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Substrate spectrum of mandelate racemase Part 1: Variation of the α-hydroxy acid moiety

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Abstract

Enzymatic racemization of mandelic acid derivatives modified at the α-hydroxy acid moiety was achieved using mandelate racemase [EC 5.1.2.2]. Whereas α-amino acid derivatives, such as phenyl glycine and mandelic acid hydrazide were not accepted, the mandelic acid amide was racemized at an acceptable rate. The latter was significantly enhanced by an electron-withdrawing substituent in the phenyl moiety. Based on the catalytic mechanism of the enzyme, the relative activities of non-natural substrates could be explained by steric and electronic reasons.

Keywords: Mandelate racemase; Racemization; Mandelic acid amide; *Pseudomonas putida* ATCC 12633; *p*-Bromo mandelic acid amide

1. Introduction

The interconversion of enantiomers–racemization is an entropically 'downhill reaction' going in hand with a loss of enantiomerically enriched material. As a consequence, it has been largely regarded as an undesired side reaction rather than a synthetically useful transformation. It was only recently, that its importance was recognized for the so-called deracemization processes [1], which allow the transformation of a racemate into a single stereoisomeric product in 100% theoretical yield. In this context, the controlled racemization of organic compounds [2] constitutes the key to dynamic (kinetic) resolution [3,4] or stepwise deracemization [5], which consists of the coupling of a racemization reaction to an enantioselective transformation. Unfortunately, the majority racemization methods described so far require extreme pH and/or temperatures, which makes them incompatible with the presence of an enantioselective reaction. This disadvantage may be overcome by enzymatic racemization which takes place under mild reaction conditions [6]. We focused our interest on mandelate racemase [EC 5.1.2.2] [7] for the following reasons: the cofactor-independent inducible enzyme can be obtained in large amounts by fermentation from *Pseudomonas putida* ATCC 12633 [8] [1], immobilization leads to an enhanced activity and facilitates its recovery [9], and the catalytic efficiency of this enzyme is exceptional and it achieves a turnover frequency of approx. $1000\,s^{-1}$ [10]. Most importantly, it promotes a reaction which is (almost) impossible by

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[1] Mandelate racemase has been recently made available in large quantities through enzyme induction (23×10^6 U from a 101 fermentation). This activity allows the racemization of ~1.5 t of mandelate within ~8 h.

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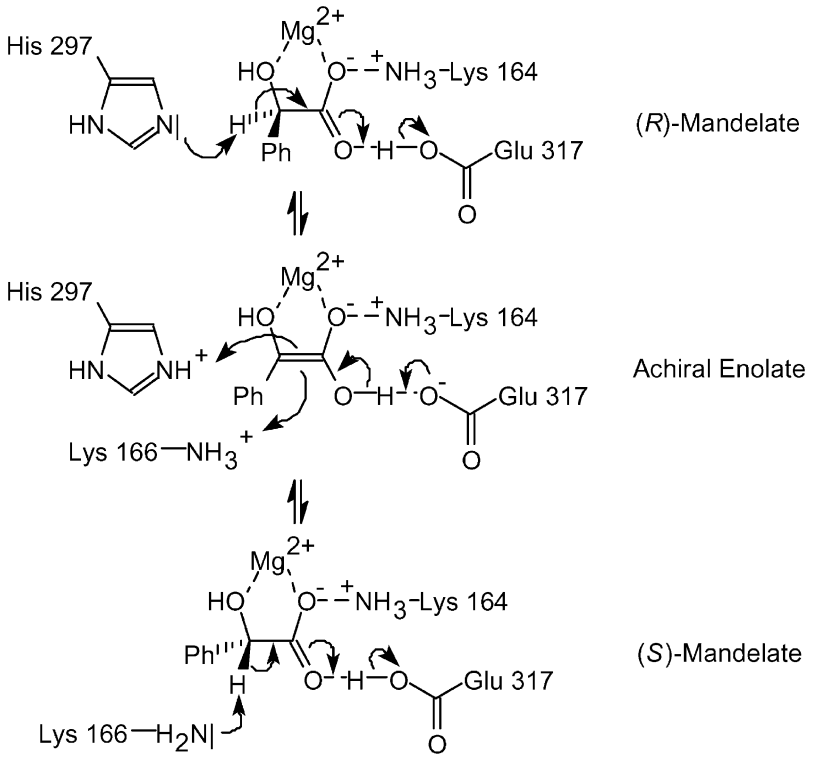


Scheme 1. Schematic mechanism of mandelate racemase.

chemical means, i.e. the base-catalyzed racemization of an α-hydroxycarboxylic acid–mandelate (**1**).

From a biochemical point of view, mandelate racemase is a well-studied enzyme and its two-base mechanism has been elucidated based on X-ray structures [11] and structure–function studies [7] (Scheme 1). Binding of both enantiomers occurs via a tight network of salt bridges and hydrogen bonds onto two Brønsted acids: Lys 164 and Glu 317 in their protonated form and a Lewis acid — an essential Mg^{2+} atom. The latter arrangement depletes the electron-density at the α-hydroxy acid moiety and, as a consequence, the pK_a value of the α-H is reduced to such an extent,[2] that it can be abstracted by a base going in hand with the formation of an achiral enol-intermediate. Deprotonation is effected by two enantiomer-specific bases juxtaposed on either side of the chiral carbon atom, i.e. His 297 and Lys 166 for (*R*)- and (*S*)-mandelate, respectively. Despite of the detailed mechanistic knowledge, very little is known on the substrate tolerance [13–15]. In order to elucidate the scope and limitations of mandelate racemase for the deracemization of non-natural compounds, we investigated the substrate-tolerance of this enzyme. In this paper, we wish to present possible variations of the α-hydroxy acid moiety[3] Fig. 1.

2. Experimental

A partially purified preparation of mandelate racemase was prepared as previously described [8] having a specific activity of 107 μmol mg^{-1} min^{-1}. Relative rates of racemization were determined by an assay in HEPES-buffer (50 mM, pH 7.5, 3.3 mM $MgCl_2.6H_2O$) based on the online measurement of the decline of

[2] The pK_a value of the α-H has been estimated to be 22 and 29 for mandelic acid and mandelate, respectively [12].

[3] For possible variations of the aryl-moiety and influences of substitutents on carbanion stabilization see [29].

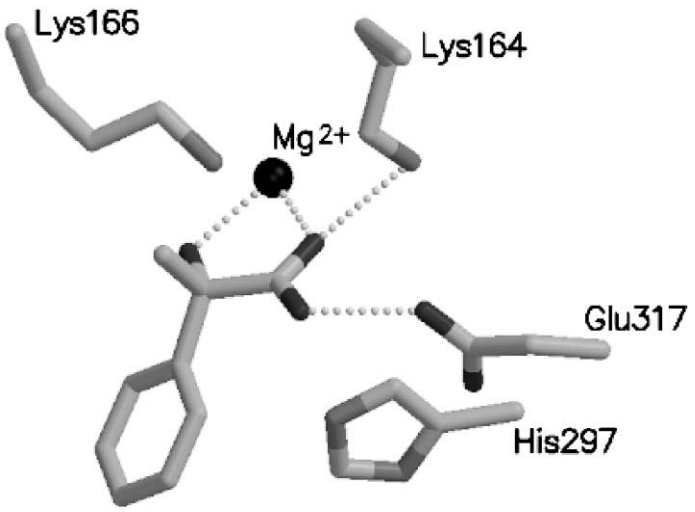


Fig. 1. X-ray structure of the active site of mandelate racemase containing (*S*)-atrolactate as inhibitor [16] (Fig. 1 was generated using the structure of mandelate racemase at 2.10 Å resolution containing (*S*)-atrolactic acid as inhibitor in the active site. Data are available at the Protein Data Bank (1MDR, release date 31 Aug 1994): http://www.rcsb.org/pdb/.). Dotted lines highlight the substrate-binding, α-H abstraction occurs via two bases (Lys 166, His 297).

optical rotation versus time [17]. Optical rotations were measured on a Polarimeter 341 of Perkin-Elmer (1 ml cuvette, $l = 10$ cm). The absence of spontaneous racemization under the reaction conditions employed was verified for all substrates in the absence of biocatalyst and in the presence of heat-denatured enzyme preparation and was proven to be <0.5% within 48 h. ^{1}H- and ^{13}C-NMR were recorded on a Bruker 360 MHz spectrometer using TMS as internal standard.

(*R*)-mandelic acid **1** (e.e. 98%) was purchased from Fluka and (*R*)-phenylglycine **2** (e.e. 98%) from Aldrich. (*R*)-*N*-acetylphenylglycine **3** [18,19], (*R*)-2-hydroxy-2-phenyl-acetamide (mandelic acid amide) **4** [20] and (*R*)-2-hydroxyphenylacetic acid hydrazide (mandelic acid hydrazide) **5** [21] were synthesized according to literature procedures.

2.1. *rac*-(4-Bromophenyl)-2-hydroxy-acetamide *rac*-**6**

Sulfuric acid (5.5 ml, 8.8 M) was added dropwise to a mixture of *p*-bromobenzaldehyde (5.02 g, 27.1 mmol) and potassium cyanide (1.76 g, 27.0 mmol) in water (6.7 ml) and ethanol (1.4 ml) at 0°C. After the mixture was stirred at room temperature for 2 h, *p*-bromobenzaldehyde cyanohydrin was extracted with diethyl ether (2 × 30 ml), the combined organic phases were dried (Na_2SO_4) and the organic solvent evaporated under reduced pressure. The crude cyanohydrin (5.56 g) was hydrolyzed in situ without further purification by rapid stirring with concentrated sulfuric acid (58 ml) at room temperature for 20 min. The solution was poured onto ice, the aqueous phase was extracted with diethyl ether (2 × 150 ml), the combined organic phases were dried (Na_2SO_4) and the organic solvent was removed under reduced pressure. Recrystallization of the residue from petroleum ether–ethyl acetate gave (±)-**6** as white crystals (3.8 g, 59%). Melting point 133–135°C ([22,23] 141°C). ^{1}H-NMR (D_6-DMSO): δ 7.51 (2H, d, J 8.4 Hz, Ar), 7.40 (2H, d, J 8.4 Hz, Ar), 7.21 (2H, s, NH_2), 4.85 (1H, s, CHOH). ^{13}C-NMR: δ 174.1 (C=O), 140.8 (*i*-Ar), 130.7 (*m*-Ar), 128.6 (*o*-Ar), 120.4 (*p*-Ar), 72.7 (CHOH).

2.2. (*R*)-(4-bromophenyl)-2-hydroxy-acetamide (*R*)-**6**

Kinetic resolution of *rac*-**6** was achieved by *Pseudomonas* sp. lipase-catalyzed *O*-acylation in analogy to a known procedure [24]. A solution of *rac*-**6** (0.2 g, 0.82 mmol) in diisopropyl ether (30 ml) and vinyl

acetate (2.4 ml) was agitated with lipase 'Amano P' (0.3 g) at room temperature on an orbit shaker. After 5 days, the reaction ceased at 50% conversion, indicating excellent enantioselectivity (E > 200). The enzyme was filtered, the organic solvent was removed under reduced pressure and the crude product was purified by column chromatography (silica gel, CH_2Cl_2/MeOH = 20:1) to give (*S*)-(4-bromophenyl)-2-acetoxy-acetamide (not isolated) and remaining non-acetylated (*R*)-**6** as white crystals (0.1 g, 49%); $[\alpha]_D^{20}$ −70.6° (*c* 0.11, H_2O); e.e. 99% (HPLC-analysis). The absolute configuration of **6** was assumed to be (*R*) for the following reasons: (i) the Kazlauskas rule [25] for *Pseudomonas* sp. lipase predicts the (*S*)-enantiomer to react faster, which proved to be correct for mandelic acid derivatives; (ii) like (*R*)-mandelic acid amide **4** [20], the optical rotation of the remaining *p*-bromo amide is (−), and both derivatives show the same elution order on HPLC (see below).

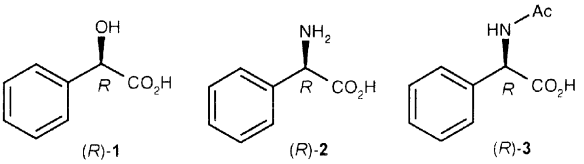
Enantiomeric compositions were determined on a Chiralpak OD-H column (Daicel, 0.46 cm × 25 cm) using a Jasco HPLC-system (pumps PU-980, multi-wavelength-detector MD-910, autosampler AS-950, degasser CMA/260). For *rac*-**1**: flow 0.5 ml min^{-1}, eluent heptane/^{i}propanol/trifluoroacetic acid = 90:10:0.1; 23.6 min (*S*), 27.7 min (*R*). For *rac*-**3**: flow 0.5 ml min^{-1}, eluent heptane/^{i}propanol/trifluoroacetic acid = 80:20:0.1; 15.7 min (*S*), 20.7 min (*R*). For *rac*-**4**: flow 0.7 ml min^{-1}, eluent heptane/^{i}propanol/trifluoroacetic acid = 90:10:0.1; 14.7 min (*R*), 17.4 min (*S*). For *rac*-**5**: flow 0.5 ml min^{-1}, eluent heptane/^{i}propanol/trifluoroacetic acid = 80:20:0.1; 16.5 min (*R*), 19.5 min (*S*). For *rac*-**6**: flow 0.7 ml min^{-1}, eluent heptane/^{i}propanol/trifluoroacetic acid = 90:10:0.1; 16.8 min (*R*), 23.3 min (*S*).

3. Results and discussion

3.1. Variation of the α-hydroxy moiety

The mode of binding of the substrate and its corresponding achiral enol-form within the active site [7] (Scheme 1) suggests a substitution of the α-hydroxy moiety with a nitrogen-analogue. The reduced electronegativity and the improved donor capabilities of nitrogen (as compared to O) would enable good binding to the metal ion Mg^{2+}. Thus, the non-natural amino acid phenylglycine (*R*)-2 was tested as substrate.[4] D-Phenylglycine is used for the industrial-scale production of carbacephalosporin antibiotics such as cephalexin, cefaclor, its stable analogue loracarbef [26], and the β-lactam antibiotic ampicillin [26–28].

3.2. Compounds *1–3*



To our disappointment, (*R*)-**2** was not racemized at all by mandelate racemase. A possible explanation for this failure is the fact that the optimum pH of mandelate racemase is at around pH 7.5, where the α-amino group is almost completely protonated and therefore positively charged. Thus, it is repelled by Mg^{2+} and racemization cannot take place. To overcome this problem, the corresponding *N*-acetyl derivative **3** was synthesized. However, also (*R*)-**3** was not transformed as well, possibly due to steric hindrance.

3.3. Variation of the carboxylic acid moiety

When the carboxylic acid moiety CO–OH is replaced by an amide group CO–NH_2 (substrate **4**), the amide nitrogen remains unprotonated at pH 7.5. Although substrate binding might be impeded to some extent by the NH_2 group, no obvious steric restrictions do apply. Indeed, a relative activity of 15% was found in comparison to (*R*)-mandelic acid **1** using a polarimetric assay [17].[5] This encouraging result led us to replace the NH_2 of the amide moiety by the larger $NHNH_2$ group to give mandelic hydrazide (*R*)-**5**. The latter was not accepted by the enzyme, presumably due to the increased size of the hydrazide-functionality.

[4] For all racemization experiments the corresponding (*R*)-enantiomer was used.

[5] Due to the moderate activity, the exact value for the racemization rate was confirmed in an independent experiment lasting 22 h using HPLC for the determination of the decline of e.e.

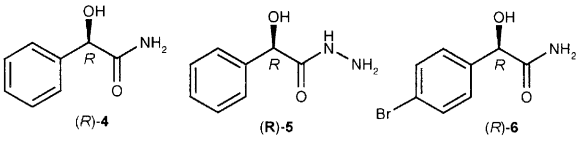
Table 1
Relative racemization rates of mandelic acid derivatives by mandelate racemase

Substrate	α-Moiety	Acid moiety	e.e. (%)[a]	Relative activity (%)[b]
(*R*)-**1**	OH	COOH	98	100
(*R*)-**2**	NH_2	COOH	98	<1
(*R*)-**3**	NHAc	COOH	>99	<1
(*R*)-**4**	OH	$CONH_2$	98	15
(*R*)-**5**	OH	$CONHNH_2$	>99	<1
(*R*)-**6**	OH	$CONH_2$	>99	22

[a] Enantiomeric excess of starting material.
[b] Relative activity (%) with respect to the natural substrate mandelate set as standard (100%).

From previous studies [13], it is known that electron-withdrawing substituents in the aromatic moiety, such as chloro or bromo, stabilize the negative charge on the α-carbon atom [7], which results in significantly enhanced racemization rates. For instance, *p*-chloro- and *p*-bromomandelate were racemized at relative rates of 326 and 376%, respectively, compared to mandelate [13]. This led us to assume that the *p*-bromo-carboxamide (*R*)-6 should be racemized at a faster rate than the corresponding non-substituted derivative (*R*)-**4**. This assumption proved to be correct and a relative rate of 22% for (*R*)-**6** was found, which accounts to an activity-enhancement of approximately 50% (Table 1). One has to remember that the turn-over-frequency (TOF) of mandelate racemase for its natural substrate 1 is about $1000\,s^{-1}$, thus, e.g. 15% of the activity equals still an impressive TOF of $150\,s^{-1}$.

3.4. Compounds **4–6**



In summary, we have shown for the first time that mandelate racemase [EC 5.1.2.2] is able to catalyze the racemization of mandelic acid derivatives modified at the α-hydroxy acid moiety. Whereas α-amino acid derivatives and mandelic acid hydrazide were not accepted, the corresponding mandelic amide proved to be a moderate substrate. The racemization rate of the latter was significantly enhanced by an additional electron-withdrawing substituent in the phenyl moiety. Based on the catalytic mechanism of the enzyme, the relative activities of non-natural substrates could be explained by steric and electronic reasons.

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