Enzymatic Racemisation and its Application to Synthetic Biotransformations

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Abstract: In contrast to kinetic resolution, where only 50% of the racemic starting material can be converted into the desired product and where the remaining 'wrong' enantiomer has to be considered as waste, so-called deracemisation processes allow the production of a single stereoisomeric product from racemic starting material. In this context, the use of environmentally benign methods for biocatalytic racemisation holds great potential. The small and largely overlooked group of racemases (EC 5.1.X.X), which are increasingly being used for dynamic kinetic resolution or in auxiliary biocatalytic recycling processes, are reviewed with respect to their properties, their substrate tolerance and their biocatalytic potential.

Keywords: biocatalysis; dynamic kinetic resolution; enzymes; racemase; racemisation

1 Introduction

Racemisation, in general, is an energetic 'downhill' reaction due to an increase of entropy[1] and thus has been considered more often as an undesired side reaction rather than a synthetically useful transformation. As a consequence, the controlled racemisation of organic compounds has been scarcely studied deliberately and a significant part of the data available to date stems from industrial research predominantly reported in the patent literature. It was only recently, that – driven by the demand to improve the economic balance of kinetic resolution processes – the importance of synthetic protocols for the controlled racemisation of organic compounds under carefully controlled reaction conditions has been recognised, as emphasised by the seminal review of Zwanenburg and co-workers.[2] A detailed investigation of the data available to date reveal that chemical racemisation techniques largely depend on harsh reaction conditions, predominantly (i) thermal racemisation as well as (ii) strong acid or (iii) base catalysis[3] and (iv) *via* chirally labile intermediates.[2] Overall, *ca.* 75% of all racemisations fall under these categories. As a consequence, process-control over chemical racemisation is very limited and undesired side-reactions, such as elimination, condensation and rearrangement/decomposition set a low ceiling on the preparative utility of these processes. It was only recently that milder methods for chemical racemisation were brought to our attention.[4] For instance, chiral *sec*-alcohols can be racemised *via* a transition metal-catalysed oxidation-reduction sequence[5] or *via* Pd^{II}-catalysed allylic rearrangement of the corresponding acetate esters.[6] Amino acid amides or esters can be racemised *via* Schiff base derivatives with aromatic aldehydes involving the α-amino group.[7]

In contrast, enzymatic racemisation takes place at intrinsic mild reaction conditions – e.g., at room temperature, atmospheric pressure and neutral pH – and is therefore highly chemoselective and the formation of by-products is avoided. [8]

Due to the fact that biochemical processes are predominantly stereospecific, Nature has faced little need for racemisation and, as a consequence, racemases are a small group of enzymes, which have been biochemically classified as a subgroup of the isomerases (EC 5.1.X.X). Nevertheless, as outlined in this review, they hold great synthetic potential as they can catalyse several reactions which are 'chemically impossible' by conventional methodology.

(Bio)catalytic racemisation becomes useful only in combination with an enantioselective (catalytic) trans-

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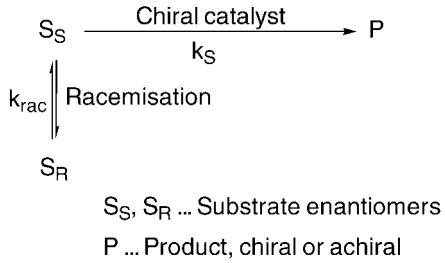


Scheme 1. Substrate-racemisation in deracemisation (P being chiral) or in auxiliary processes (P being achiral).

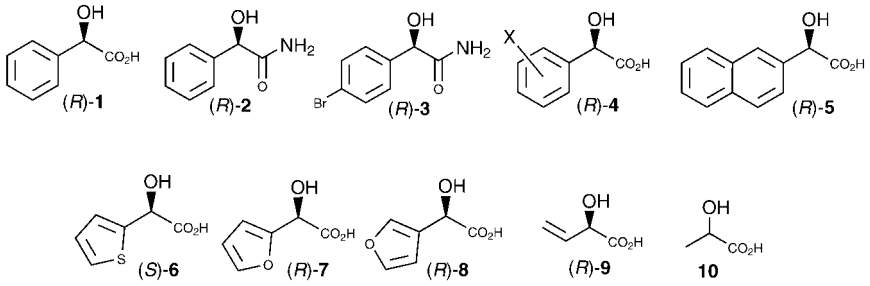
formation[9] to furnish a so-called deracemisation,[10] as outlined in Scheme 1 (P being chiral).

Three main concepts have been described so far:

(i) Stepwise racemisation (chiral P): It has been common practice to racemise the unwanted, non-reacted enantiomer S_R from a kinetic resolution after its separation from the formed product P and to subject this material again to the resolution process in a subsequent cycle, until virtually all of the racemic starting material has been converted into stereoisomer P. At a first glance, the stepwise nature of this protocol appears suboptimal, bearing in mind that an infinite number of cycles are theoretically required to transform all of the racemic starting material into a single stereoisomer. On a closer look, however, it becomes a viable option considering that only five cycles are required to produce P in >95% (theoretical) yield, provided that both reactions – i.e., kinetic resolution and racemisation – are essentially 'clean' without loss of material.[11,12]

(ii) *In situ* racemisation (chiral P): A more elegant process – termed dynamic kinetic resolution (DKR) – can be devised when the racemisation of the non-reacting enantiomer S_R is achieved *in situ*, i.e., in the presence of the enantioselective reaction forming P. Thus, when S_S becomes depleted during the course of the enantioselective reaction, the relative concentrations of S_R and S_S become unequal, and, as a consequence, racemisation takes place. Overall, the slow-reacting substrate enantiomer S_R is converted to the desired product P *via* its mirror-image S_S. As a consequence, P is obtained in 100% chemical and optical (theoretical) yield.[4,13–18]

(iii) Auxiliary racemisation (achiral P): Both enantiomers of a racemic starting material are converted into non-chiral products, e.g., DL-glutamate is transformed into NH_3 and α-ketoglutarate. In this context, racemisation represents an auxiliary process, which delivers (achiral) reagents from a racemate in 100% theoretical yield for a (bio)catalytic reaction sequence (see below).



Scheme 2. Substrates for mandelate racemase.

In order to evaluate the applicability of an enzyme for the biocatalytic transformation of non-natural compounds, its substrate-tolerance is of primary importance. In the following, various racemases are discussed in this context along with some considerations of their mechanism of action. Caution has to be exercised with racemases regarding activity data. In contrast to other enzyme classes, there are two ways to determine their activity, i.e., (i) the formation of the mirror image enantiomer (e.g., S_S from S_R), and (ii) the formation of racemate from either enantiomer (e.g., *rac*-S_S/S_R from S_S) over a given time.[1]

This review gives an overview on the application of racemases already in use, and illustrates the potential of hitherto unexplored candidates.

Racemases are grouped in this review according to their substrate pattern. The two main groups are:
(i) Racemases for α-hydroxy carbonyl derivatives and
(ii) Racemases for α-amino acid derivatives.

Each section is subdivided according to substrate derivatives or the substitution pattern, respectively.

2 α-Hydroxy Carbonyl Derivatives

The number of enzymes known to be capable of racemising α-hydroxy carbonyl compounds is rather small. Among them, mandelate racemase is a well known enzyme, which has been (biochemically) investigated in great detail, while data on lactate racemase are rather scarce. The existence of an acetoin racemase has been assumed but has not yet been fully proven.

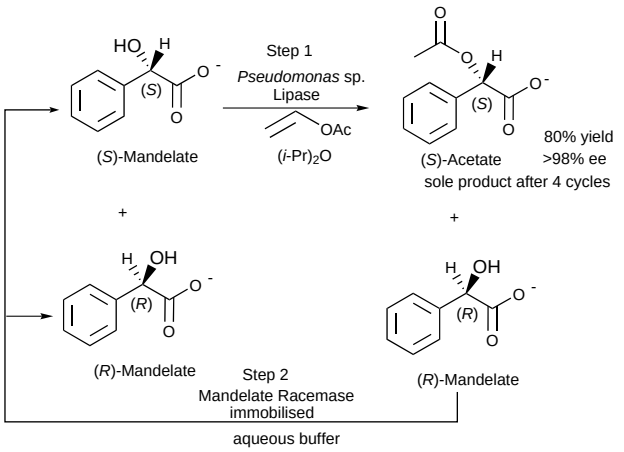
2.1 Mandelate Racemase

2.1.1 Substrates

The substrate spectrum of the inducible divalent metal ion-dependent mandelate racemase (EC 5.1.2.2) from *Pseudomonas putida* ATCC 12633 is rather broad (Scheme 2, Table 1): besides its natural substrate mandelate **1**, it effects also the enzymatic racemisation of the corresponding amide derivative **2** at a fair rate. The enzyme is a very fast catalyst, as the turnover frequency (TOF) of mandelate racemase for its natural substrate **1** is approx. 1000 s^{-1},[19] meaning 1.0 g of the enzyme racemises approximately 1.7 kg of mandelic acid per hour. Electron-withdrawing substituents in the *para*-position, such as *p*-Cl and *p*-Br in mandelic acid derivatives of type **4** or *p*-Br in amide **3** increase the activity of mandelate racemase, while electron-donating substituent (*p*-OH, *p*-OMe) decrease the activity; *meta*-derivatives, like *m*-Cl-**4**, are racemised at a lower rate compared to the *para*-analogues, which might be due to steric hindrance. Steric reasons are definitely responsible for the non-acceptance of *o*-chloromandelate. Furthermore, the aromatic system of mandelic acid may be expanded to a naphthyl system to give substrate **5**, which is still racemised with good activity (TOF = 260 s^{-1}). In addition, heteroaromatic analogues of mandelate, e.g., thienyl (substrate **6**) and furyl analogues (substrates **7**, **8**) are well accepted by the enzyme. Even vinyl glycolate **9** can be racemised, which proves that the aromatic system may be 'minimised' to a single C=C bond in the β,γ-position. However, in the absence of π-electrons in this position, such as for lactate (substrate **10**), no racemisation occurs.

Table 1. Relative activities of mandelate racemase for various substrates.

Substrate	X	Rel. Activity [%]	Ref.
(*R*)-**1**	–	100	–
(*R*)-**2**	–	15	[20]
(*R*)-**3**	–	22	[20]
(*R*)-**4**	*p*-MeO	17	[21]
(*R*)-**4**	*p*-OH	45	[21]
(*R*)-**4**	*p*-F	96	[22]
(*R*)-**4**	*p*-Br	376	[21]
(*R*)-**4**	*p*-Cl	326	[21]
(*R*)-**4**	*m*-Cl	61	[22]
(*R*)-**4**	*o*-Cl	< 1	[22]
(*R*)-**5**	–	26	[22]
(*S*)-**6**	–	27	[22]
(*R*)-**7**	–	14	[22]
(*R*)-**8**	–	23	[22]
(*R*)-**9**	–	35	[23]
10	–	< 0.01	[21]



Scheme 3. Application of mandelate racemase in stepwise deracemisation.

2.1.2 Application

The applicability of mandelate racemase for preparative-scale biotransformations was demonstrated in the stepwise deracemisation of mandelic acid as model substrate (Scheme 3). The process starts with the enantioselective acylation of (±)-mandelic acid employing *Pseudomonas* sp. lipase in an organic solvent using vinyl acetate as the acyl donor. After the kinetic resolution came to a standstill at 50% conversion, the enzyme was filtered off and the organic solvent and excess acyl donor were removed by evaporation. Then, the non-reacted remaining (*R*)-mandelate was racemised without prior separation from the formed (*S*)-*O*-acetate in aqueous buffer employing immobilised mandelate racemase, while the *O*-acylated product remained untouched. After filtration of the enzyme and lyophilisation to remove the aqueous solvent, the cycle was resumed. After four cycles in total, (*S*)-*O*-acetylmandelic acid was obtained in 80% yield and >98% ee as sole product.[12]

2.1.3 The Enzyme

In contrast to the majority of racemases, the catalytic mechanism of mandelate racemase has been studied in great detail. The enzyme requires a divalent metal, such as Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+} or Ni^{2+} for activity[24] and it functions according to a so-called 'two–base mechanism', as elucidated by X-ray structure analysis[25] and structure-function studies (Figure 1).[26] Binding of both enantiomers occurs *via* a tight network of salt bridges and hydrogen bonds onto two Brønsted acids – Lys164 and Glu317 in their protonated form – and a Lewis acid, i.e., Mg^{2+} in the native enzyme. The latter arrangement depletes the electron density at the α-hydroxy acid moiety to such an extent that the pK_a value of the α-H is

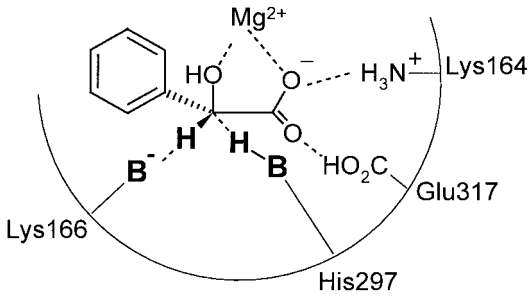


Figure 1. Schematic representation of the active site of mandelate racemase.

reduced to allow its abstraction by an adjacent 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., His297 and Lys166 for (*R*)- and (*S*)-mandelate, respectively. The simplicity of this mechanism is impressive bearing in mind that (for isolated mandelic acid and mandelate) the pK_a value of the α-H has been estimated to be 22 and 29, respectively.[27]

The inducible octameric enzyme (subunit 39 kDa)[28] can be obtained in large amounts by fermentation from *Pseudomonas putida* ATCC 12633;[29] its immobilisation leads to enhanced activity and facilitates its recovery.[30]

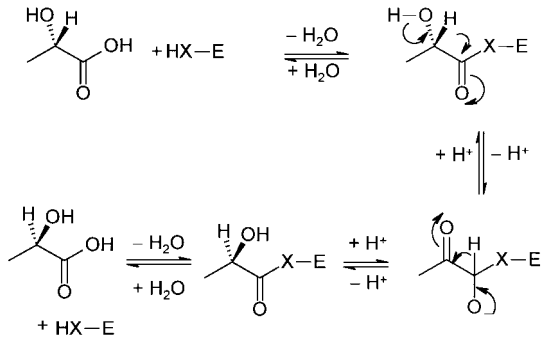
2.2 Lactate Racemase

Lactate racemase has not been described as well as mandelate racemase. Until now it is only known to interconvert the enantiomers of lactic acid, which indicates that a β,γ-double bond is not essential for racemisation (c.f. mandelate racemase).

2.2.1 The Enzyme

In contrast to mandelate racemase, less is known about lactate racemases, which were isolated and characterised, for instance, from *Lactobacillus curvatus*,[31] *Lactobacillus sake*[32] and *Haloverax volcanii*.[33] In all cases the enzyme appeared as a monomer with a molecular weight between 25 and 82 kDa.

No structure of a lactate racemase is available to date and the proposed mechanism for the reaction involves an internal hydride transfer,[8] during which the α-H of lactate is transferred onto the C-1 atom. Random return leads to racemisation (Scheme 4). The chemical operator X within the active site of the enzyme is postulated to be a thiol and the proposed symmetrical α-carbonyl intermediate has been trapped as an enzyme-bound oxime.[34,35] The substrate tolerance of lactate racemase is unknown.



Scheme 4. Postulated mechanism of lactate racemase.

2.3 Acetoin Racemase

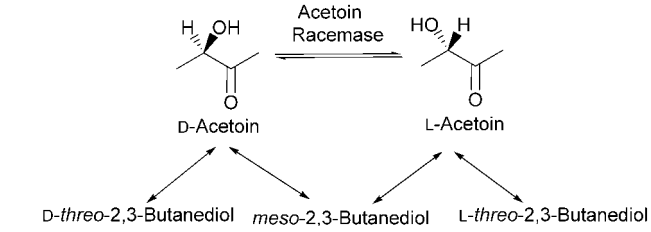
The existence of an acetoin racemase (EC 5.1.2.4) has been proposed in order to provide an explanation for the interconversion of both enantiomers of *threo*-2,3-butanediol by *Klebsiella pneumoniae* ATCC 8724 *via* acetoin (see Scheme 5).[36,37] However, despite the still unexplored substrate tolerance of this enzyme, it appears that racemases are not limited to α-hydroxy acids and α-amino acids (see below), but may encompass also α-hydroxy ketones, thus widening the spectrum of applications for racemases.

3 α-Amino Acids and Derivatives

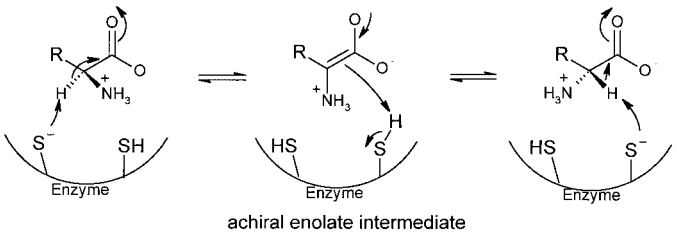
Although the biosynthesis of α-amino acids is highly stereospecific with respect to the L-stereoisomers, an impressive number of D-analogues have been found in various biological sources – usually as components of highly potent natural products.[38,39] Instead of a *de novo* biosynthesis *via* 'mirror – image' metabolic pathways, D-amino acid isomers are generally obtained *via* biocatalytic racemisation and kinetic resolution and thus it is not surprising that racemases acting on α-amino acids and derivatives are quite common.

3.1 Racemases Acting on Underivatised α-Amino Acids

In Nature, racemisation of underivatised 'free' α-amino acids can be achieved *via* two different methods, i.e. (i) *via* a two-base mechanism without cofactor and (ii) *via* a pathway involving a chirally unstable Schiff base intermediate with an aromatic aldehyde serving as cofactor – pyridoxal 5′-phosphate (PLP). It is interesting to note that this chemical trick employing an aromatic aldehyde has been copied by chemists for the chemocatalysed racemisation of amino acids to effect dynamic kinetic resolution.[40] Thus, α-amino acid racemases are subdivided into (i) PLP-independent and (ii) PLP-dependent racemases.



Scheme 5. Proposed interconversion of D- and L-*threo*-2,3-butanediol involving a putative acetoin racemase in *Klebsiella pneumoniae*.



Scheme 6. Schematic representation of the two-base mechanism of PLP-independent α-amino acid racemases.

3.1.1 Pyridoxal 5′-Phosphate (PLP) Independent α-Amino Acid Racemases

Among the PLP-independent amino acid racemases, only glutamate racemase (EC 5.1.1.14),[41] and proline racemase (EC 5.1.1.4)[42] are discussed in more detail. For other racemases of this group, such as aspartate racemase (EC 5.1.1.13)[43,44] and 4-hydroxyproline racemase (EC 5.1.1.8, more exactly an epimerase acting on diastereomers rather than enantiomers), only limited data are available.

All PLP-independent α-amino acid racemases follow a 'two – base mechanism', which utilises two cysteine residues as conjugated catalytic acid and base for the abstraction of the α-H at either enantiomer (Scheme 6).[45,46] The deprotonated thiolate group of a cysteine residue acts as a catalytic base by abstracting the α-proton of the substrate, while the Cys-thiol on the opposite side acts as a conjugated acid by donating a proton to the achiral enolate-type intermediate.

Despite the fact that an explanation for how the carbanionic intermediate is stabilised (in the absence of a PLP-cofactor or metal ion) is still lacking, a strong mechanistic relationship to mandelate racemase (c.f. Figure 1) seems clear.

The biocatalytic significance of various PLP-independent α-amino acid racemases will now be dealt with more detail.

3.1.1.1 Glutamate Racemase

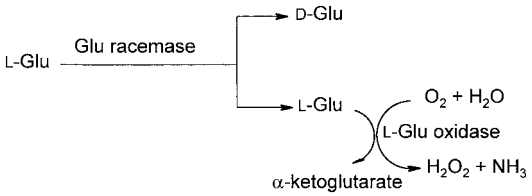
The substrate spectrum of glutamate racemase is very narrow and is limited to L and D-glutamate, receptively.

Application

A method for the synthesis of D-glutamate, an important intermediate for the production of pharmaceuticals, such as penicillin derivatives,[47] starts from cheap L-glutamate obtained by fermentation, which is enzymatically racemised to the DL-form using glutamate racemase (Scheme 7). The L-isomer thereof is then enantioselectively oxidised by the action of glutamate oxidase using molecular oxygen as oxidant, while the remaining non-reacting D-Glu can easily be separated.[48] Since this process constitutes a kinetic resolution, it certainly lacks synthetic elegance in view of the maximum of 50% theoretical yield.

A more elegant application for glutamate racemase (GluR) is the production of various D-amino acids by a multi-enzyme system, consisting of GluR, a thermostable D-amino acid aminotransferase, glutamate dehydrogenase and formate dehydrogenase (Scheme 8). In the main step, the α-amino group of D-Glu is enzymatically transferred onto the carbonyl moiety of a pyruvate derivative with high D-specificity under the action of the aminotransferase. The α-ketoglutarate thus formed as a co-product is enzymatically recycled *via* a reductive amination involving an NADH-dependent glutamate dehydrogenase and NH_3. Since the latter enzyme is L-specific, the 'recycled' Glu has the wrong absolute configuration for the transferase. This problem is solved by the aid of glutamate racemase.

The system was employed for the production of aromatic D-amino acids, such as D-phenylalanine and D-tyrosine from the corresponding α-keto acids, i.e., phenyl pyruvate and *p*-hydroxyphenyl pyruvate. Overall, 58 g/L of D-Phe[49] and 60 g/L of D-Tyr[50] were produced in 100% of optical purity from equimolar amounts of *p*-hydroxyphenyl pyruvate. In this process, the racemisation served as auxiliary step to provide the biological ammonium source (D-glutamate) for the aminotransferase.



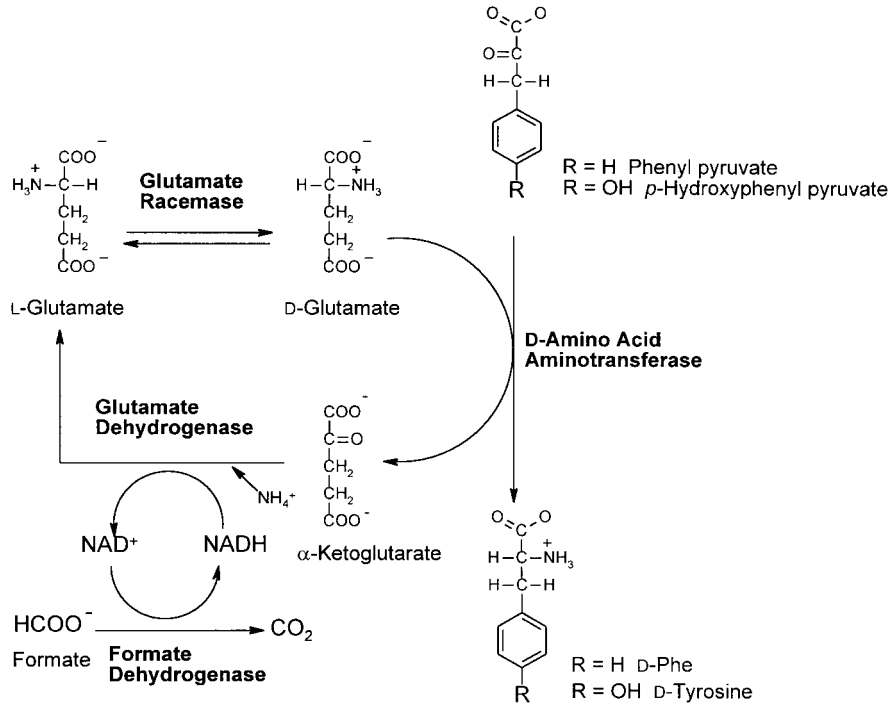
Scheme 7. Production of D-glutamic acid involving glutamate racemase.

The Enzyme

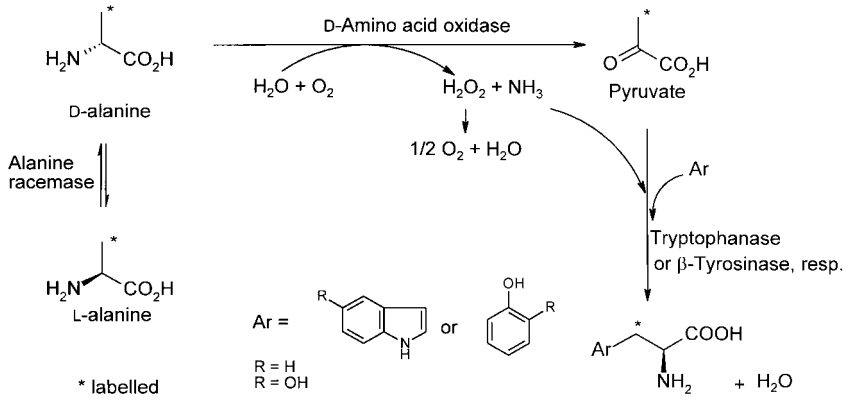
Glutamate racemase from *Aquifex pyrophilus* MurI forms a dimer from subunits of a molecular weight of 28 kDa.[51,52] In contrast, glutamate racemase from *Bacillus SK-1* is a monomeric protein with a molecular weight of 29 kDa and is active within a remarkably wide pH range of 6.0 – 11.5.[49]

3.1.1.2 Proline Racemase

Proline racemase has been used for the production of D-proline from the L-isomer *via* enantioselective enzymatic oxidation of the L-enantiomer in a process



Scheme 8. Use of glutamate racemase in an auxiliary process for the production of D-Phe and D-Tyr.



Scheme 9. Use of alanine racemase in an auxiliary process to produce ammonium and C-3-labelled pyruvate from L-Ala.

described above for D-glutamate (see Scheme 7).[47] *Clostridium stricklandii* has been reported as a possible source of proline racemase.[53,54]

3.1.2 Pyridoxal 5′-Phosphate (PLP) Dependent α-Amino Acid Racemases

The most important enzymes of this group from a biocatalytic point of view are alanine (EC 5.1.1.1) and amino acid racemases (EC 5.1.1.10). Other racemases of this group are, e.g., methionine racemase (EC 5.1.1.2), lysine racemase (EC 5.1.1.5), threonine racemase (EC 5.1.1.6, more exactly an epimerase acting on diastereomers rather than enantiomers), arginine racemase (EC 5.1.1.9), and ornithine racemase (EC 5.1.1.12). All of them have in common, that prior to the actual racemisation – again by base-catalysed α-proton abstraction – a chirally labile Schiff base intermediate is formed from the α-amino acid and the aromatic aldehyde whereby PLP is required as cofactor (Scheme 9). Again, there are close mechanistic similarities to the previously discussed racemases acting *via* a two-base mechanism.

3.1.2.1 Alanine Racemase

Among the PLP-dependent racemases, alanine racemase (AlaR) has been studied in most detail and it was found that in general the enzyme exclusively catalyses the interconversion of L- and D-alanine. However, the enzyme from *Salmonella typhimurium* has a relaxed substrate specificity as it also accepts L-serine, L-homoserine and L-cysteine as substrates.[55] In Nature, AlaR is required for the construction of bacterial cell walls.[56] Since no human alanine racemase is known to date, microbial alanine racemases are an ideal target for the design of new antibiotics directed to inhibit the biosynthesis of bacterial cell walls.

Application

Alanine racemase has been applied to the preparation of labelled aromatic amino acids, such as L-Tyr, L-DOPA, L-Trp and 5-hydroxy-L-tryptophan[57,58] using C-3-labelled L-alanine as starting material in order to obtain NH_3 and (C-3-labelled) pyruvate as an intermediate for the C–C bond forming enzyme tyrosinase (or tryptophanase) for the enzymatic synthesis of (hetero)aromatic α-amino acids (Scheme 9). In this multi-enzyme process, alanine racemase catalyses an auxiliary process to produce D-Ala from the (cheap) L-isomer, the former is enzymatically degraded by a D-specific amino acid oxidase to provide pyruvate and ammonium.

The Enzyme

Alanine racemase was found in many bacteria, in particular within the Actinomycetes, such as *Bacillus* sp.,[59] *Pseudomonas putida*[60] and *Streptococcus faecalis*[61] to name just a few. All of the latter racemases have been purified and characterised; the enzymes described to date from different sources are highly homologous. For example, alanine racemase from *Bacillus stearothermophilus* is a homodimer composed of subunits of 43 kDa.[62]

Alanine racemase was found to be a bifunctional enzyme, i.e., besides racemisation it also catalyses transamination at low pH (pH ~6).[63,64] However, since the pH optimum for racemisation is in the alkaline region (pH 9 – 10), no interfering transamination was observed at this pH.

The mechanism of alanine racemase follows a two-base mechanism[65,66] (Scheme 10). In the absence of substrate, PLP is 'chemically stored' in the active site *via* binding though an internal Schiff-base (aldimine) formation involving the ε-NH_2-group of Lys39. When the substrate alanine enters the active site, PLP is transferred from the ε-NH_2-group of Lys39 onto the α-amino moiety Ala to form an external Schiff base. In this intermediate, α-proton abstraction is facilitated and

racemisation proceeds *via* two steps: (i) abstraction of the α-hydrogen atom, to furnish a resonance-stabilised quinoid-type carbanion intermediate, followed by (ii) reprotonation of the α-carbanion on the opposite[67] or the original side.[68] Whereas Lys39 is the catalytic base for the conversion of D- into L-alanine, Tyr265 is the catalytic counterpart for the transformation *vice-versa*.

3.1.2.2 Amino Acid Racemase

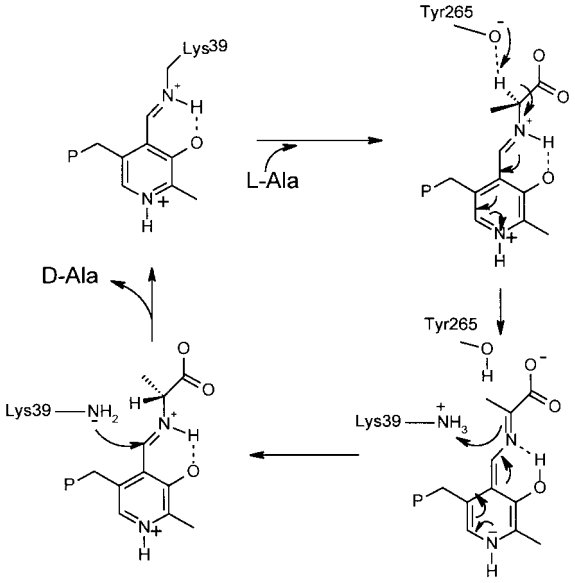
In contrast to the amino acid racemases discussed so far, which are largely restricted to a particular amino acid as substrate, the amino acid racemase (EC 5.1.1.10) of *P. putida* shows a great potential for preparative biotransformations, as it exhibits an exceptionally wide substrate tolerance, which encompasses the following amino acids: D-Arg, D-Asp, D-Gln, D-Phe, D-Ala, L-Lys, L-Met, L-norLeu, L-Orn, L-Ser, L-Thr, N^6-acetyl-L-Lys, *S*-methyl-L-Cys.[69]

Application

The biocatalytic synthesis of L-tryptophan was performed by a coupled reaction of tryptophan synthase and amino acid racemase starting from DL-serine and indole in a dynamic kinetic resolution process (Scheme 11). Whereas tryptophan synthase (EC 4.2.1.20) of *E. coli* enantioselectively catalysed the β-substitution reaction of L-serine to form L-tryptophan, the amino acid racemase provided D-serine from the racemate simultaneously in the same reactor. Under optimised conditions the production was carried out in a 200-L reactor using whole cells of *E. coli* and *P. putida*. After 24 h of incubation with intermittent indole feeding, 110 g/L of L-tryptophan was formed in yields of 91 and 100% for added DL-serine and indole, respectively.[70]

The Enzyme

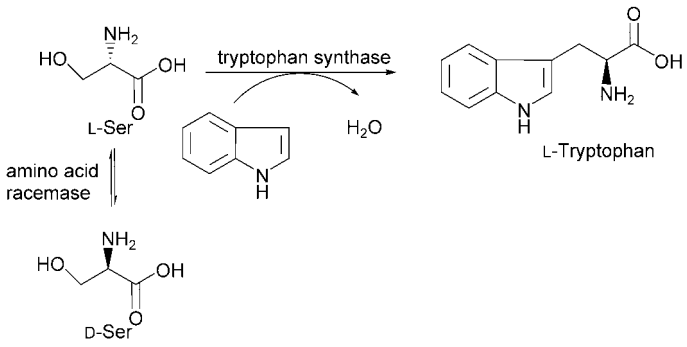
PLP-dependent amino acid racemases showing a wide substrate tolerance have been isolated from *P. striata*[71–74] and *P. putida*.[69,70,72,75] In earlier reports,[76] the enzyme mechanism was assumed to proceed according to a one-base mechanism, but recent results argue against this idea and rather support a two-base mechanism in analogy to those racemases described above. This change of concepts was based on the fact that only one of the bases undergoes proton exchange with the solvent while the amino acid is bound onto the enzyme. The holoenzyme from *P. putida* contains two units of PLP per protein.[71]



Scheme 10. Two-base mechanism of PLP-dependent alanine racemase.

3.1.2.3 Arginine Racemase

An arginine racemase isolated from *Pseudomonas graveolens* shows also a broad substrate spectrum. The best substrates are listed in Table 2.[77] Most remarkably, the enzyme shows a higher activity for L-lysine (110%)



Scheme 11. Synthesis of L-trypthophan in a dynamic kinetic resolution process using amino acid racemase.

Table 2. Relative activities of arginine racemase for various amino acids.

Substrate	Rel. Activity [%]	Substrate	Rel. Activity [%]
L-Lysine	110	Homoarginine	25
Arginine	100	Canavanine	19
ε-*N*-Acetyllysine	86	2,4-Diaminobutyric acid	18
Ornithine	44	Ethionine	13
2,3-Diaminopropionic acid	40	Citrulline	12

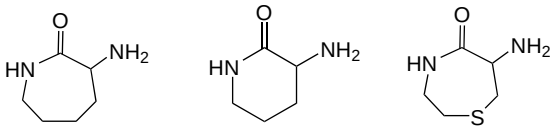


Figure 2. Substrates for α-amino-ε-caprolactam racemase.

than for arginine (100%) and possesses also transamination activity like alanine racemase (see above).[78]

3.2 Phenylalanine Racemase

The only racemase which follows a completely different mechanism of action is phenylalanine racemase (EC 5.1.1.11). For activation of the substrate, either D- or L-phenylalanine, ATP is required as cosubstrate and the reaction it actually catalyses is:

D- or L-Phenylalanine + ATP + H_2O

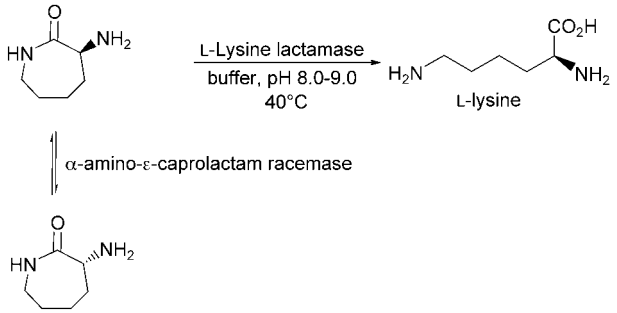
⟶ L- or D-Phenylalanine + AMP + diphosphate

The enzyme activates both enantiomers of phenylalanine by forming phenylalanyl adenylate bound to the enzyme from which the L- or D-Phe moiety is transferred onto an essential thiol residue within the active site of the enzyme, which allows inversion of configuration. Although the D-isomer has the more favourable configuration within the active site,[79,80] the equilibrium of D- versus L-isomer strongly depends on the pH. Thus, in a strict sense, this enzyme does not represent a true racemase – which, by definition, converts either substrate enantiomer to the corresponding racemate – but it resembles more an isomerase. Other substrates which are accepted by the enzyme are L-tyrosine and *p*-fluoro-L-phenylalanine.

3.3 Racemases Acting on α-Amino Acid Derivatives

3.3.1 Racemisation of α-Amino Lactams

The only α-amino lactam racemase which is of biocatalytic relevance is 2-aminohexano-6-lactam racemase (EC 5.1.1.15), commonly denoted as 'α-amino-ε-caprolactam racemase'. The substrate spectrum of this racemase is rather narrow including only α-amino-ε-caprolactam **11**,[81] α-amino-δ-valerolactam **12**[82] and α-amino-3-thia-ε-caprolactam **13** (Figure 2).[83] Possible sources of this racemase are the bacteria *Achrobacter obae*[81] and *Pseudomonas* sp.[84]



Scheme 12. Industrial production of L-lysine by dynamic resolution of (±)-α-amino-ε-caprolactam employing 2-aminohexano-6-lactam racemase.

3.3.1.1 Application

α-Amino-ε-caprolactam racemase of *Achromobacter obae* is industrially utilised to produce L-lysine, a nutrient and food supplement on a level of 4000 t per annum. Starting from synthetic DL-α-amino-ε-caprolactam, a one-pot dynamic resolution is performed (Scheme 12). Thus, whole cells of *Cryptococcus laurentii* possessing a lactamase (L-α-amino-ε-caprolactamase) enantioselectively hydrolyse the L-lactam from the racemate while whole cells of *Achromobacter obae* (containing the above mentioned racemase) catalyse the interconversion of the substrate enantiomers to furnish L-lysine in high yields with an ee >99.5%.[82,85]

3.3.1.2 The Enzyme

α-Amino-ε-amino-caprolactam racemase is a monomer with an approximate molecular weight of 49–51 kDa.[82,86] Similar to other amino acid racemases described above, PLP is required as cofactor for activity. Although a one-base mechanism has been proposed,[86] details on the mechanism or the enzyme's structure are not yet available.

3.3.2 Racemisation of N-Acetylamino Acids

The racemisation of *N*-acetylamino acids is catalysed by *N*-acetylamino acid racemases (NAAR). However, according to metabolic investigations, the natural role of a so-called *N*-acetylamino acid racemase from an *Amycolatopsis* sp.[87] is that of an *o*-succinylbenzoate synthase.[88] For the sake of clarity, this enzyme is referred to as *N*-acetylamino acid racemase in this article. *N*-Acylamino acid racemase activity was found by Tokuyama et al. in various Actinomycetes strains.[89,90] Among them, NAAR from *Streptomyces atratus* Y-53, *Amycolatopsis* sp. TS-1-60[90] and *Amycolatopsis orientalis* subsp. *lurida* have gained importance.[91]

By comparing the activity of NAAR from *A. orientalis* and *Amycolatopsis* sp. TS-1-60, a significant difference

Table 3. Relative activity of NAAR from *Amycolatopsis orientalis* and *A.* sp. TS-1-60 for different enantiomers.

	Amycolatopsis orientalis[a, b]		*A.* sp. TS-1-60[a, c]	
Substrate	D-Isomer [%]	L-Isomer [%]	D-Isomer [%]	L-Isomer [%]
N-Acetylmethionine	100	130	100	150
N-Acetylphenylalanine	76	30	69	28
N-Acetylvaline	83	22	27	83

[a] The activity of *N*-acetyl-D-methionine was set to 100% as standard.
[b] Data from ref.[91]
[c] Data from ref.[90]

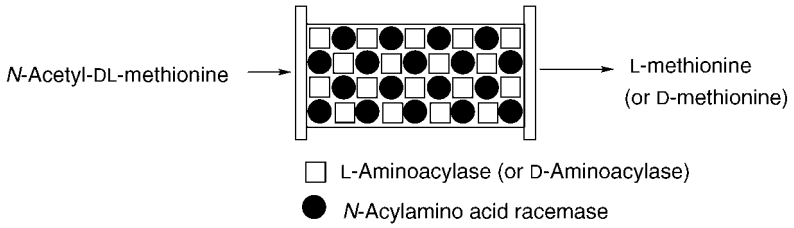


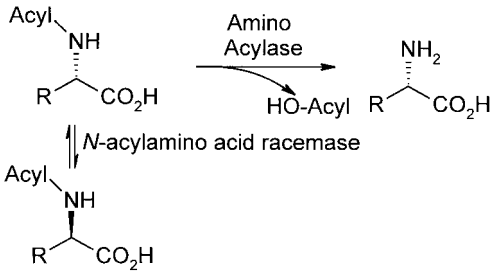
Figure 3. Sketch of bioreactor with immobilised enzymes for continuous production of L- (or D-)methionine starting from racemic starting material.

in the relative activity for the D- and L-enantiomers of selected *N*-acylamino acids (Table 3) was observed, which possibly indicates that the natural role of this enzyme *per se* is not necessarily racemisation. The best substrate is *N*-acetylmethionine; further substrates, which were racemised by NAAR from *A. orientalis* with moderate activity are *N*-acetyl-D-alanine, *N*-acetyl-D-α-aminobutyric acid, *N*-acetyl-L-tyrosine, *N*-acetylvaline and *N*-chloroacetyl-L-phenylalanine (Table 3). However, *N*-acetyl-L-*tert*-leucine, *N*-benzyloxycarbonyl-L-phenylalanine and *N*-acetyl-D-naphthylalanine are not transformed by this enzyme.

The NAAR from *Amycolatopsis* sp. TS-1-60 catalysed the racemisation of *N*-acetyl-L-tyrosine and leucine.[90] In addition, variation of the *N*-acetyl moiety by propionyl-, butyryl- or chloracetyl-groups was tolerated. Quite remarkably, this enzyme also catalysed the racemisation/epimerisation of the dipeptide L-alanyl-L-methionine. However, *N*-unsubstituted α-amino acids, *N*-alkyl-α-amino acids and ethyl esters of *N*-acetyl-D- and -L-methionine, were not racemised.

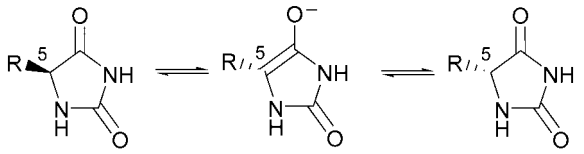
3.3.2.1 Application

Since these enzymes only act on *N*-acylamino acids but not on the corresponding 'free' amino acids they are top candidates for application to the one-pot dynamic kinetic resolution of *N*-acylamino acids using a two-enzyme system (Scheme 13): Thus, whereby an L- (or D-) selective amino acylase hydrolyses the acyl moiety to give the unprotected L- (or D-)amino acid, NAAR serves for the *in-situ* interconversion of the substrate enantiomers. A process of this type has been recently

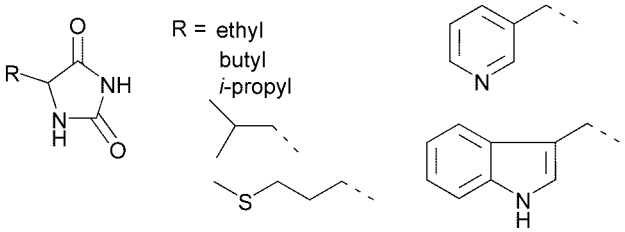


Scheme 13. Dynamic kinetic resolution of *N*-acylamino acids using a two-enzyme system.

established at Degussa Co. (Germany) based on the NAAR from *Amycolatopsis orientalis* subsp. *lurida* and an amino acylase.[92] Currently, several hundred tons of L-methionine are produced annually by kinetic resolution in an enzyme membrane reactor.[93] Previously, kinetic resolution of *N*-acetyl-DL-amino acids was achieved by using an enantiospecific L-amino acylase from *Aspergillus oryzae* which hydrolyses only the L-enantiomer to produce a mixture of the corresponding L-amino acid and unreacted *N*-acetyl-D-amino acid. After separation of the formed L-amino acid by crystallisation, the remaining *N*-acetyl-D-amino acid was recycled by thermal racemisation under drastic conditions according to a classic protocol. In contrast, the dynamic process employing the NAAR from *A. orientalis* subsp. *lurida* allows one to reduce costs and time and makes the production more efficient. Furthermore, limiting problems of substrate inhibition and the requirement for elevated concentrations of divalent metal ions (which limit the use of other *N*-acylamino



Scheme 14. Chemical racemisation of 5-substituted hydantoins *via* enolates.



Scheme 15. Substrates for hydantoin racemases from *Arthrobacter* spp.

acid racemases) can be overcome by the enzyme from *A. orientalis* subsp. *lurida*.

The production of D- or L-methionine is industrially performed in a continuous bioreactor which is fed with *N*-acetyl-DL-methionine, containing NAAR and L-amino acylase from *Streptomyces atratus* Y-53[94] or D-amino acylase from *Amycolatopsis* sp. TS-1-60[95], respectively, which were immobilised onto DEAE-Toyopearl (Figure 3). For instance, L-methionine was continuously produced in a yield of >99% from *N*-acetyl-DL-methionine for more than 25 h.

3.3.2.2 The Enzymes

The physicochemical characteristics of the purified enzyme of *A. orientalis* subsp. *lurida*[91] were found to be similar to those from *Amycolatopsis* sp. TS-1-60 and *S. atratus*,[95] i.e., a pI of 4.4, pH optimum of about 8, and thermal stability at 50 °C for 30 min. The molecular weight of the NAAR of *A. orientalis* is about 300 kDa for the native enzyme and 40 kDa for the subunit suggesting that the enzyme is a homooctamer, while the one of *S. atratus* occurs as hexamer (244 kDa). The octameric NAAR from *Amycolatopsis* sp. TS-1-60 is slightly more stable than that of *S. atratus* (at 55 °C for 30 min). For this enzyme, no significant sequence homology was found between the DNA sequence or the deduced amino acid sequence with that of other racemases or epimerases. However, comparison of the amino acid sequences of mandelate racemase and NAAR showed that both enzymes share a partial homology with respect to the catalytic and metal ion binding sites. Whereas mandelate racemase requires metals, such as Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+} or Ni^{2+} for activity,[24] NAAR of *Amycolatopsis* sp. TS-1-60 depends on Co^{2+}, Mn^{2+} or Fe^{2+}. Thus, it was speculated that the catalytic mechanism of NAAR is related to that of mandelate racemase due to the similar metal-ion requirement of both enzymes.[90]

3.3.3 Racemisation of Hydantoins

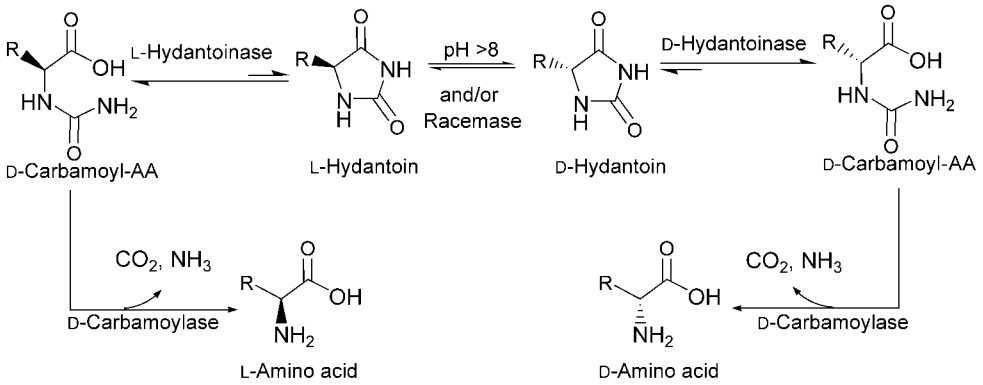
Monosubstituted hydantoins bearing an aromatic substituent in position 5, e.g., phenylhydantoin (Scheme 14, R = Ph) spontaneously racemise under weakly alkaline conditions.[96] It has been discussed that the velocity of chemical racemisation is influenced by the electronic nature of the substituent, such as its electronegativity, and the presence of adjacent π-systems, which facilitates enolate stabilisation due to participation of mesomeric structures.[97,98] As a consequence, 5-alkyl- or 5,5-arylalkyl'-substituted hydantoins are chirally stable and spontaneous reacemisation is too slow to allow dynamic kinetic resolution. For these types of substrates, hydantoin racemases were found in *Arthrobacter* and *Pseudomonas* species which enzymatically catalyse the interconversion of enantiomers.

The hydantoin racemase from *Arthrobacter aurescens* DSM 3747 shows a preference for hydantoins with arylalkyl side chains.[99] Further examples of substrates for hydantoin racemases from *Arthrobacter* spp. DSM 9771[92] and DSM 3745[100] are depicted in Scheme 15.

3.3.3.1 Application

Hydantoin racemases find their application in the so called hydantoinase-process for the industrial-scale production of either D- (or L-)amino acids, depending on the follow-up 'hydantoinase – enzymes' used.[101] Dynamic kinetic resolution of racemic hydantoins to either the D- (or L-)amino acid consists of three steps (Scheme 16): (i) First, ring-opening hydrolysis of the hydantoin is performed by a D- (or L-)specific hydantoinase, followed by (ii) carbamoylase-catalysed hydrolysis of the resulting *N*-carbamoylamino acid produced, which shifts the equilibrium towards completion. (iii) In order to achieve complete transformation of the *rac*-starting material in a dynamic kinetic resolution, substrate racemisation is performed *via* chemical and/or enzymatic catalysis using a hydantoin racemase. Since three enzymes are involved in this process, the relative activity (or expression level) of the enzymes must match the kinetic criteria,[102] i.e., (i) racemisation of the hydantoin must be fast enough to meet (or exceed) the rate of hydantoin hydrolysis and (ii) the degradation of the carbamoylamino acid must be equal or higher than its formation to avoid its accumulation which would cause reformation of the hydantoin.

The classic hydantoinase process was first introduced in the 1970 s for the production of D-amino acids such as D-phenylglycine and D-*p*-OH-phenylglycine.[103] Today,



Scheme 16. Hydantoinase process for production of D- (or L-)amino acids (AA) by dynamic kinetic resolution.

it is commercially applied at a scale of >1000 tons per year for the above-mentioned amino acids which are used as side chains for β-lactam antibiotics, such as ampicillin and amoxicillin, respectively. It was only recently that the hydantoin process became also feasible on a large scale for L-amino acids due to improvement of the productivity by using a recombinant whole-cell biocatalyst,[92] which allows commercialisation even for low-priced amino acids such as L-methionine.

3.3.3.2 The Enzyme(s)

Despite their importance for the industrial production of amino acids, remarkably little is known about the biochemical properties of hydantoin racemases. So far, these enzymes have been purified and characterised from *Arthrobacter*[97,99,104] and *Pseudomonas* sp.[105,106] The hydantoin racemase from *Arthrobacter aurescens* DSM 374 consists of 236 amino acids corresponding to 25.1 kDa.[99] The optimal conditions for racemase activity were found to be pH 8.5 and 55 °C with L-5-benzylhydantoin as substrate. Enzymatic racemisation of D-5-indolylmethylenehydantoin in D_2O followed by NMR analysis showed that the hydrogen at the chiral centre of the hydantoin underwent deuterium exchange with the solvent during racemisation, which was taken as an indication for a two-base mechanism. However, the exact enzyme mechanism remains to be elucidated.

3.3.4 Racemisation of α-Aminonitriles

Whole cells of *Actinomadura madurae* ATCC 15904 were found to racemise D-2-amino-2-phenylethanenitrile, which is a precursor for the important amino acid phenylglycine, in phosphate buffer at 30 °C to a certain extent.[107] However, in view of the scarce data available, the question whether this activity can be associated to a corresponding 'α-aminonitrile racemase' is unsolved.

4 Conclusion

Processes for the complete transformation of racemate mixtures into a single stereoisomeric product in 100% theoretical yield without the occurrence of an 'unwanted' stereoisomer are becoming increasingly important. This may be achieved *via* deracemisation[10] or dynamic kinetic resolution.[13–18] For the latter the use of racemases for the chemo-selective interconversion of enantiomers in the absence of side-reactions holds a great potential for the biotransformation of non-natural compounds on a preparative-scale. The aspect of biocatalytic racemisation under mild and environmentally benign conditions matches well with the rising general awareness for environmental issues and economic factors in the (bio)chemical production of organic compounds in non-racemic form. As a consequence, it is expected that these largely overlooked enzymes have a great chance for increased applicability in the future.

Acknowledgements

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