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Asymmetric ketone reduction by a hyperthermophilic alcohol dehydrogenase. The substrate specificity, enantioselectivity and tolerance of organic solvents

Dunming Zhu, Hiba T. Malik and Ling Hua*

Department of Chemistry, Southern Methodist University, Dallas, TX 75275, USA

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Abstract—In an effort to search for effective biocatalysts for asymmetric ketone reduction, the substrate specificity and enantioselectivity of an alcohol dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus* have been evaluated. This hyperthermophilic alcohol dehydrogenase catalyzes the reduction of various ketones including aryl ketones, α- and β-ketoesters. Interestingly, aryl ketones, phenyl-substituted α- and β-ketoesters were reduced to the corresponding chiral alcohols in an enantiomerically pure form, while the substrates lacking phenyl groups were reduced with a moderate enantioselectivity. It thus suggests that a phenyl group next to the carbonyl group could be very helpful for achieving an excellent enantioselectivity, and this could provide valuable guidance for the future application of this useful enzyme through rational substrate engineering. The reaction temperature increased the enzyme activity, but exerted no effect on the enantioselectivity. This alcohol dehydrogenase also showed a high tolerance of organic solvents such as dimethyl sulfoxide, *iso*-propanol, methyl *tert*-butyl ether, and hexane, a particularly important and useful feature for the reduction of ketones with a low solubility in aqueous buffers.

1. Introduction

Asymmetric reduction of ketones is a very important transformation in organic synthesis because chiral carbinols are useful bioactive compounds and their precursors. Among the methodologies available, asymmetric biocatalytic reduction has attracted a great attention from the green chemistry perspective.[1–5] Thus, there is a constant demand for efficient biocatalysts for this vital transformation, and many new biocatalytic systems both in whole cell and isolated enzyme forms have recently been developed.[6–12] As part of our own effort to develop an effective ketoreductase tool-box of isolated enzymes, we are especially interested in ketoreductases which complement the currently available ones.[13–15] More specifically, we have particularly been searching for ketoreductases with the following properties, such as anti-Prelog enantioselectivity,[16] broader substrate specificity,[17] larger active site for bulky substrates,[17,18] higher thermostability[18] and tolerance of organic solvents. Enzymes from hyperthermophiles, that is, microorganisms that grow optimally above 80 °C, usually display not only an extreme stability at a high temperature and high pressure, but also a high tolerance of chemical denaturants such as organic solvent.[19] In addition, the hyperthermophilic enzymes can be subjected to heat treatment, which is an easy handling and effective protein purification process. These unique features are desired for certain applications. Therefore, hyperthermophilic enzymes are interesting from both a scientific point of view and industrial applications.[20,21] Recently, Machielsen et al. have cloned and biochemically characterized an alcohol dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*.[22] This enzyme shows a high resistance to thermal inactivation, which has a half-life of 130 min at 100 °C. In addition, this alcohol dehydrogenase shows a preference for the less expensive co-factor NADH, although it takes both NADH and NADPH as a co-factor. These unique properties make this enzyme a potential biocatalyst for practical use and stimulated us to further assess its substrate specificity, stereoselectivity, and tolerance of organic solvents. Herein we report our findings on this unique alcohol dehydrogenase, which would provide useful guidance for the future application of this enzyme.

* Corresponding author. Tel.: +1 214 768 1609; fax: +1 214 768 4089; e-mail: lhua@smu.edu

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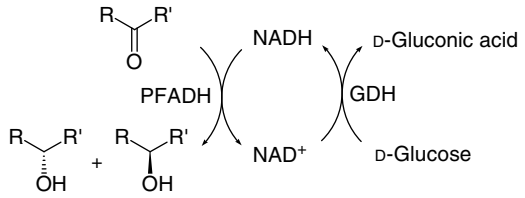
2. Results and discussion

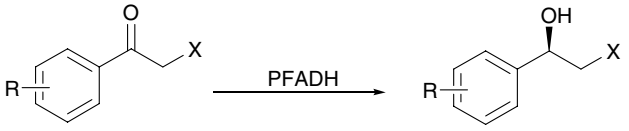
The alcohol dehydrogenase gene *adhD* from the hyperthermophilic archaeon *P. furiosus* was cloned and expressed in *Escherichia coli* and the recombinant enzyme was purified by heat treatment, and lyophilized (see Section 4). The obtained alcohol dehydrogenase (**PFADH**) was stable for months at 4 °C. The enzyme activity was assayed toward various ketones including aryl ketones and α- and β-ketoesters by spectrophotometrically measuring the oxidation of NADH at 340 nm. The enantioselectivity for the reduction of various ketones catalyzed by **PFADH** was evaluated using NADH as a co-factor, which was regenerated with a recycling system comprising glucose dehydrogenase and glucose (Scheme 1). The results are presented in Tables 1–3.



Scheme 1. Reduction of various ketones catalyzed by **PFADH** with NADH regeneration system.

From Table 1, it can be seen that the alcohol dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus* catalyzed the reduction of acetophenone derivatives to the corresponding (*S*)-chiral alcohols in an enantiomerically pure form. The substituents on the benzene ring of the aryl ketones exerted some effect on the enzyme activity, although the influence was not dramatic. The enantioselectivity of the reduction was not affected by the substituents and pattern of the substitution. The α-chlorinated acetophenone showed a much higher activity than the unsubstituted one (more than 10 times).

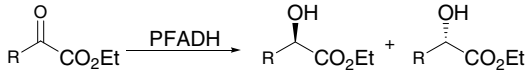
Table 1. Reduction of aryl ketones catalyzed by **PFADH**



X	R	Relative activity[a]	ee (%)
H	4′-H	100	99 (*S*)
H	4′-F	162	99 (*S*)
H	4′-Cl	131	99 (*S*)
H	4′-Br	77	99 (*S*)
H	4′-CH_3	108	99 (*S*)
H	4′-CF_3	146	99 (*S*)
H	2′-Cl	231	99 (*S*)
H	3′-Cl	285	99 (*S*)
H	3′-CH_3	154	99 (*S*)
H	2′-OCH_3	157	99 (*S*)
H	3′,5′-$(CF_3)_2$	131	99 (*S*)
Cl	4′-H	1331	99 (*R*)

[a] The specific activity of acetophenone was 13 nmol min^{-1} mg^{-1} at 37 °C and its relative activity was defined as 100.

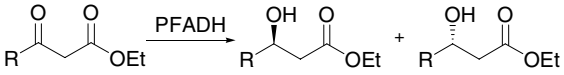
Table 2. Reduction of α-ketoesters catalyzed by **PFADH**



R	Relative activity[a]	ee (%)
C_6H_5	754	99 (*R*)
4′-F–C_6H_4	631	99 (*R*)
4′-Cl–C_6H_4	915	99 (*R*)
4′-Br–C_6H_4	1092	99 (*R*)
4′-CH_3–C_6H_4	523	99 (*R*)
4′-CN–C_6H_4	1469	99 (*R*)
3′,5′-F_2–C_6H_4	646	99 (*R*)
3′,4′-Cl_2–C_6H_4	769	99 (*R*)
iso-C_3H_7	946	44 (*S*)
tert-C_4H_9	654	71 (*S*)

[a] The specific activity of acetophenone was 13 nmol min^{-1} mg^{-1} at 37 °C and its relative activity was defined as 100.

Table 3. Reduction of β-ketoesters catalyzed by **PFADH**



R	Relative activity[a]	ee (%)
CH_3	285	95 (*S*)
C_2H_5	162	60 (*S*)
C_3H_7	215	24 (*R*)
iso-C_3H_7	108	16 (*S*)
CH_2Cl	1092	4 (*R*)
C_6H_5	154	99 (*S*)

[a] The specific activity of acetophenone was 13 nmol min^{-1} mg^{-1} at 37 °C and its relative activity was defined as 100.

Table 2 summarizes the results of the **PFADH**-catalyzed reduction of aromatic and aliphatic α-ketoesters at 37 °C. It can be seen that **PFADH** was more active toward the reduction of α-ketoesters than the above discussed aryl ketones. The enzyme activity was affected by the substituents on the benzene ring of aromatic α-ketoesters. For example, the enzyme activity increased when the *para*-substituents were F, Cl, and Br. The aromatic α-ketoester with *para*-CN substituent showed the highest activity. For aliphatic α-ketoesters, the enzyme activity decreased when *iso*-propyl was replaced with *tert*-butyl, suggesting that the bulkiness of the R group might inhibit the enzyme activity. It is interesting to note that the alcohol dehydrogenase from *P. furiosus* catalyzed the reduction of aromatic α-ketoesters to the corresponding enantiomerically pure (*R*)-configured α-hydroxyesters, while the enantioselectivity of the aliphatic α-ketoester reduction was moderate with the major products being (*S*)-enantiomer. Similar enantiopreference reversals have previously been observed in the reduction of α-ketoesters catalyzed by a carbonyl reductase from *Sporobolomyces salmonicolor* AKU4429 (**SSCR**) and enzyme–substrate docking studies showed that the opposite orientations of aromatic and aliphatic α-ketoesters in the active cavity of **SSCR** enzyme was responsible for the enantiopreference flip-flop.[17] Aromatic and aliphatic α-ketoesters may also take opposite orientations in the active

site of the **PFADH** enzyme, thus the different enantiomers were obtained as the major products. The excellent enantioselectivity for aromatic α-ketoesters also suggested that the phenyl group next to the carbonyl functionality might play a critical role in the substrate–protein interaction to distinguish the faces at which the hydride transfer occurred.

The **PFADH**-catalyzed reduction of several β-ketoesters was also studied (Table 3). Ethyl 3-oxo-butyrate was reduced to ethyl (*S*)-3-hydroxybutyrate in a high enantiomeric purity. As the size of the R group increased, the enantioselectivity of the reductions decreased, with the exception of R being a phenyl group, in which the product ethyl (*S*)-3-hydroxy-2-phenylpropionate was obtained in 99% ee. Although the size of R groups did not significantly affect the activity, an electron-withdrawing group such as Cl greatly enhanced the enzyme activity. This is similar to the above observation for α-chloroacetophenone (Table 1).

Since the alcohol dehydrogenase from the hyperthermophilic archaeon *P. furiosus* showed high resistance to thermal inactivation,[22] it would be interesting to study the temperature effects on the reactivity and enantioselectivity of this enzyme. Therefore, ethyl 2-(4-cyanophenyl)-2-oxo-acetate was used as the substrate for the reduction at different temperatures. The reductions were performed under two conditions, that is, (a) 2 equiv of NADH and (b) a catalytic amount of NADH with a regeneration system consisting of D-glucose dehydrogenase and D-glucose. The reactions were quenched after 1 h. The reaction mixtures were extracted with methyl *t*-butyl ether. The conversion and ee values were then determined by HPLC analysis. The conversions are presented in Figure 1. It can be seen from the results that the enzyme activity enhanced as the reaction temperature increased. Interestingly, ethyl (4-cyanophenyl)-2-hydroxyacetate was obtained in an essentially enantiomerically pure form at all temperatures. This is in contrast to the previous observation that the enantioselectivity decreases at higher reaction temperatures.[23]

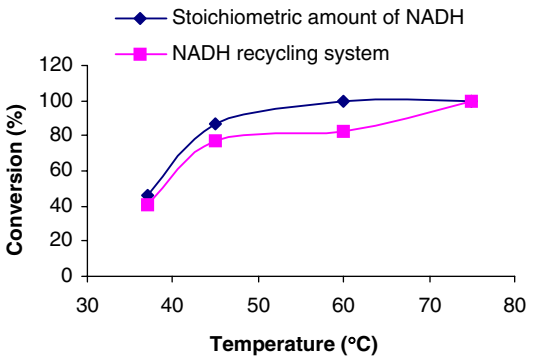


Figure 1. Temperature effect on the enzyme activity of the alcohol dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus* toward the reduction of ethyl 2-(4-cyanophenyl)-2-oxo-acetate.

Enzymatic reduction of poorly water-soluble ketones in aqueous media is usually hampered by the low availability of substrates. One strategy to overcome this limitation is the introduction of organic solvents, which improve the solubility of hydrophobic ketones.[24] However, the presence of organic solvents deactivates the enzymes in many cases. Enzymes, which are tolerant of organic solvents, will thus be of great interest from both scientific and practical points of view. Therefore, the reductions of ethyl 2-(4-cyanophenyl)-2-oxo-acetate in various organic solvents were examined to assess the organic solvent tolerance of the alcohol dehydrogenase from the hyperthermophilic archaeon *P. furiosus*. The reductions were carried out under two conditions: (a) 2 equiv of NADH and (b) a catalytic amount of NADH with a regeneration system consisting of D-glucose dehydrogenase and D-glucose. The reaction mixtures were incubated at 30 °C for 16 h. The products and the recovered substrates were extracted into methyl *tert*-butyl ether. The conversion and ee values were determined by chiral HPLC analysis. The conversions are presented in Figure 2. The results showed that the alcohol dehydrogenase from the hyperthermophilic archaeon *P. furiosus* was active in reaction media, with up to 30% of water-miscible solvents, such as DMSO and *iso*-propanol. The low conversion with NADH recycling system in the buffer–DMSO mixture (3 in Fig. 2) should be due to the inactivation of D-glucose dehydrogenase, because 100% conversion was obtained with a stoichiometric amount of NADH. This hyperthermophilic alcohol dehydrogenase was also active in two phase systems containing methyl *tert*-butyl ether or hexane, but the presence of toluene, ethyl acetate or *n*-butyl acetate inactivated this enzyme. It was worth noting that an excellent enantioselectivity of 99% ee was observed in all the cases. This indicated that the alcohol dehydrogenase from the hyperthermophilic archaeon *P. furiosus* was a very robust enzyme in some organic solvents. From a synthetic point of view, this property was particularly important and useful for the reduction of ketones with a low solubility in aqueous buffers.

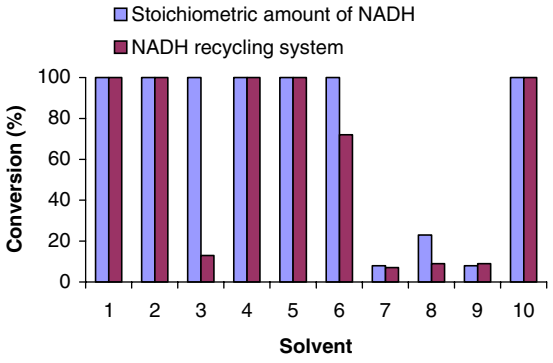


Figure 2. **PFADH**-catalyzed reduction of ethyl 2-(4-cyanophenyl)-2-oxo-acetate in various organic solvents. (1) Potassium phosphate buffer; (2) 15% DMSO; (3) 30% DMSO; (4) 15% *iso*-propanol; (5) 30% *iso*-propanol; (6) 30% methyl *tert*-butyl ether; (7) 30% toluene; (8) 30% ethyl acetate; (9) 30% *n*-butyl acetate; (10) 30% hexane.

3. Conclusion

The substrate specificity and enantioselectivity of an alcohol dehydrogenase from the hyperthermophilic archaeon *P. furiosus* (**PFADH**) have been assessed toward a variety of ketones including aryl ketones, α- and β-ketoesters. This hyperthermophilic alcohol dehydrogenase catalyzed the

asymmetric reduction of these ketones to the corresponding chiral alcohols. The temperature-dependent studies indicated that the enzyme activity enhanced as the reaction temperature increased. Surprisingly, the enantioselectivity did not change at higher temperatures. The latter was not consistent with the previous observations that the enantioselectivity decreased as the reaction temperature increased.[23] This was a useful feature for certain applications requiring asymmetric reduction at high temperatures. Another interesting observation was that aryl ketones, phenyl-substituted α- and β-ketoesters were reduced to the corresponding chiral alcohols in an enantiomerically pure form, while the substrates without phenyl groups were reduced with a moderate enantioselectivity. This suggested that a phenyl group next to the carbonyl group might be required to achieve an excellent enantioselectivity. The chloro group at the α-carbon of the carbonyl group improved the enzyme activity as shown in the reductions of α-chloroacetophenone and ethyl 4-chloro-3-oxobutyrate. These observations will provide useful guidance for the future application of this unique enzyme through rational substrate design. The present alcohol dehydrogenase also showed a high tolerance of organic solvents, such as dimethyl sulfoxide, *iso*-propanol, methyl *tert*-butyl ether, and hexane. This feature would be particularly important and useful for the reduction of hydrophobic ketones with a low availability in pure aqueous buffers.

4. Experimental

The chiral HPLC analysis was performed on an Agilent 1100 series high-performance liquid chromatography system with (*S*,*S*)-Whelk-O 1 column (25 cm × 4.6 mm, Regis Technologies Inc.). The chiral GC analysis was performed on a Hewlett–Packard 5890 series II plus gas chromatograph equipped with autosampler, EPC, split/splitless injector, FID detector, and CP-Chirasil-Dex CB chiral capillary column (25 m × 0.25 mm). The alcohol dehydrogenase activities toward the reduction of ketones were assayed using SpectraMax M2 microplate reader (Molecular Devices). All the ketones and alcohol standard samples were purchased from Sigma–Aldrich or prepared as reported previously.[17,18]

4.1. Gene expression and purification of alcohol dehydrogenase

The alcohol dehydrogenase gene *adhD* from the hyperthermophilic archaeon *P. furiosus* (GenBank Accession number AE010289) was amplified from genomic DNA (gift from Frank T. Robb) by forward primer 5′-GCGCGCCATGGCAAAAAGGGTAAATGCATTCAACGA-3′ (the *Nco* I restriction site is underlined) and the reverse primer 5′-TTTTGGGATCCTCACACACACCTCCTTGCCATCT-3′ (the *Bam*H I restriction site is underlined). The amplification was performed in a final volume of 100 μl, and the reaction mixtures contained 100 ng of genomic DNA, 50 pmol of each primer, 200 μM of dNTP, 1 × PCR buffer, 1.25 U of *Pfx* DNA polymeras and 1 mM $MgSO_4$. The *adhD* gene was cloned into pET15b expression vector at the (*Nco*I/*Bam*H I) sites and the plasmid DNA containing this alcohol dehydrogenase gene was transformed into Rosetta2(DE3)pLysS *E. coli* strain (Novagen). Overnight pre-cultures were diluted into LB containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol, the cells were induced with 0.5 mM of IPTG when optical density at 595 nm was 0.62. The bacterial cultures were incubated at 30 °C on an orbital shaker at 180 rpm for another 5 h.

The cultures of *E. coli* Rosetta2(DE3)pLysS were harvested by centrifugation. The cell pellet was re-suspended in potassium phosphate buffer (20 mM, pH 7.3), and the cells were lysed by homogenizer. The cell-free extract was obtained after being centrifuged at 11,000 rpm (~15,000*g*) for 30 min. The resulting cell-free extract was heated in a water bath at 80 °C for 30 min, and centrifuged at 4100 rpm for 30 min. The obtained supernatant was concentrated and centrifuged at 4100 rpm for 15 min. The resulting enzyme solution was lyophilized. The lyophilized PFADH enzyme was confirmed by protein gel analysis and used for enzymatic reactions.

4.2. Activity assay of the alcohol dehydrogenase

The activity of alcohol dehydrogenase toward the reduction of ketones (Tables 1–3) was determined by spectrophotometrically measuring the oxidation of NADH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) in the presence of excess ketones. The activity was measured at 37 °C in a 96-well plate, in which each well contained ketone substrates (6.25 mM), NADH (0.25 mM) in potassium phosphate buffer (100 mM, pH 6.5, 180 μl). The reaction was initiated by the addition of alcohol dehydrogenase PFADH (20 μl solution containing 20 or 40 μg of enzyme). The specific activity was defined as the number of nmol of NADH converted in one minute by 1 mg of enzyme ($nmol \times min^{-1} \times mg^{-1}$).

4.3. Enantioselectivity of ketone reduction catalyzed by alcohol dehydrogenase

The enantioselectivity of the enzymatic reduction of ketones was studied using an NADH recycling system. The general procedure was as follows: D-glucose (4 mg), D-glucose dehydrogenase (0.5 mg), NADH (0.5 mg), alcohol dehydrogenase (1 mg) and ketone solution in DMSO (50 μl, 0.25 M) were mixed in a potassium phosphate buffer (1 ml, 100 mM, pH 6.5) and the mixture was shaken overnight at 37 °C. The mixture was extracted with methyl *tert*-butyl ether (1 ml). The organic extract was dried over anhydrous sodium sulfate and subjected to either chiral HPLC or GC analysis to determine the enantiomeric excess. The absolute configurations of product alcohols were identified by comparing the chiral HPLC or GC data with the standard samples as reported previously.[17,18,25]

4.4. The reduction of ethyl 2-(4-cyanophenyl)-2-oxo-acetate in various organic solvents

The reductions of ethyl 2-(4-cyanophenyl)-2-oxo-acetate in various organic solvents were performed as follows: (a) NADH (14 mg), alcohol dehydrogenase (1 mg) and ketone

solution in DMSO (40 μl, 0.25 M) were mixed in a mixture (1 ml) of potassium phosphate buffer (100 mM, pH 6.5) and appropriate amount of solvent; (b) D-glucose (4 mg), D-glucose dehydrogenase (0.5 mg), NADH (0.5 mg), alcohol dehydrogenase (1 mg) and ketone solution in DMSO (40 μl, 0.25 M) were mixed in a mixture (1 ml) of potassium phosphate buffer (100 mM, pH 6.5) and appropriate amount of solvent. The mixture was shaken at 30 °C for 16 h. The mixture was extracted with methyl *tert*-butyl ether (1 ml). The organic extract was dried over anhydrous sodium sulfate and subjected to chiral HPLC analysis to determine the conversion and enantiomeric excess.

4.5. The reduction of ethyl 2-(4-cyanophenyl)-2-oxo-acetate at different temperatures

The reductions of ethyl 2-(4-cyanophenyl)-2-oxo-acetate at 37, 45, 60, and 75 °C were performed as follows: (a) NADH (14 mg), alcohol dehydrogenase (0.5 mg) and ketone solution in DMSO (40 μl, 0.25 M) were mixed in a mixture (1 ml) of potassium phosphate buffer (100 mM, pH 6.5); (b) D-glucose (4 mg), D-glucose dehydrogenase (0.5 mg), NADH (0.5 mg), alcohol dehydrogenase (0.5 mg), and the ketone solution in DMSO (40 μl, 0.25 M) were mixed in a mixture (1 ml) of potassium phosphate buffer (100 mM, pH 6.5). The mixture was shaken at the specified temperature for 1 h. The mixture was extracted with methyl *tert*-butyl ether (1 ml). The organic extract was dried over anhydrous sodium sulfate and was subjected to chiral HPLC analysis to determine the conversion and enantiomeric excess.

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