al., 2003). The integrity of the construct was verified by DNA sequencing. The culture of the *E. coli* cells, induction of the recombinant enzyme, and preparation of the cell extract were carried out as described previously (Nakanishi et al., 1995; Usami et al., 2003). Recombinant RatHCR was purified from the cell extract by essentially the same procedures for the purification of rabbit HCR (Imamura et al., 1999).

Results

Species difference of stereoselectivity in the reduction of 4-BP

Table 1 summarizes the stereoselectivity in the reduction of 4-BP in cytosolic fractions from the heart of 9 vertebrates. In the cytosolic fractions from the heart of pig, rabbit and guinea pig, 4-BP was preferentially reduced to *S*(-)-PPOL at the percentage of 94.7, 80.5 and 83.7%, respectively. Furthermore, the formation of *S*(-)-PPOL in mouse heart cytosol was significantly larger than that of *R*(-)-PPOL. However, the stereoselectivity in the reduction of 4-BP was little observed in the cytosolic fractions from the heart of rat, chicken, soft-shelled turtle, frog and flounder.

Strain difference of stereoselectivity in the reduction of 4-BP

Of mammalian heart cytosol tested in this study, only rat heart cytosol had little ability to reduce stereoselectively 4-BP, as described above. The data of the rat in Table 1 are those obtained from a rat strain (Wistar strain). Thus, the stereoselectivity in the reduction of 4-BP was further examined in the cytosolic fractions from the heart of several rat strains other than Wistar strain (Table 2). However, the stereoselectivity was little observed even in these cytosolic fractions, indicating that there is no strain difference of stereoselectivity in the reduction of 4-BP in rat heart cytosol.

Deduced amino acid sequence of RatHCR

We cloned the cDNA for RatHCR by rapid amplification of cDNA end (RACE) and RT-PCR methods. Amino acid sequence of RatHCR was deduced from the cloned cDNA (Fig. 2). The amino acid sequence of RatHCR was 79% identical with that of PigHCR, which shows a high stereoselectivity in the reduction of 4-BP, as reported previously.

Stereoselective reduction of 4-BP by recombinant RatHCR

Recombinant RatHCR was isolated from the cell extract of *E. coli* transfected with the plasmids harboring its cDNA. Furthermore, the ability of recombinant RatHCR to reduce stereoselectively 4-BP was evaluated. When 4-BP was incubated in the reaction mixture containing recombinant RatHCR and NADPH for 10 min, the reduction product appeared as a main peak corresponding to *S*(-)-PPOL on HPLC, as

shown in Fig. 3. This result indicates that recombinant RatHCR stereoselectively reduces 4-BP to *S*(-)-PPOL with a high optical purity [82.3% enantiomeric excess (ee)].

Discussion

It has been reported that 4-BP is stereoselectively reduced to *S*(-)-PPOL in the cytosolic fraction from the heart of pig (Shimada et al., 2004). However, the results of the present study using cytosolic fractions from the heart of 9 vertebrates (pig, rabbit, guinea pig, rat, mouse, chicken, soft-shelled turtle, frog and flounder) have demonstrated that there is a large species difference of stereoselectivity in the reduction of 4-BP. In particular, the cytosolic fraction from the heart of rat, unlike those of the other mammalian species, had little ability to reduce stereoselectively 4-BP. Furthermore, there was no difference of stereoselectivity in the reduction of 4-BP in the cytosolic fractions from the heart of different rat strains.

In an attempt to elucidate the reason for the large species difference of stereoselectivity in the reduction of 4-BP, amino acid sequence of RatHCR was deduced from the cloned cDNA and compared with that of PigHCR reported previously (Usami et al., 2003). RatHCR showed a high identity with PigHCR in amino acid sequence. In addition, the residues involved in the coenzyme binding (Ala-21, Gly-25, Gly-27) and active (Ser-151, Tyr-164, Lys-168) sites of the SDR family proteins (Jörnvall et al., 1995; Oppermann et al., 2001; Tanaka et al., 2001) were conserved in the sequences of both RatHCR and PigHCR. These results suggest that RatHCR, like

PigHCR, can catalyze efficiently the stereoselective reduction of 4-BP. In fact, recombinant RatHCR was confirmed to reduce 4-BP to *S*(-)-PPOL with a high optical purity comparable to recombinant PigHCR: the optical purities of *S*(-)-PPOL formed by recombinant RatHCR and PigHCR were 82.3 and 87.2% ee, respectively. Based on these results, we propose the possibility that constitutive reductase other than RatHCR present in rat heart cytosol counteracts the stereoselective reduction of 4-BP to *S*(-)-PPOL, by catalyzing the reduction of 4-BP to the *R*(+)-enantiomer.

4-BP was stereoselectively reduced to *S*(-)-PPOL in the cytosolic fraction from the heart of pig. Interestingly, the amount of *R*(+)-enantiomer formed in pig heart cytosol was similar to that formed in rat heart cytosol. However, since the amount of *S*(-)-PPOL formed in pig heart cytosol is much larger than that of the *R*(+)-enantiomer formed simultaneously, it is reasonable to assume that the *R*(+)-enantiomer has little effect on the stereoselectivity in the reduction of 4-BP to *S*(-)-PPOL in pig heart cytosol.

In the cytosolic fractions from the heart of vertebrates other than mammalian species, the stereoselectivity in the reduction of 4-BP was little observed. This may be because the carbonyl reductase catalyzing the reduction of 4-BP to *S*(-)-PPOL and enzyme with the opposite stereospecificity present in the heart of these vertebrates counteract each other the stereoselectivity in the reduction of 4-BP, as described above. We are currently investigating characteristics of carbonyl reductase purified from the heart of these vertebrates.

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Figure legends

Fig. 1. Stereoselective reduction of 4-BP to *S*(-)-PPOL by recombinant PigHCR.

Fig. 2. Amino acid sequence alignment of RatHCR with PigHCR. Asterisks show the residues in the coenzyme binding (Ala-21, Gly-25, Gly-27) and active (Ser-151, Tyr-164, Lys-168) sites.

Fig. 3. HPLC chromatogram of the reduction products of 4-BP. The peaks *S* and *R* correspond to authentic *S*(-)-PPOL and *R*(+)-PPOL, respectively. The optical purity of *S*(-)-PPOL was 82.3% ee.