The catalytic mechanism of amidase also involves nitrile hydrolysis

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Abstract The amidase from *Rhodococcus rhodochrous* J1, which hydrolyzes an amide to an acid and ammonium, was surprisingly found to catalyze the hydrolytic cleavage of the C-N triple bond in a nitrile to form an acid and ammonium stoichiometrically. The amidase exhibited a K_m of 3.26 mM for benzonitrile in contrast to that of 0.15 mM for benzamide as the original substrate, but the V_{max} for benzonitrile was about 1/6000 of that for benzamide. A mutant amidase containing alanine instead of Ser^{195}, which is essential for amidase catalytic activity, showed no nitrilase activity, demonstrating that this residue plays a crucial role in the hydrolysis of nitriles as well as amides.

Key words: Amidase; Amide; Nitrile; Hydrolysis

1. Introduction

An amide bond is of considerable importance in biochemistry since many C-terminal amide-containing peptides act as hormones [1]. Amidase, which catalyzes the hydrolysis of amide to acid and ammonium, has recently attracted a great deal of attention in diverse fields [2] such as neurobiochemistry [3,4], plant physiology [5–7] and applied microbiology [8–10].

Our amidase [11] is involved in nitrile metabolism of an industrially adapted *Rhodococcus rhodochrous* J1 strain [8–10]. This *Rhodococcus* amidase [2], whose gene exists in close linkage with two other structural genes encoding cobalt transporter [12] and cobalt-containing nitrile hydratase [13], shows sequence similarity to indoleacetamide hydrolase [5,6], oleamide hydrolase [3], a vitamin D3 hydroxylase-associated protein [14,15], a nylon oligomer-degrading EI enzyme [16], allophanate hydrolase [17], acetamidase [18], and other amidases (coupled with nitrile hydratases) [19–22], but not aliphatic amidase from *Pseudomonas aeruginosa* [23,24]. We initially identified the catalytically active serine residue conserved in all amidases reported to date, which would play a role as the nucleophile attacking the amide carbon atom [2].

On the other hand, we have also studied nitrile metabolism from both applied and academic viewpoints [8–10,25]. The microbial degradation of nitriles proceeds through two distinct enzymatic pathways: nitrilase catalyzes the direct hydrolysis of nitrile to acid plus ammonium [25,26], whereas nitrile hydratase catalyzes the hydration of nitrile to amide, followed by its conversion to acid plus ammonium by amidase [8–10]. We are also interested in how C-N hydrolases (e.g. amidase and nitrile-degrading enzymes) evolved. Both amide and nitrile contain a C-N bond, although the numbers of covalent bonds between the carbon and nitrogen atoms are different: $CO\text{-}NH_2$ in the former and $C \equiv N$ in the latter. We noted the previously known similar properties (e.g. inhibition by sulfhydryl compounds and hydrolytic cleavage of a C-N bond in each substrate) of both amidase and nitrilase despite the sequence differences between both enzymes. Since no amidases identified to date have been reported to act on nitriles, we investigated the nitrilase activity of amidase. Here, we describe unusual substrate specificity characteristics of amidase in that this enzyme catalyzes the hydrolysis of nitriles to acids and ammonia at the same catalytic site as used in the amide hydrolysis reaction. The unique catalytic mechanism of this process will also be discussed.

2. Materials and methods

2.1. Enzyme preparation

Amidase was purified from cells (wet cell mass 22.5 g) of *Escherichia coli* JM109 harboring pALJ30, which carried the *R. rhodochrous* J1 amidase gene, as described previously [2]. The S195A mutant amidase, in which Ser^{195} (crucial for amidase activity) was replaced with alanine, was also purified, as described previously [2].

2.2. Enzyme assay

The amidase activity of the purified amidase was assayed by the method described previously [2]. Amidase activity was defined as the amount of enzyme that catalyzed the formation of benzoic acid from benzamide as the substrate under the above conditions.

Velocities of amidase-catalyzed nitrilase activity were expressed as μmol benzoic acid formed/min/mg protein from benzonitrile.

2.3. Analytical methods for amidase

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [27]. Proteins were stained either with Coomassie brilliant blue R-250 or with silver according to Oakley et al. [28].

The anti-(nitrilase) antiserum was raised in young white male New Zealand rabbits immunized with the nitrilase purified from *R. rhodochrous* J1 [29] as follows: the purified nitrilase (5.1 mg) was emulsified with an equal volume of Freund's complete adjuvant (Difco, Michigan, USA) and injected into the neck of the rabbits. Booster injections of 1 mg of antigen in Freund's incomplete adjuvant (Difco) were given monthly. On the 8th day after the booster injection, blood was collected and the serum was prepared. Prior to first antigen injections, rabbits were bled to obtain normal control serum; the control serum did not react with the nitrilase antigen. Immunoblotting of SDS-PAGE gels was performed according to Kyse-Andersen [30].

3. Results and discussion

The *R. rhodochrous* J1 amidase was highly purified from the *E. coli* transformant containing only the enzyme gene, as described in Section 2. Neither gels stained with Coomassie brilliant blue nor gels stained with silver after SDS-PAGE showed protein bands other than the amidase band. No immunoreactive bands were detected (data not shown) in the Western blots for the above SDS-PAGE with the anti-(nitrilase) antiserum which was found to immunoreact with the *R. rhodochrous* J1 nitrilase. Physicochemical properties (such

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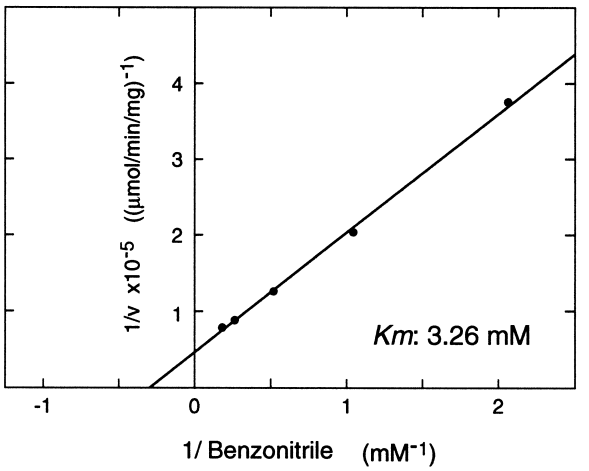
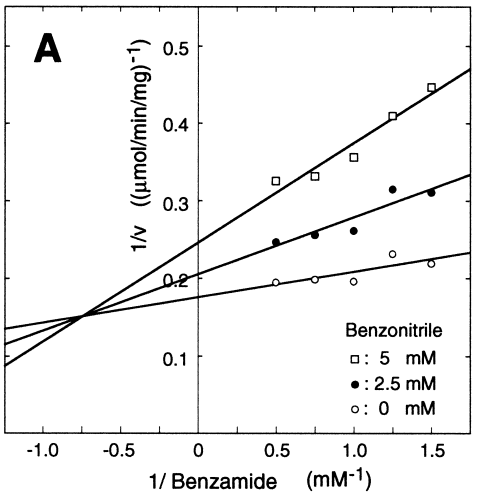


Fig. 1. Effects of substrate concentration on nitrilase activity by the amidase. The reaction was carried out at 30°C in the reaction mixture (3 ml) containing the amidase (81.5 nmol) and benzonitrile (0.48, 0.76, 1.92, 3.84 and 5.76 mM) in the buffer (pH 7.5).



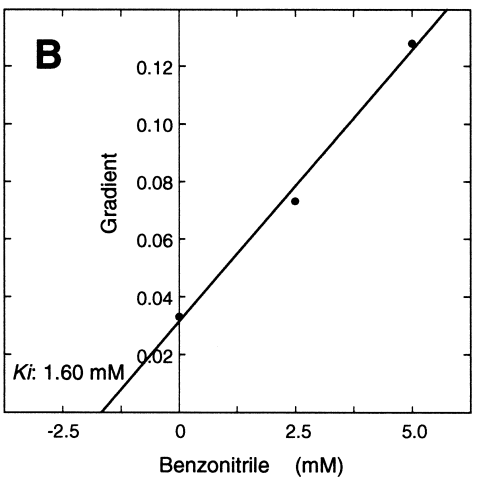


Fig. 2. Inhibition of the amidase reaction by benzonitrile. A: Inhibition of the amidase reaction by benzonitrile; the *R. rhodochrous* J1 amidase was incubated with 0.667, 0.8, 1, 1.33 or 2 mM benzamide in the presence of benzonitrile (0, 2.5 and 5 mM), and then its activity was measured. B: A replot of the slope versus the millimolar concentration of benzonitrile.

as molecular mass, optimum temperature and pH) of the amidase purified here from the *E. coli* transformant were in agreement with those of the purified amidase reported previously [2]. Therefore, the amidase prepared here had very high purity and did not contain any other contaminant proteins exhibiting nitrilase activity.

Amidase-catalyzed nitrilase activity was demonstrated with benzonitrile as the substrate. The reaction was carried out at 30°C using the purified *R. rhodochrous* J1 amidase (81.5 nmol) in the standard reaction mixture (3 ml) for amidase as described in Section 2, with the exception of the replacement of benzamide with benzonitrile as a substrate. Aliquots (0.1 ml) of the reaction mixture were taken at various time points (i.e. 0.5, 1, 2 and 3 h), and the reaction was terminated by adding 10 μl of 1 M HCl. Throughout the experiment, benzonitrile was almost stoichiometrically hydrolyzed, with the concomitant formation of benzoic acid and ammonium. Significant amounts of benzamide were not detected even with large amounts of the amidase. The activity of the amidase toward benzonitrile was 1.33×10^{-3} μmol/min/mg protein, while the activity toward benzamide was 7.95 μmol/min/mg protein [11]: nitrilase activity corresponded to about 1/6000 of the original amidase activity. A double reciprocal plot of velocity and benzonitrile concentration gave a straight line (Fig. 1). K_m and V_{max} were calculated from the intercept to be 3.26 mM and 2.09×10^{-3} μmol/min/mg protein, respectively, while those for benzamide as the substrate were 0.15 mM [11] and 8.15 mmol/min/mg protein, respectively. These kinetic values were determined under the following conditions: substrate range, 0.48–5.76 mM (for the assay of nitrilase activity) or 0.05–6 mM (for the assay of amidase activity); pH 7.5; 30°C; enzyme amount, 81.5 nmol (for the assay of nitrilase activity) or 0.05 nmol (for the assay of amidase activity) in 3 ml of reaction mixture; reaction time, 2 h (for the assay of nitrilase activity) or 10 min (for the assay of the amidase activity).

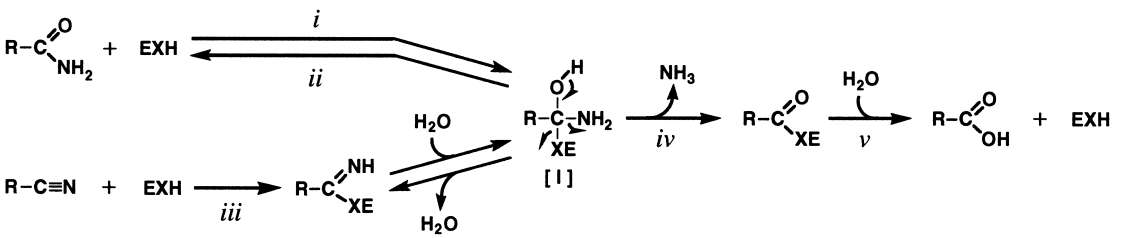


Fig. 3. Proposed catalytic mechanism for the amidase reaction. The tetrahedral intermediate is indicated by [I].

When the enzyme was first boiled for 5 min and then used in the above reaction mixture, benzoic acid was not formed from benzonitrile, demonstrating that the above nitrile degradation was not spontaneous. The purity of benzonitrile (Aldrich Chemical Company, USA), which was used as the substrate in the following experiments, was also very high (i.e. $>99.9\%$). Neither benzoic acid nor benzamide was detected in the control experiment in which the enzyme was not added to the reaction mixture containing benzonitrile as the substrate. Together with the above high purity of the amidase used in the experiments, these results demonstrated that nitrilase activity in the amidase was not due to impurities in either the enzyme or the substrate. This was also supported by the observation that the *R. rhodochrous* J1 $Ser^{195} \rightarrow Ala$ mutant amidase, in which Ser^{195} (crucial for amidase activity) was replaced with alanine, showed no nitrilase activity, as discussed below.

We next investigated whether benzonitrile could also be an inhibitor of amidase activity (Fig. 2). The *R. rhodochrous* J1 amidase (0.081 nmol) was incubated at 30°C with 0.667, 0.8, 1, 1.33 and 2 mM benzamide in the presence of benzonitrile (0, 2.5, and 5 mM) for 15 min (pH 7.5; 3 ml reaction mixture), and then its activity was measured. The $K_{i(app)}$ value was determined to be 1.60 mM on the assumption that benzonitrile was a dead-end inhibitor, although it was surely a substrate for the amidase. This $K_{i(app)}$ was of the same order of magnitude as the K_m value (3.26 mM) for benzonitrile [11]. Benzonitrile apparently showed its inhibition effect in a competitive/non-competitive mixed-type manner. The inactivation process was found to be reversible; even after the amidase had been treated with benzonitrile (1, 6 and 25 mM) (resulting in remaining amidase activity of 90%, 58% and 41%, respectively, in comparison with the non-treated enzyme), the resultant enzyme dialyzed against 0.01 M potassium phosphate buffer (pH 7.5) exhibited full amidase activity. The mixed type of inhibition could have generally resulted from either reversible binding of the apparent inhibitor to a region at or near the active site, which precludes substrate binding, or binding of the inhibitor to the enzyme to form a dead-end species (*EI*) at a region other than the active site, accompanied by a conformational change of the enzyme preventing formation of the ternary complex, *ESI*. Considering the functions of benzonitrile as both a substrate and an inhibitor for the amidase, and its mixed type of inhibition, benzonitrile may also be a kind of suicide substrate in the reaction.

Although the amidase family has long been studied, the reaction mechanism has not yet been clarified. The members of this family have been shown to operate through an acyl-enzyme mechanism with a tetrahedral intermediate [3,31] when showing acyl transferase activity [11,31]. We have previously found that both Ser^{195} and Asp^{191} of the *R. rhodochrous* J1 amidase are crucial for amidase catalysis but Cys^{203} (which is highly conserved among members of this family and was a possible candidate for the active site) is not [2], although the amidases had been generally classified as belonging to a branch of the sulfhydryl enzymes. The *R. rhodochrous* J1 amidase shows acyl transferase activity [11], and its reaction seems to proceed through an acyl-enzyme intermediate when Ser^{195} functions as the nucleophile for the amide bond within the substrate, amide [2].

On the other hand, the reaction mechanism of nitrilase, which contains an active cysteine residue, has been proposed [25,32–35] to be the nucleophilic attack on a nitrile carbon atom by the sulfhydryl group of the nitrilase (in this case, X of EXH is S in Fig. 3) leading to the formation of a tetrahedral intermediate ([I] in Fig. 3) via an enzyme-thioimidate (= enzyme-iminothiol ether) intermediate (route *iii* in Fig. 3). Ammonium is then removed from the tetrahedral intermediate to yield an acyl enzyme (route *iv*), followed by hydrolysis of the latter to an acid (route *v*). Particularly the second step of the nitrilase reaction, in which attack by water on the covalently attached thioimidate requires activation of the water to transform it into an adequate nucleophile, is unique to this enzyme, unlike in the case of the cysteine protease, papain [36].

Considering the acyl-enzyme mechanism of the amidase and its nitrilase activity, we propose here a reaction mechanism of the amidase, which is likely to be analogous to that of the nitrilase (Fig. 3), although each active nucleophile of both enzymes is different. When an amide is used as the substrate in the amidase reaction, the carbonyl group of the amide would undergo attack by the nucleophilic amino acid of the amidase (EXH in Fig. 3) (route *i*), resulting in the formation of a tetrahedral intermediate ([I]). With removal of ammonium, the intermediate is converted into an acyl-enzyme (route *iv*), which is then hydrolyzed to an acid. As for route *v* (i.e. from acyl-enzyme to the acid) after route *iv*, this cannot be reversible; neither benzonitrile nor benzamide was formed from benzoic acid, when the latter's ammonium salt was used as the substrate even in the presence of a large amount of the amidase (81.5 nmol enzyme in 3 ml of the reaction mixture).

We detected no nitrilase activity of the *R. rhodochrous* J1 $Ser^{195} \rightarrow Ala$ mutant amidase, in which Ser^{195} (crucial for amidase activity) was replaced with alanine. Therefore, even when a nitrile is used as the substrate in the amidase reaction, the same amino acid residue (Ser^{195}) as used in the above reaction (with amide as the substrate) would be the nucleophile for the C-N triple bond of the substrate (route *iii*).

As the *R. rhodochrous* J1 amidase-dependent nitrile-degrading activity resulted only in the formation of benzoic acid, but not in that of benzamide, this reaction is completely different from nitrile hydratase reaction [8–10]. We also did not detect formation of benzonitrile from benzamide as the substrate. These findings demonstrated that the catalytic reactions of the amidase are one way from either benzamide or benzonitrile finally to benzoic acid.

Although there is no homology between the amidase and nitrilase families, comparison of their reaction mechanisms could provide novel insight for the construction of novel catalysts for the hydrolysis of a C-N triple bond and an amide bond. Further studies on the three-dimensional structures of both families would shed new light on the evolution of their specificities, and also stimulate 'carbon-nitrogen cleavage biochemistry' (in addition to 'peptide-bond cleavage biochemistry' on which a great deal of attention has been focused).

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