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Dynamic Kinetic Resolution of α-Aminonitriles to Form Chiral α-Amino Acids

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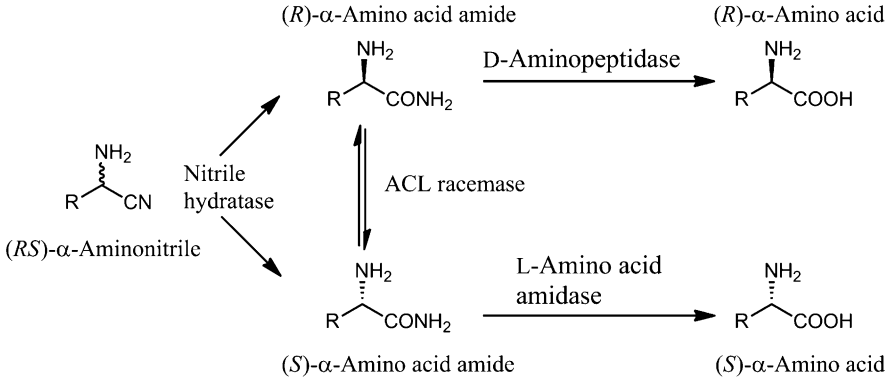
Abstract: We have succeeded in the enzymatic synthesis of (*R*)-α-aminobutyric acid from racemic α-aminobutyronitrile. This has been demonstrated by the use of non-stereoselective nitrile hydratase (NHase) from *Rhodococcus opacus* 71D, D-aminopeptidase from *Ochrobactrum anthropi* C1-38 and α-amino-ε-caprolactam (ACL) racemase from *Achromobacter obae*. Racemic α-aminobutyronitrile was completely converted in 6 h at 30 °C to (*R*)-α-aminobutyric acid whose optical purity was more than 99%. (*S*)-α-Aminobutyric acid was also synthesized from α-aminobutyronitrile by NHase, ACL racemase and L-amino acid amidase from *Brevundimonas diminuta* TPU 5720. In a similar manner, other (*R*)- or (*S*)-α-amino acids with more than 97.5% *ee* could be synthesized from the corresponding α-aminonitriles. This is the first report on the dynamic kinetic resolution (DKR) of α-aminonitriles to form chiral α-amino acids. The key enzyme in this DKR is non-stereoselective NHase, which had been newly screened from soil samples, and its gene cloned.

Keywords: amino acids; biotransformation; dynamic kinetic resolution; nitrile hydratase

Optically active α-amino acids are important additives for animal feed and infusion as well as building blocks for pharmaceuticals, agrochemicals, and artificial sweeteners. Racemic α-amino acids have been synthesized by chemical hydrolysis of α-aminonitriles, but biologically important chiral (*R*)- or (*S*)-α-amino acids cannot be produced in such a way.

Most published works on stereoselective enzymatic conversions of racemic α-aminonitriles have dealt with the direct production of optical pure α-amino acids *via* a nitrilase-catalyzed hydrolysis of α-aminonitriles, forming α-amino acids such as (*S*)-alanine,[1] (*S*)-leucine,[2] and (*S*)-phenylglycine.[3] Only very little work has been done on the conversion of mainly phenylglycinonitrile to (*S*)- or (*R*)-phenylglycine by kinetic resolution (KR) in from 25% up to 50% yield *via* phenylglycinamide with a nitrile hydratase-amidase system.[4–7] For example, Ewert et al.[4] reported that nitrile hydratase (NHase) from *Klebsiella oxytoca* preferentially converted the *S*-enantiomer to (*S*)-phenylglycinamide with an *E* value of 7.8, and in the presence of an *S*-stereoselective amidase, 1 mM (*RS*)-phenylglycinonitrile was converted to (*S*)-phenylglycine with >99% *ee* and (*R*)-phenylglycinamide with >99% *ee* by KR. These KR of α-aminonitriles only leave unreacted substrate or by-product in the reaction mixture. We focused on a more efficient and powerful method, dynamic kinetic resolution (DKR) consisting of resolution and racemization steps of the substrate in the reaction system. This DKR process transforms a racemic starting material into only a single chiral product in >99% theoretical yield. We previously reported that α-amino-ε-caprolactam (ACL) racemase catalyzes the racemization of α-amino acid amides as new substrates and is effective for the DKR of α-amino acid amides, when used with *R*- or *S*-stereoselective amino acid amide hydrolase.[8–10] Our purpose in this study is to obtain optically pure α-amino acids in theoretical yield with high enantiomeric excess by the DKR of racemic α-aminonitriles. This DKR process consists of three steps: (i) hydrolysis of both enantiomer of racemic α-aminonitrile by non-stereoselective NHase, (EC 4.2.1.84), (ii) stereoselective hydrolysis of the resulting racemic α-amino acid amide by stereoselective amino acid amide hydrolase, shifting the equilibrium toward the product, and at the same time, (iii) racemization of



Scheme 1. Dynamic kinetic resolution of α-aminonitriles to form chiral α-amino acids.

the remaining α-amino acid amide by ACL racemase (Scheme 1). A non-stereoselective NHase is a key enzyme in this method, and most of the enzymes utilized have been discovered and characterized during our original studies.

Asano et al. have isolated various nitrile-degrading microorganisms.[11,12] They first purified, characterized, and named "nitrile hydratase" from *Rhodococcus rhodochrous* (formerly *Arthrobacter* sp.) J-1.[13,14] They also found that *Pseudomonas chlororaphis* B23 accumulates large quantities of amides from nitriles and is suitable for the industrial production of acrylamide from acrylonitrile.[11–14] Moreover, nicotinamide and 5-cyanovaleramide are also industrially produced by NHase.[15,16] NHase has become one of the most important enzymes in the chemical industry.

In this report, we have isolated a new bacterial strain of *Rhodococcus opacus* 71D from soil exhibiting NHase activity with very low stereoselectivity toward α-aminobutyronitrile and purified and characterized its NHase (see Supporting Information for detaile). The specific activity of the purified enzyme was 1040 U mg^{-1} protein, when (*RS*)-α-aminobutyronitrile was used as a substrate. The amino acid sequence of this NHase showed that it would be a cobalt-containing enzyme with the amino acids sequence of CTLCSCY motif (see Supporting Information). One of the characteristics of Co-type NHase is that it is capable of hydrating aliphatic, aromatic, and heterocyclic nitriles, whereas no Fe-type NHase has been reported to have an activity toward aromatic and heterocyclic substrates.[17–19] In the past, several NHase have been purified and characterized from various microorganisms. However, none of these has been explored in detail for the catalytic activity to hydrolyze α-aminonitriles, probably because of the lack of availability of pure α-aminonitriles. While Bhalla et al. reported the substrate specificity of nitrilase from *R. rhodochrous* PA-34[2] on α-aminonitriles such as alaninonitrile, α-aminobutyronitrile, α-amino-*n*-valeronitrile, leucinonitrile, α-amino-*n*-capronitrile, and phenylglycinonitrile, they did not report *E* values and K_m values for the α-aminonitriles. We show that NHase has a wide substrate specificity toward aliphatic nitriles such as acrylonitrile (1,520 U mg^{-1}), butyronitrile (3,770 U mg^{-1}), alaninonitrile (770 U mg^{-1}), α-aminobutyronitrile, valinonitrile (157 U mg^{-1}) and leucinonitrile (88.5 U mg^{-1}). Moreover, aromatic nitrile such as benzonitrile (758 U mg^{-1}), phenylglycinonitrile (303 U mg^{-1}), phenylalaninonitrile (446 U mg^{-1}) and manderonitrile (860 U mg^{-1}) were also shown to be good substrates. Kinetic parameters for the several racemic α-aminonitriles were calculated by Hanes–Woolf plots. The K_m values for alaninonitrile, α-aminobutyronitrile, valinonitrile, leucinonitrile, *tert*-leucinonitrile, phenylglycinonitrile, and phenylalninonitrile were calculated to be 17.3, 4.33, 0.20, 1.15, 1.59, 3.63, and 1.58 mM, respectively. Compared with K_m values for substrates with high affinities of known NHases such as *P. chlororaphis* B23,[17] *Arthrobacter* sp. J-1,[14] and *R. rhodochrous* J-1,[23] these K_m value for α-aminonitriles of NHase from *R. opacus* 71D revealed similar affinities.

Generally, NHases are rather not enantioselective with enantiomeric compounds. It has been shown that some NHases show moderate stereoselectivity, only when synthetic substrates were specifically designed to fit the active sites. For instance, it has been reported that NHase from *R. equi* A4 show slight *S*-selectivity toward 2-(4-isobutylphenyl)-2-methylpropionitrile, 2-(4-chlorophenyl)-2-methylpropionitrile and 2-(4-methoxyphenyl)-2-methylpropionitrile, and NHase in *Rhodococcus* sp. HT 40-6 has *S*-stereoselective activity toward mandelonitrile.[20] NHase from *P. putida* 2D-11-5-1b and *P. putida* 5B-MNG-2P act on the *R*-enantiomer of 2-(4-isobutylphenyl)-2-methylpropionitrile.[20] Thus, the stereoselectivity of NHase has been a very interesting topic, although there has been no report on competent stereoselectivity of an NHase active toward α-aminonitrile. On the other hand, we here demonstrate a successful utilization of non-stereoselective NHase in DKR. The purified NHase hy-

drated alaninonitrile, α-aminobutyronitrile, valinonitrile, leucinonitrile, phenylglycinonitrile and phenylalninonitrile with *E* values[21] of 1.2 (*S*), 1.2 (*S*), 1.1 (*S*), 2.1 (*S*), 1.8 (*S*) and 1.0, respectively.

It is important that NHase should be resistant to cyanide inhibition for biotransformation of α-aminonitriles using NHase, because an α-aminonitrile is spontaneously decomposed into aldehyde and cyanide by a retro-Strecker reaction in aqueous solution, and cyanide strongly inhibits NHase activity. Fe-type NHase from *P. chlororaphis* B23[17] and *Brevibacterium* sp. R312[22] were only slightly inhibited by cyanide, while Co-type NHase from *Arthrobacter* sp. J-1,[14] *R. rhodochrous* J-1,[23] and *Rhodococcus* sp. YH3-3[24] were strongly inhibited by cyanide. NHase from *R. opacus* 71D showed a high performance toward α-aminonitrile hydrolysis more than the usual Co-type NHases showing high resistanse toward cyanide even in 1 mM cyanide solution (data not shown).

This NHase has been proven to be very suitable for conversion of several α-aminonitriles to α-amino acid amides. The time course of (*R*)-α-aminobutyric acid (ABA) conversion from racemic (*RS*)-α-aminobutyronitrile sulfuric acid salt (ABN) by purified NHase, D-aminopeptidase (DAP) from *O. anthropi* C1-38[25] and ACL racemase is shown in Figure 1.

In the initial phase of the reaction, the NHase rapidly converted ABN to racemic α-aminobutyramide (ABA-NH_2) with an *E* value of 1.2. After 20 min, ABN was completely converted to (*R*)- and (*S*)-ABA-NH_2. (*R*)-ABA-NH_2 was hydrolyzed by DAP, while the remaining (*S*)-ABA-NH_2 was racemized by ACL racemase. The concentration of (*R*)-ABA was the same as that of the ABN originally present in the reaction mixture. All of (*RS*)-ABN was converted to (*R*)-ABA in 6 hours (conversion >99% with >99% *ee*). (*S*)-ABA also could be obtained by DKR of racemic ABN by purified NHase, L-amino acid amidase ($LaaA_{Bd}$) from *B. diminuta* TPU 5720[26] and ACL racemase (see Supporting Information for details). All of (*RS*)-ABN was converted to (*S*)-ABA in 12 hours (conversion >99% with >99% *ee*).

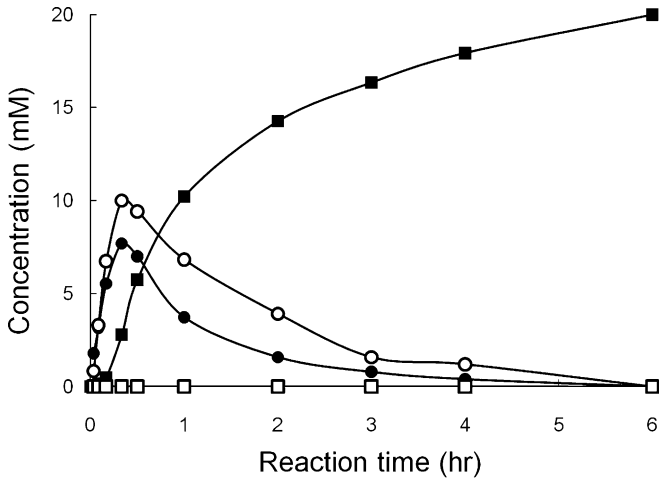


Figure 1. The enzymatic conversion of (*RS*)-α-aminobutyronitrile to (*R*)-α-aminobutyric acid. The reaction mixture (total volume 1.0 mL) was composed of 50 mM potassium phosphate buffer, pH 7.0, 20 mM (*RS*)-α-aminobutyronitrile, 500 nM pyridoxal phosphate, NHase (1.0 U), DAP (0.52 U), and ACL racemase (1.6 U) at 30 °C. The NHase was added to initiate the reaction. Symbols: (*R*)-α-aminobutyramide (●), (*S*)-α-aminobutyramide (○), (*R*)-α-aminobutyric acid (■), and (*S*)-α-aminobutyric acid (□).

As a proof of principle, the feasibility of achieving the sequential conversion of racemic α-aminonitriles to chiral α-amino acids in a one-pot enzymatic reaction was demonstrated by using non-stereoselective NHase, ACL racemase and *R*- or *S*-stereoselective amidase.

We have reported the discoveries and properties of *R*-stereoselective amino acid amidases such as DAP, D-amino acid amidase (DaaA),[27] and alkaline D-peptidase[30] and *S*-steroselective amino acidc amide hydrolases such as $LaaA_{Bd}$ and L-amino acid amidase ($LaaA_{Pa}$) from *P. azotoformans* IAM 1603.[29] By combination of non-stereoselective NHase, ACL racemase and *R*- or *S*-stereoselective amidase, it is possible to construct an efficient system to produce the corresponding (*R*)- or (*S*)-α-amino acids from racemic α-aminonitriles. Actually, (*S*)-alanine with a yield of 99% and 99% *ee* was converted from 50 mM alaninonitrile using NHase (1.6 U), ACL racemase (0.5 U), and $LaaA_{Pa}$ (2 U) in 3 hours. Moreover, using NHase, ACL racemase, and $LaaA_{Bd}$, 10 mM valinonitrile, 30 mM leucinonitrile, and 10 mM phenylalaninonitrile were converted to the corresponding *S*-enantiomer of the α-amino acids with yields of 99% (Table 1). $LaaA_{Bd}$ acted on not only (*S*)-alaninamide and (*S*)-phenylalaninamide, but also on *R*-form alaninamide and slightly on phenylalaninamide.[26] (*S*)-Phenylalanine was produced from phenylalaninonitrile with a little bit lower enantiomeric excess. (*R*)-Amino acids also could be produced from several racemic α-aminonitriles. DAP acted on (*R*)-amino acid amides with small substituents, such as (*R*)-alaninamide and (*R*)-α-aminobutyramide. On the other hand, DaaA showed hydrolyzing activity toward bulky amino acid amides like (*R*)-leucinamide and (*R*)-phenylalaninamide. Alaninonitrile (50 mM), 30 mM leucinonitrile, and 20 mM valinonitrile were converted to the corresponding (*R*)-α-amino acids (Table 2).

Nitrilases have been used to produce only (*S*)-α-amino acids from corresponding α-aminonitriles in the kinetic resolution reaction, while a nitrilase-mediated *R*-stereoselective hydrolysis of an α-aminonitrile has not been reported. To the best of our knowledge, this is the first report on the dynamic kinetic resolution of racemic α-aminonitriles to not only the corresponding (*R*)-α-amino acids but also to (*S*)-α-amino

Table 1. Conversion of α-aminonitrile to (*S*)-α-amino acid by DKR. The reaction (1 mL) was carried out using 1.6 U NHase, 0.5 U ACL racemase, and 1.8 U $LaaA_{Bd}$ at 30 °C.

Substrate	Concentration [mM]	Reaction Time [h]	Yield [%]	Enantiomeric Excess [% *ee*]
alaninonitrile[a]	50	3	>99	>99
α-aminobutyronitrile	50	3	>99	>99
valinonitrile	10	6	99.0	>99
leucinonitrile	30	9	>99	>99
phenylalininonitrile[b]	10	12	>99	97.5

[a] The reaction (1 mL) was carried out using 1.6 U NHase, 0.5 U ACL racemase, and 2 U $LaaA_{Pa}$.
[b] The reaction (1 mL) was carried out using 1.6 U NHase, 1.0 U ACL racemase, and 1 U $LaaA_{Bd}$. The yield and enantiomeric excess were determined by HPLC (see Supporting Information).

Table 2. Conversion of α-aminonitrile to (*R*)-α-amino acid by DKR. The reaction (1 mL) was carried out using 1.6 U NHase, 0.5 U ACL racemase, and 1.9 U DaaA at 30 °C.

Substrate	Concentration [mM]	Reaction Time [h]	Yield [%]	Enantiomeric Excess [% *ee*]
alaninonitrile[a]	50	3	>99	>99
α-aminobutyronitrile[a]	50	3	>99	>99
valinonitrile	20	20	>99	>99
leucinonitrile	30	9	>99	>99

[a] The reaction (1 mL) was carried out using 1.6 U NHase, 0.5 U ACL racemase, and 1.8 U DAP. The yield and enantiomeric excess were determined by HPLC (see Supporting Information).

acids. This new method of dynamic kinetic resolution has the potential to be developed for the large-scale production of optically active α-amino acids.

Experimental Section

Enzyme Reaction and Activity Assay

The standard reaction mixture (total volume 1.0 mL) for assaying the nitrile hydratase activity contained 50 mM KPB, pH 7.0, 20 mM (*RS*)-α-aminobutyronitrile, 0.1 mM $CoCl_2 \cdot 6H_2O$ and the appropriate amount of the enzyme. One unit (U) of the enzyme activity was defined as the amount of enzyme solution which catalyzed the formation of 1 μmol of α-aminobutyramide from α-aminobutyronitrile per min at 30 °C.

One unit (U) of ACL racemase activity was defined as the amount of enzyme solution which catalyzed the formation of 1 μmol of (*S*)-α-aminobutyramide from (*R*)-α-aminobutyramide per min at 30 °C.[8]

The D-aminopeptidase activity was assayed by the formation of (*R*)-α-aminobutyric acid from (*R*)-α-aminobutyramide as follows. The reaction mixture contained 50 mM KPB (pH 7.0), 50 mM (*R*)-α-aminobutyramide, and an appropriate amount of the enzyme. One unit (U) of D-aminopeptidase activity was defined as the amount of the enzyme solution which catalyzed the formation of 1 μmol of (*R*)-α-aminobutyric acid per min at 30 °C.[25]

L-Amino acid amidase activity was assayed by measuring the production of (*S*)-α-aminobutyric acid from (*S*)-α-aminobutyramide. One unit (U) of L-amino acid amidase activity was defined as the amount of the enzyme solution which catalyzed the formation of 1 μmol of (*S*)-α-aminobutyric acid per min at 30 °C.[26]

Analytical Procedures

(*R*)- or (*S*)-α-aminobutyramides were determined with an HPLC equipped with a Crownpak CR (+) column at a flow rate of 0.3 mL min^{-1}, using a solvent (60 mM $HClO_4$). (*R*)- and (*S*)-α-aminobutyric acids were determined with HPLC equipped with a SUMICHIRAL OA-5000 column at a flow rate of 1.0 mL min^{-1}, using a solvent system of 2 mM $CuSO_4$ in 5% 2-propanol.

The identity of the (*R*)-α-aminobutyric acid formed by NHase, ACL racemase, and DAP was confirmed by its isolation. The reaction mixture (40 mL) contained 2.0 mmol KPB (pH 8.0), 4.0 mmol (*RS*)-α-aminobutyronitrile sulfuric acid salt (0.532 g), 80 nmol pyridoxal phosphate, 4.0 μmol $CoCl_2 \cdot 6H_2O$, 120 U of NHase, 39 U of ACL racemase, and 93 U of DAP. After the mixture was incubated at 30 °C for 10 h, (*R*)-α-amiobutyric acid formed was isolated by Dowex-X8 column chromatography and recrystallized from water-methanol-ether. The optical purity of the isolated (*R*)-α-aminobutyric acid was more than 99% *ee* with an isolated yield of 68% (0.28 g, 2.72 mmol); Optical rotations were measured on a SEPA-300 (Horiba, Ltd., Kyoto, Japan) instrument: $[\alpha]_D^{20}$: −19.7° (*c* 1.00, 5 mol dm^{-3} HCl) {ref.[30], $[\alpha]_D^{20}$: −20.5° (*c* 1.00, 5 mol dm^{-3} HCl)}: ^{1}H NMR (D_2O, 400 MHz): δ = 3.60 (t, 1H, *J* = 5.8 Hz), 1.79 (dq, 2H, *J* = 5.8, 7.5 Hz), 0.87 (d, 3H, *J* = 7.5 Hz); ^{13}C NMR (400 MHz, D_2O): δ = 174.8, 55.8, 23.6, 8.5; MS (microTOF): *m*/*z* = 104.0731, calcd. for $C_4H_{10}N_1O_2$ $[M+H]^+$: 104.0706. HPLC elution profile and ^{1}H NMR, ^{13}C NMR, MS and IR spectra of the isolated (*R*)-α-aminobutyric acid are described in the Supporting Information.

Supporting Information

Experimental details of the purification, characterization and cloning of the enzyme are described in the Supporting Information file.

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