A Nitrile Hydratase with a Wide Substrate Spectrum Produced by a *Brevibacterium* sp.

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A mutant strain, *Brevibacterium* 19, defective for the enzyme nitrile hydratase but retaining all the amidase activity of the wild-type *Brevibacterium* R312, was isolated. This is genetic evidence in favour of the hypothesis that all the nitrile-hydratase activity of the wild-type was due to a single enzyme, the structural gene of which was lost in the mutant strain 19.

The specific activities and K_m of the nitrile hydratase were determined for 12 different substrates. The affinity of the enzyme increased as the number of hydrogen-bonding positions of the substrate increased, and decreased with more spatial crowding of the hydrocarbon chain. The specific activity of the enzyme for a substrate was enhanced by the nucleophilic and hydrophilic properties of the hydrocarbon side chain of that substrate.

INTRODUCTION

The wild-type strain *Brevibacterium* R312 (Arnaud *et al.*, 1976*a, b*) was able to hydrolyse all water-soluble nitriles via the activities of its nitrile hydratase (Arnaud *et al.*, 1977) and its amidase (Jallageas *et al.*, 1978*a*). A mutant strain, *Brevibacterium* A4, unable to hydrolyse acetamide, was described by Jallageas *et al.* (1979) and Arnaud *et al.* (1980). Unlike the wild-type R312 (Arnaud *et al.*, 1976*c*), this mutant could no longer hydrolyse many amides such as the aliphatic amides, α -aminopropionamide, lactamide, benzamide and the D- α -aminopropionamides. This strain retained, however, the ability to hydrolyse L- α -aminoamides (Kieny-L'Homme *et al.*, 1981). The enzyme no longer synthesized by the mutant A4 strain had previously been described and named acetamidase (Jallageas *et al.*, 1978*a*).

In the present work, we report the isolation of a mutant strain, *Brevibacterium* 19, which had lost its ability to hydrate nitriles, including cyanide, while retaining its amidase activity. Considering the industrial potential for the use of the nitrile hydratase enzyme (Bui *et al.*, 1982), we also studied the effect of the structure of the substrate nitriles on the activity of hydration of nitriles by the wild-type *Brevibacterium* R312. The K_m and V_{max} of the nitrile hydratase were determined for 12 different substrates.

METHODS

Bacterial strain. The wild-type strain used was *Brevibacterium* R312 (Arnaud *et al.*, 1976*a, b*).

Media. The composition of the basal minimal medium was as previously described (Kieny-L'Homme *et al.*, 1981). Ammonium sulphate (5 g l⁻¹) and glucose (10 g l⁻¹) were added to the minimal medium. Slant cultures were maintained on YMPG-agar, the composition of which was given by Jallageas *et al.* (1978*a*).

Growth. Cultures were grown at 28 °C in conical flasks filled to 10% (v/v) of their capacity and shaken at 80 r.p.m.

Enzyme extraction. Disruption of bacteria by grinding, and preparation of extracts, were performed as previously described (Jallageas *et al.*, 1978*a*). Determinations of K_m values were performed using the S₂ supernatant obtained by centrifugation at 180 000 g for 90 min.

Protein assay. Proteins were assayed by the biuret method according to Stickland (1951), with bovine serum albumin as standard.

Enzyme assay. Enzyme activity assays for most nitrile or amide substrates were performed by GLC according to the technique of Jallageas *et al.* (1978*b*). Cyanide, α -aminonitriles and α -hydroxynitriles were determined by the colorimetric technique of Szewczuk (1959) as modified by Buti (1975), Bridi (1980) and L'Homme (1980).

Origin of nitriles and amides used. The α -aminonitriles were synthesized according to the classical methods of Marvel & Brubacker (1948) and Béjaud *et al.* (1975). All other nitriles and amides used were commercially available.

RESULTS AND DISCUSSION

Isolation and study of a nitrile hydratase defective mutant

The isolation of a nitrile hydratase defective mutant was based on the following principle: in the presence of the wild-type, a halogenated nitrile would be hydrated into a halogenated amide, which would then be hydrolysed into a halogenated acid. Such compounds are toxic to some micro-organisms (Apirion, 1965; Hynes & Pateman, 1970; Clarke & Tata, 1973). All colonies capable of growing on a solid medium in the presence of such a halogenated nitrile would be potentially nitrile hydratase defective mutants.

Chloroacetonitrile was chosen for our purposes. It is a substrate for the *Brevibacterium* nitrile hydratase. The toxicity of chloroacetonitrile for the wild-type *Brevibacterium* R312 was tested using a solid agar medium with acetamide (5 g l^{-1}) as a source of carbon and nitrogen. There was no growth of the wild-type with chloroacetonitrile at 0.6 g l^{-1} and above.

The isolation scheme is shown in Table 1. The wild-type was grown on minimal medium containing glucose (10 g l^{-1}) and acetamide (5 g l^{-1}). The cells were then spread on to minimal agar medium containing glucose (10 g l^{-1}), acetamide (5 g l^{-1}), and chloroacetonitrile (0.6 g l^{-1}). All colonies which developed on this medium were selected and maintained on YMPG agar slants. The selected strains were then inoculated into minimal medium containing acetonitrile as sole nitrogen source. No growth was observed for strain 19, obtained without mutagenic treatment, and for six other strains, obtained following treatment with EMS.

The *Brevibacterium* strain 19 showed the same morphological and biological properties as the wild-type. Its nitrile hydratase activity spectrum was compared with that of the wild-type (Arnaud *et al.*, 1976*a, b*) for the following compounds: KCN, acetonitrile, propionitrile, α -hydroxypropionitrile, β -hydroxypropionitrile, α -aminopropionitrile, β -aminopropionitrile, acrylonitrile, butyronitrile, isobutyronitrile, valeronitrile, pivalonitrile and benzonitrile. Whole cells and a cell-free preparation of the mutant strain were unable to hydrate the above nitriles, while the amidase activity of the mutant remained identical to that of the wild-type. The mutation was stable for at least 50 generations of vegetative growth.

Determination of K_m and V_{max} values of nitrile hydratase on different substrates

This general nitrile hydratase enzyme was previously described as acetonitrilase (Arnaud *et al.*, 1977). Its optimum pH is 7.0 and its optimum temperature is 30 to 35 °C for acetonitrile. The enzyme is thermolabile, losing all activity on heating at 45 °C for 5 min. The K_m determined for acetonitrile is $2.5 \times 10^{-2} \text{ M}$. The active site of the enzyme contains $-\text{OH}$, $-\text{NH}_2$ and $-\text{SH}$ groups. As this enzyme could be used in bioconversion to hydrate many nitriles into amides, its hydration parameters for 12 different substrates have been determined.

We found during preliminary experiments that the enzyme activity of our cell-free preparation was very high. For some substrates, this preparation had to be diluted up to 100 times for precise kinetic measurements. As genetic results showed that only one enzyme was involved, we did not find it necessary to purify the preparation for the determination of K_m and relative specific activities for different substrates. Only the V_{max} results would be affected by the relative amount of the hydratase in the extract. Thus all K_m and specific activities were determined using the supernatant following centrifugation at 180000 *g* for 90 min.

The K_m and V_{max} of the nitrile hydratase for different substrates, determined from Lineweaver-Burk plots, are summarized in Table 2. Also included in this table are the results obtained by Bridi (1980) for cyanide and Arnaud *et al.* (1977) for acetonitrile. The hydration

Table 1. Isolation scheme for a nitrile hydratase defective mutant

Medium	Wild-type	Mutant
Minimal medium + Glucose + acetamide	Growth	Growth
Minimal medium + Glucose + acetamide + Chloroacetonitrile →	No growth Chloroacetamide ↓ Chloroacetic acid (toxic)	Growth
Minimal medium + Glucose + acetonitrile	Growth	No growth (no nitrogen source available)

Table 2. K_m and V_{max} of nitrile hydratase for different substrates

The K_m and V_{max} values listed in columns 2 and 3 were determined from more than one batch of enzyme preparation. Because of variations in the amount of the hydratase in the different batches, the V_{max} values sometimes differed. Therefore, the relative specific activities (column 4) were calculated from V_{max} values determined using the same enzyme preparation for each of the different substrates; the V_{max} for propionitrile (100%) was $30 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

	Substrate	K_m (mM)	V_{max} [$\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$]	Relative specific activity
C ₁	KCN*	2.4	0.36	2
C ₂	Acetonitrile†	25	3.0	10
	Propionitrile	6.4	31	100
		5.2	30	
	α -Hydroxypropionitrile	10	25	116
	(DL-lactonitrile)	12	40	
	β -Hydroxypropionitrile	64	11	35
		49	9.5	
C ₃	α -Aminopropionitrile	1.0	27.4	90
		1.5		
	β -Aminopropionitrile	28	1.9	2.7
		14	1.1	
	Acrylonitrile	15	10	58
		14	16	
	Butyronitrile	1.1	5.2	33
		0.8	8.4	
C ₄	Isobutyronitrile	3.9	34	113
		2.2	28	
	Valeronitrile	1.0	1.1	2.7
		1.4	1.3	
C ₅	Pivalonitrile	6.6	5.6	5.3
		6.5	5.2
Benzonitrile	1.0	1.0	1.3
		0.6	0.9	

* Bridi (1980).

† Arnaud *et al.* (1977).

reactions rapidly ceased with lactonitrile, α -aminopropionitrile or isobutyronitrile as substrate.

The K_m results for the linear aliphatic nitrile series (i.e. cyanide, acetonitrile, propionitrile, butyronitrile, valeronitrile) showed that this enzyme had the lowest affinity for acetonitrile (K_m 25 mM). The affinity was 10 times greater for cyanide and also increased as the hydrocarbon side chain became longer. Except for cyanide, the length of the hydrocarbon side chain seemed to enhance the affinity of the enzyme for the substrate. This is probably due to the increase of the number of hydrogen-bonding positions between the enzyme and the hydrocarbon side chain.

Considering the substrate series acetonitrile, propionitrile, isobutyronitrile, pivalonitrile, the longer the hydrocarbon chain the lower was the K_m for the substrate. The affinities of the enzyme for propionitrile, isobutyronitrile and pivalonitrile were four to eight times greater than

for acetonitrile. The methyl groups on the hydrocarbon chain thus increased the affinity of the enzyme for these substrates, probably again due to a greater number of hydrogen bonds which could be established. On the other hand, this increase in affinity was counterbalanced by the spatial crowding effect of the same methyl groups, which decreased the affinity of the enzyme. This was especially apparent for the K_m of pivalonitrile (6.3 mM) and of isobutyronitrile (3.0 mM).

Also, the linear-chained substrates butyronitrile and valeronitrile had K_m values three to six times smaller than their corresponding branched isomers, isobutyronitrile and pivalonitrile. The difference in affinity increased as the number of methyl groups increased. This confirmed the supposition that molecular crowding near the $-C\equiv N$ terminal caused a decrease in the affinity of the enzyme for the nitrile substrates. Nitriles having the form $R-CH_2-C\equiv N$ can also temporarily exist in the form $R-CH=C=NH$ (Allinger *et al.*, 1983); in this form access to the active site of the enzyme would be facilitated.

The K_m values for α - and β -hydroxypropionitrile and α - and β -aminopropionitrile showed that the affinity of the enzyme was 5 to 15 times greater when the amino or the hydroxy group was in the α position as compared to the β position. The propionitrile derivatives with an amino group had K_m values five to eight times smaller than those with a hydroxy group substituted at the same position, α or β . These results agree with the reports by Arnaud *et al.* (1977) and L'Homme (1980) that there are $-OH$ and $-NH_2$ groups at the active site of this enzyme, and with the hypothesis that the affinity of the enzyme increases as more hydrogen bonds are established between the enzyme and its substrates.

It was noteworthy that the K_m for benzonitrile was similar to those for butyronitrile and valeronitrile, despite its aromatic ring. This was probably due to the flatness of this molecule, which permitted a closer contact with the enzyme as compared to the spatial interference of the methyl groups of the branched aliphatic nitriles.

Determination of relative specific activities

In order to obtain specific activities comparable to each other, the same enzyme preparation was used to determine the V_{max} of different substrates and of propionitrile in the same experiment. The results are given in Table 2, where the relative specific activities are expressed as the percentage of that obtained for propionitrile, $30 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

Except for lactonitrile (α -hydroxypropionitrile) and isobutyronitrile, which will be discussed further, the greatest specific activity was found for propionitrile. The specific activities of the branched substrates isobutyronitrile and pivalonitrile were greater than those of their respective isomers, butyronitrile and valeronitrile. The hydrocarbon side chains of these branched substrates were more nucleophilic than their isomers.

In the nitrile substrate class with three carbon atoms in each molecule, the presence of a hydroxy group enhanced the hydration rate compared to that observed with an amino group substituted at the same position. Also, substrates with an active group substituted at the β position had smaller specific activities than those with the same active group substituted at the α position. The specific activity obtained for acrylonitrile, with its double bond, was very close to that for β -hydroxypropionitrile. These results suggest that a hydrophilic hydrocarbon side chain, especially at the α position, enhances hydration catalysed by this enzyme.

The specific activity determined for benzonitrile was smaller than those found for the aliphatic nitriles used in this work. However, the activity was strong enough to be easily measured, confirming the reports of Arnaud *et al.* (1976*a, b*) that this enzyme has a wide activity spectrum, including aromatic as well as aliphatic nitriles.

The above results agree with the reaction mechanism proposed by Asano *et al.* (1982) for a nitrile hydratase:

(1) The thiol enzyme (ESH) initiates a nucleophilic attack on the nitrile substrate carbon and yields an imino-enzyme complex. An electrophilic hydrocarbon side chain, R-, would tend to draw electrons away from the triple bond of the nitrile substrate and slows down this reaction step.

(2) The imino-enzyme complex is hydrated, yielding the intermediary (I). This step is favoured by the presence of a hydrophilic hydrocarbon side chain, R-, which would facilitate the approach of a water molecule required for the reaction.

(3) The intermediary (I) decomposes yielding an amide product and the original thiol enzyme.

The specific activity measured for each substrate resulted from the combined contrary effects of their electrophilic and hydrophilic properties on the reaction rate.

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