APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1997, p. 3390–3393 Vol. 63, No. 9
0099-2240/97/$04.00+0

Formation of a Chiral Hydroxamic Acid with an Amidase from *Rhodococcus erythropolis* MP50 and Subsequent Chemical Lossen Rearrangement to a Chiral Amine

BEATE HIRRLINGER AND ANDREAS STOLZ*

Institut für Mikrobiologie der Universität Stuttgart, 70569 Stuttgart, Germany

Received 16 January 1997/Accepted 6 June 1997

The amidase from *Rhodococcus erythropolis* MP50 demonstrated, in the presence of hydroxylamine, acyltransferase activity and catalyzed the formation of hydroxamates from amides and hydroxylamine. The rates of acyltransferase activity of the purified amidase for the substrates acetamide, phenylacetamide, and 2-phenylpropionamide were higher than the corresponding rates for the hydrolysis reactions. With the substrate 2-phenylpropionamide the hydrolysis reaction and the acyltransferase activity were highly enantioselective. The optically active 2-phenylpropionhydroxamate was converted by a chemical Lossen rearrangement in an aqueous medium into the enantiopure *S*-1-phenylethylamine.

We have recently described the isolation of new bacterial strains with the ability to produce almost pure *S*-naproxen [*S*-2-(6-methoxy-2-naphthyl)propionic acid] from racemic naproxen nitrile (16). The isolate *Rhodococcus erythropolis* MP50 hydrolyzed (*R,S*)-naproxenamide highly enantioselectively to *S*-naproxen. A purified amidase from *R. erythropolis* MP50 converted racemic 2-phenylpropionamide, naproxen amide [2-(6-methoxy-2-naphthyl)propionamide], and ketoprofen amide [2-(3′-benzoylphenyl)propionamide] to the corresponding S-acids with enantiomeric excesses that were >99% at almost 50% conversion of the racemic amides. The enzyme also hydrolyzed different α-aminoarylacetamides with different degrees of enantioselectivity (9). It has been previously shown that different amidases transfer, in the presence of hydroxylamine, the acyl groups of their substrates to hydroxylamine, which results in the formation of the corresponding hydroxamates (1, 5, 6, 14, 20, 21). In the present study it was shown that the enantioselective amidase from strain MP50 also catalyzed the almost strict enantioselective formation of the corresponding hydroxamates and that this reaction can be used for the production of optically pure amines.

MATERIALS AND METHODS

Bacterial strain and growth media. The isolation and characterization of *R. erythropolis* MP50 has been described before (16). The strain has been deposited at the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany, as DSM 9675. The composition of the culture media and the methods for growth measurement have been described before. The cells were grown with succinate, nutrient broth, and ketoprofen amide (9).

Determination of enzyme activities with resting cells. The reaction mixture contained, in Tris-HCl buffer (pH 7.5, 30 mM), resting cells of strain MP50 and a 0.1 M concentration of the respective amide. These cell suspensions were incubated on a rotary shaker (30°C, 150 rpm). Usually, every 10 min aliquots (0.2 to 0.5 ml) were taken, the cells were removed by centrifugation at room temperature (3 min, 14,000 × *g*), and the supernatants were analyzed by high-performance liquid chromatography (HPLC). For the determination of acyltransferase activity hydroxylamine (0.5 M) was added. The formation of the hydroxamates was determined spectrophotometrically at a λ of 500 nm (see below).

Determination of the amidase (amide-hydrolyzing) activity with cell extracts or the purified amidase. The preparation of cell extracts, the quantification of protein, and the purification of the amidase were performed as described before (9). The standard assay mixture for amide-hydrolyzing activity determination was composed of (in a total volume of 0.5 ml) 12 μmol of Tris-HCl buffer (pH 7.5), 2.5 μmol of phenylacetamide (stock solution; 50 mM in methanol), and different amounts of protein (1 to 10 μg). The reaction was performed at room temperature in a plastic reaction tube. After different time intervals aliquots were taken (100 μl each), the reaction was stopped by the addition of 10 μl of 1 M HCl, and the precipitated protein was removed by centrifugation (5 min, 14,000 × *g*). The formation of the corresponding acids was determined by HPLC.

The hydrolysis of acetamide was measured as previously described (9). A commercially available ammonia test kit was used (Spectroquant 14572; Merck, Darmstadt, Germany).

Analytical methods. The concentrations of amides and acids were quantified by HPLC (HPLC Millennium Chromatography Manager 2.0, equipped with a programmable multiwavelength detector [model 486] and an HPLC pump [model 510]; Waters Associates, Milford, Mass.).

For the analysis of phenylacetamide and phenylacetohydroxamate, racemic 2-phenylpropionate, 2-phenylpropionamide, and 2-phenylpropionhydroxamate, a reverse-phase column (Grom-Sil C_8; Grom, Herrenberg, Germany) was used. The aqueous solvent systems contained different portions of methanol (3 to 15% [vol/vol]) plus Na-phosphate buffer (pH 2, 10 mM) and an ion pair reagent (PicB6; Waters) according to the instructions of the manufacturer.

For separation of the enantiomers of 2-phenylpropionamide and 2-phenylpropionhydroxamate a Chiral-HSA column (with guard column) was used (column size: 150 by 4 mm; 5-μm silica gel particles with human serum albumin; ChromTech, Norsborg, Sweden). The eluent was Na-phosphate buffer (pH 5.0, 100 mM), and the flow rate was 0.5 ml/min. Individual compounds were detected at 210 nm. With this chromatographic system a complete separation of the two enantiomers of 2-phenylpropionhydroxamate was achieved (R_t = 7.7 and 11.9 min).

The separation of the enantiomers of (*R,S*)-phenylethylamine was done with a Crownpak Cr(+) column (with guard column; column size: 150 by 4 mm; Baker, Groß Gerau, Germany). The solvent was $HClO_4$ (5.15 g/liter, pH 1.5), and the flow rate was 0.8 ml/min. The column was incubated at a temperature of 25°C, and individual compounds were detected at 210 nm.

Acyltransferase activity. The acyltransferase activity was determined by a modification of the method of Brammar and Clarke (3). The substrate mixture contained, in 1 ml of Tris-HCl buffer (30 mM, pH 7.5), 5 μmol of amide (stock solution; 50 mM in methanol), 0.5 mmol of hydroxylamine, and resting cells, cell extract, or purified amidase. The hydroxylamine stock solution (2 M) was freshly prepared daily. Immediately before the start of the assay an aliquot (250 μl) was taken from this stock solution, neutralized by the addition of NaOH (10 μl, 10 M), mixed with Tris-HCl buffer, resting cells or enzyme, and amide. Aliquots (250 μl each) were taken after 5, 10, and 15 min and were mixed with 250 μl of an acid $FeCl_3$ solution (0.1 M $FeCl_3$ plus 0.08 M HCl, filtered through an 0.45-μm-pore-size filter [11]). The formation of the hydroxamates was assayed spectrophotometrically at 500 nm.

For the chiral analysis of the reaction, the purified amidase (2.6 μg of protein) was incubated with 2-phenylpropionamide (5 mM) and hydroxylamine (0.5 M) in Na-phosphate buffer (10 mM, pH 7.5) in a total volume of 1 ml. Every 10 min aliquots (50 μl each) were taken and the reaction was terminated by the addition of HCl (5 μl, 1 M). The denatured protein was removed by centrifugation (5 min, 14,000 × *g*), and the concentrations of 2-phenylpropionamide and 2-phenylpropionhydroxamate were determined by ion pair chromatography. The enantiomeric excess of the 2-phenylpropionhydroxamate was determined by chiral HPLC on a Chiral-HSA column.

* Corresponding author. Mailing address: Institut für Mikrobiologie der Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany.

Spectrophotometric quantification of the acyltransferase activity. The molar extinction coefficients of the Fe(III)-hydroxamic acid complexes were determined at a λ of 500 nm for acetohydroxamate, phenylacetohydroxamate, and 2-phenylpropionhydroxamate as ε_{500nm} = 3,500, 3,800, and 4,500 liters mol^{-1} cm^{-1}, respectively.

Lossen rearrangement. After the amidase had converted about 50% of the initial amount of 2-phenylpropionamide (usually about 5 mM), the reaction was stopped by the addition of HCl (final pH about 1.0). The denatured protein was removed by centrifugation (3 min, 14,000 × *g*), and the supernatant was heated for 10 min to 80°C. Under these conditions the remaining hydroxylamine was converted to the volatile compounds ammonia and N_2O (12). Then the pH of the solution was raised by the addition of NaOH to 4.5 and solid *N'*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (final concentration, 0.1 M) was added (11). After 1 h of incubation at 80°C the products were analyzed by chiral HPLC.

Chemicals. *N'*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide, *S*-(−)-1-phenylethylamine, and acetohydroxamic acid were purchased from Aldrich (Steinheim, Germany). Free hydroxylamine was prepared according to the method of DeWitt Hurd and Brownstein (7). The syntheses of 2-phenylpropionamide and 2-phenylpropionhydroxamate were done basically as described by Hall and Gisler (8) and Jones and Neuffer (13), respectively. The sources of all other chemicals have been described before (2, 15, 16).

RESULTS AND DISCUSSION

Experiments with resting cells. Cells of *R. erythropolis* MP50 were grown in the presence of ketoprofen amide to induce the amidase (9). Cells were harvested by centrifugation, and resting cells (optical density at 546 nm, 1.0) were incubated in Tris-HCl buffer (30 mM, pH 7.5) with phenylacetamide or acetamide (100 mM each) and hydroxylamine (500 mM). In control experiments the respective amides were incubated with hydroxylamine without cells. The reaction mixtures were shaken for 15 min at 30°C, and finally an acid $FeCl_3$ solution was added and the cells were removed by centrifugation. The incubation mixtures with cells turned immediately red after the addition of $FeCl_3$. In contrast, the controls without cells turned only yellow after the $FeCl_3$ solution was added. The color reaction with Fe(III) ions is characteristic of hydroxamates. Thus, the cells enzymatically formed hydroxamates from the amides and hydroxylamine.

In the following experiment the rates for the formation of phenylacetohydroxamate and phenylacetate from phenylacetamide were compared. Both reactions proceeded linearly for more than 30 min. The rate of acyltransferase activity with phenylacetamide (7.8 U/mg of protein) was higher than the rate for the hydrolysis of the same substrate to phenylacetate (0.5 U/mg of protein).

It has been demonstrated before that amidases show acyltransferase activity not only with amides but also with esters and hydroxylamine (6, 20, 21). Therefore, resting cells were incubated with a saturated solution of phenylacetic acid ethylester and hydroxylamine (500 mM), but no hydroxamate formation was observed.

Acyltransferase activity of cell extracts and the purified amidase. Acyltransferase activity was also found in cell extracts and with the purified amidase. The purified amidase showed hydrolysis and acyltransferase activities with all substrates tested. The specific activities for the hydrolysis of acetamide, phenylacetamide, and 2-phenylpropionamide were 0.83, 4.1, and 4.5 U/mg of protein, respectively. The specific activities for the formation of the corresponding hydroxamates were 3.5, 36.6, and 13.0 U/mg of protein, respectively. Thus, with all three substrates the formation of the hydroxamates proceeded with higher specific activities than those for the hydrolysis reaction.

Enantioselectivity of hydroxamate formation. It has been shown before that the amidase from strain MP50 was highly enantioselective for the hydrolysis of compounds such as 2-phenylpropionamide, naproxen amide, and ketoprofen amide (9). Whether the enzymatically formed hydroxamate from 2-phenylpropionamide was also optically active was therefore tested. 2-Phenylpropionamide was incubated with hydroxylamine and the purified amidase, and the reaction was analyzed by chiral HPLC. After 46% turnover of the substrate there was still only one enantiomer of 2-phenylpropionhydroxamate detectable by HPLC (Fig. 1). To further determine the degree of enantioselectivity of the reaction, the enzyme was incubated for a longer period with racemic 2-phenylpropionamide and hydroxylamine. Even after 80 min of incubation only 51% substrate turnover was observed.

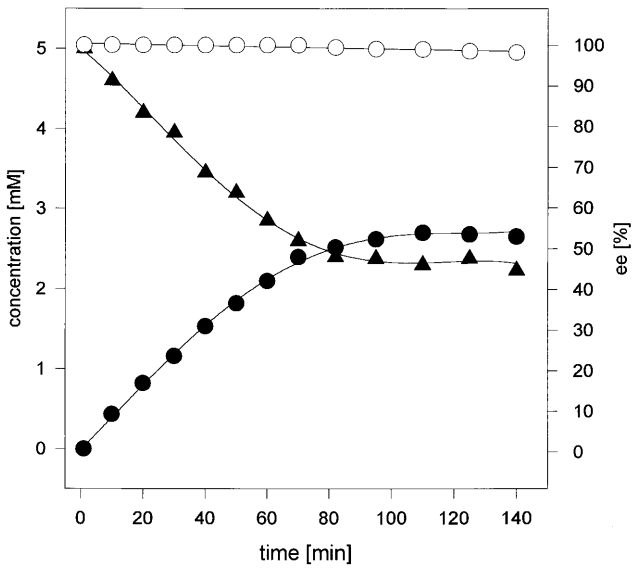

FIG. 1. Enantioselective formation of 2-phenylpropionhydroxamate by the purified enantioselective amidase from *R. erythropolis* MP50. The concentrations of 2-phenylpropionamide (▲) and 2-phenylpropionhydroxamate (●) and the enantiomeric excess (ee) of 2-phenylpropionhydroxamate (○) were determined as described in Materials and Methods.

Lossen rearrangement of the chiral 2-phenylpropionhydroxamate. The Lossen rearrangement converts a hydroxamate via the corresponding isocyanate to a primary amine. It has been shown before that in organic solvents the rearrangement proceeds with the retention of the configuration of the asymmetric carbon where the migration reaction occurs (4). Recently, a modified procedure for the Lossen rearrangement with acetohydroxamate as the substrate has been described. In this reaction the usage of water-soluble carbodiimides allows the rearrangement to proceed in aqueous media (11, 17). Therefore, an attempt was made to rearrange the chiral 2-phenylpropionhydroxamate formed in the amidase reaction to the corresponding chiral phenylethylamine. It was shown by chiral HPLC analysis that during this rearrangement only *S*-(−)-1-phenylethylamine was produced. The yield of *S*-(−)-1-phenylethylamine from the chemical rearrangement of the almost enantiopure 2-phenylpropionhydroxamate on a molar basis was 87%.

Because the Lossen rearrangement proceeds with the retention of the configuration, this reaction proved that the S-enantiomer of the hydroxamate was intermediately formed (Fig. 2). Thus, both reactions which are catalyzed by the amidase (hydrolysis and acyltransferase) produced predominantly the S-enantiomers of the respective products.

It has been previously shown that the enantioselective amidase from *R. erythropolis* MP50 converts a wide range of substrates and is highly enantioselective for various α-substituted

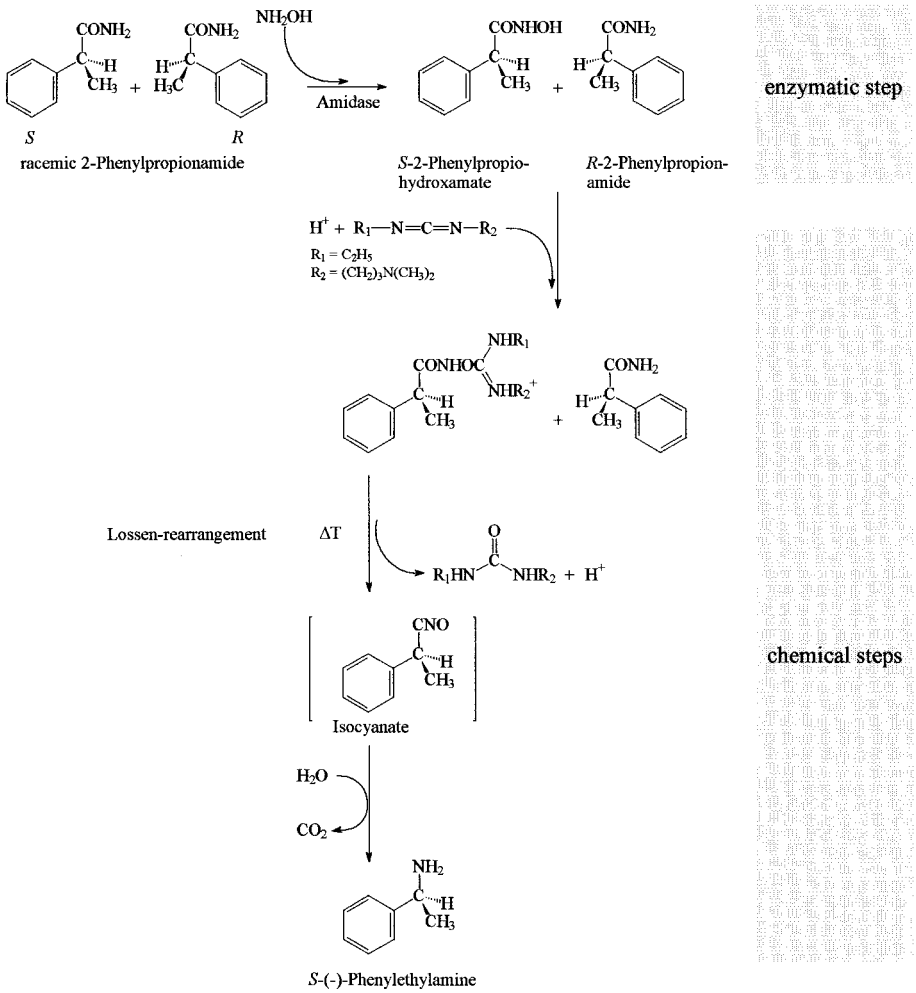

FIG. 2. Reaction scheme for the enantioselective formation of S-2-phenylhydroxamate by the amidase from *R. erythropolis* MP50 and the Lossen rearrangement of 2-phenylpropionhydroxamate to 1-phenylethylamine.

amides (9). Therefore, the reaction described here enables a new method for the synthesis of various chiral amines from racemic amide precursors (10). This synthesis differs considerably from the known enzymatic processes for the resolution of amines via enantiospecific *N*-acylation by hydrolases in organic solvents or aminotransferases (18, 19), which always require the intermediate synthesis of the racemic amine (or its *N*-acyl derivative).

REFERENCES

1. **Asano, Y., M. Tachibana, Y. Tani, and H. Yamada.** 1982. Purification and characterization of amidase which participates in nitrile degradation. Agric. Biol. Chem. **46:**1175–1181.
2. **Bauer, R., B. Hirrlinger, N. Layh, A. Stolz, and H.-J. Knackmuss.** 1994. Enantioselective hydrolysis of racemic 2-phenylpropionitrile and other (*R,S*)-2-arylpropionitriles by a new bacterial isolate, *Agrobacterium tumefaciens* strain d3. Appl. Microbiol. Biotechnol. **42:**1–7.
3. **Brammar, W. J., and P. H. Clarke.** 1964. Induction and repression of *Pseudomonas aeruginosa* amidase. J. Gen. Microbiol. **37:**307–319.
4. **Campbell, A., and J. Kenyon.** 1946. Retention of asymmetry during the Beckmann, Lossen, and Curtius changes. J. Chem. Soc. **1946:**25–27.
5. **Ciskanik, L. M., J. M. Wilczek, and R. D. Fallon.** 1995. Purification and characterization of an enantioselective amidase from *Pseudomonas chlororaphis* B23. Appl. Environ. Microbiol. **61:**998–1003.
6. **Clarke, P. H.** 1970. The aliphatic amidases of *Pseudomonas aeruginosa*. Adv. Microbiol. Physiol. **4:**179–221.
7. **DeWitt Hurd, C., and H. J. Brownstein.** 1925. The preparation of free hydroxylamine. J. Am. Chem. Soc. **47:**67–69.
8. **Hall, J. H., and M. Gisler.** 1976. A simple method for converting nitriles to amides. Hydrolysis with potassium hydroxide in *tert*-butyl alcohol. J. Org. Chem. **41:**3769–3770.
9. **Hirrlinger, B., A. Stolz, and H.-J. Knackmuss.** 1996. Purification and properties of an amidase from *Rhodococcus erythropolis* MP50 which enantioselectively hydrolyzes 2-arylpropionamides. J. Bacteriol. **178:**3501–3507.
10. **Hirrlinger, B., A. Stolz, and H.-J. Knackmuss.** August 1996. German patent application 196 34 446.8.
11. **Hoare, D. G., A. Olson, and D. E. Koshland, Jr.** 1968. The reaction of hydroxamic acids with water-soluble carbodiimids. A Lossen rearrangement. J. Am. Chem. Soc. **90:**1638–1643.
12. **Holleman, A. F., and E. Wiberg.** 1985. Lehrbuch der anorganischen Chemie. Walter de Gruyter, Berlin, Germany.
13. **Jones, L. W., and L. Neuffer.** 1917. Hydroxamic acids related to α-hydroxy acids and to acrylic acid, and a study of their rearrangements. J. Am. Chem. Soc. **39:**659–668.
14. **Kobayashi, M., H. Komeda, T. Nagasawa, M. Nishiyama, S. Horinouchi, T. Beppu, H. Yamada, and S. Shimizu.** 1993. Amidase coupled with low-molecular-mass nitrile hydratase from *Rhodococcus rhodochrous* J1. Eur. J. Biochem. **217:**327–336.
15. **Layh, N., A. Stolz, S. Förster, F. Effenberger, and H.-J. Knackmuss.** 1992. Enantioselective hydrolysis of O-acetylmandelonitrile to O-acetylmandelic acid by bacterial nitrilases. Arch. Microbiol. **158:**405–411.
16. **Layh, N., A. Stolz, J. Böhme, F. Effenberger, and H.-J. Knackmuss.** 1994. Enantioselective hydrolysis of naproxen nitrile and naproxen amide to *S*-naproxen by new bacterial isolates. J. Biotechnol. **33:**175–182.
17. **March, J.** 1984. Advanced organic chemistry: reactions, mechanisms and structure, p. 985. McGraw-Hill, New York, N.Y.
18. **Poppe, L., and L. Novák.** 1992. Selective biocatalysis, p. 131–132. VCH

Verlagsgesellschaft, Weinheim, Germany.
19. **Stirling, D. I.** 1992. The use of aminotransferases for the production of chiral amino acids and amines, p. 209–222. *In* A. N. Collins, G. N. Sheldrake, and J. Crosby (ed.), Chirality in industry. John Wiley & Sons, Chichester, United Kingdom.
20. **Thiéry, A., M. Maestracci, A. Arnaud, P. Galzy, and M. Nicolas.** 1986. Purification and properties of an acylamide amidohydrolase (E.C. 3.5.1.4) with a wide activity spectrum from *Brevibacterium* sp. R312. J. Basic Microbiol. **26:**299–311.
21. **Thiéry, A., M. Maestracci, A. Arnaud, and P. Galzy.** 1986. Acyltransferase activity of the wide spectrum amidase of *Brevibacterium* sp. R312. J. Gen. Microbiol. **132:**2205–2208.