Folia Microbiol. **21**, 178–184 (1976)

Amidase Activity of Some Bacteria

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Received October 30, 1975

ABSTRACT. The amidase activity of bacteria possessing a high nitrilase activity was found to display the same spectrum although the bacteria may belong to different taxonomic groups, *Bacillus, Bacteridium, Micrococcus, Brevibacterium.* The spectrum of amidase activity, although very broad, is more restricted than that of nitrilase activity. Internal amides as well as vinyl-bound amides are not hydrolyzed.

A great many authors have studied the metabolism of amides. Amidases have been described in various organisms, ranging from bacteria (Clarke, 1970) to yeasts (Gorr and Wagner, 1933; Joshi and Handler, 1962) to moulds (Hynes and Pateman, 1970; Hynes, 1970, 1975). The bacteria possessing amidase activity belong to the most diverse taxonomic groups: *Corynebacterium* (Grant, 1973; Grant and Wilson, 1973); *Mycobacterium* (Halpern and Grossowicz, 1957; Kimura, 1959*a,b,c,d*; Draper, 1967; Tacquet *et al.*, 1967; Viallier and Viallier, 1971; Georges and Dailloux, 1973); *Lactobacillus* (Hughes and Williamson, 1953); *Pseudomonas* (Kelly and Kornberg, 1964; Jakoby and Fredericks, 1964; Clarke, 1970, 1974; Betz *et al.*, 1974); *Bacillus* (Engelhardt, 1973).

It was intended here to study the amidase activity of previously isolated samples displaying nitrilase activity.

MATERIALS AND METHODS

The following strains were used: *Bacillus* R 332, *Bacteridium* R 340, R 341; *Micrococcus* A 111, *Brevibacterium imperiale* B 222, *Brevibacterium* A 13, B 212, C 211, R 312. The characteristics of these nine strains are described elsewhere (Arnaud *et al.*, prepared for pubcication.

Cultivation conditions. The bacteria were grown in a complete YMPG medium of the following composition: 3 g yeast extract, 3 g malt extract, 5 g bacto-peptone, 1 g glucose, per litre water. The cultures were placed in Erlenmeyer flasks in one-tenth their volume, and agitated for 16 h at 28°C (80 strokes per min at an 8 cm amplitude). The inoculation was done with a preculture in the same medium. The YMPG medium with 2.5% agar was used for maintenance of the strains.

Amides used. Besides commercially available amides, some were prepared by hydrochloric acid hydrolysis of the appropriate nitriles in formic acid (Becke *et al.*, 1971). α-Amino-ε-caprolactam was synthesized from α-chloro-ε-caprolactam through the action of ammonia (Wineman *et al.*, 1958; Francis *et al.*, 1958). The hydrolysis was carried out with 4% aqueous solutions at pH 7, using bacterial suspensions

TABLE I. Chemical shifts for various amides and acids using the peak of tertiary butyl alcohol as internal standard. Z = $-CONH_2$ for amides, and $-COOH$ for acids.

Compound	pH	Protons examined	δ (Hz)	
			Amide	Acid
$CH_3-CH(Z)(NH_2)$	10	CH_3	doublet at 6	doublet at 4
$CH_3-CH(Z)(OH)$	6	CH_3	doublet at 3.5	doublet at 2
$CH_2(Z)(OH)$	7	CH_2	singlet at 30	singlet at 27
$CH_3-CH(Z)(NH-CH_3)$	≦1	$C-CH_3$	doublet at 5	doublet at 10
$CH_3-CH(Z)(NHCHO)$	9	CH_3	doublet at 3.5	doublet at 5
CH_3-Z	9	CH_3	singlet at 22.5	singlet at 20
$(CH_3)_2-CH-Z$	9	CH_3	doublet at 4	doublet at 6

(20—40 g dry weight per litre) and agitating them for 4—5 h. After centrifugation, the supernatant was used for analysis.

Analytical techniques. Amidase hydrolysis of amides gives rise to the corresponding organic acid and to ammonia and hence either the acid or the ammonia can be used for analysis. It was preferred here, in contrast with some other authors (Jakoby and Fredericks, 1964; Clarke, 1970; Georges and Dailloux, 1973), to follow the appearance of acid. Most of the methods used permit to follow the disappearance of the corresponding amide. Nuclear magnetic resonance, gas-liquid chromatography and thin-layer chromatography were used although they were not found to be particularly convenient. Hence in some cases more traditional techniques were employed, such as extraction with organic solvents or precipitation.

NMR spectrometry. The qualitative and quantitative analysis is based on the differences of chemical shift δ of certain protons in the amides and in the acids. The supernatant was adjusted to a suitable pH before measurement, since the shifts of some protons are affected by the pH. Table I shows the shifts observed with the peak of tertiary butyl alcohol (used as internal standard) for the products studied by this method in a Varian T 60 NMR spectrometer. Using the Varian HA 100 spectrometer, it was possible to differentiate between some of the amide and acid peaks with very close values. The δ shifts shown in Table II were referred to the methyl peak of hexamethyldisiloxane used as external standard.

Gas-liquid chromatography. In some cases where the NMR gave no practicable results, gas-liquid chromatography was employed, such as in the case of crotonamide and allylamide. After acidification of the supernatant with concentrated hydro-

TABLE II. Chemical shifts for various amides and acids using the peak of CH_3 of hexamethyldisiloxane as external standard. Z = $-CONH_2$ for amides, and $-COOH$ for acids.

Compound	pH	Protons examined	δ (ppm)	
			Acid	Amide
$CH_2=CH-Z$	10	$CH_2=$	multiplet at 6.33; 6.35; 6.37 and 6.43	multiplet at 6.53; 6.54 and 6.60
		$=CH-$	multiplet at 5.88; 5.92; 5.96 and 6	multiplet at 6.05; 6.09; 6.13 and 6.17
$CH_3(Z)C=CH_2$	7	CH_3	multiplet at 2.10 (band width at mid-height) = 4 Hz	multiplet at 2.17 = 4 Hz
benzene ring with Z, H_1, H_6	7	H_1 and H_6	multiplet at 7.89; 7.90; 7.94 and 7.95	multiplet at 7.95; 7.97; 8 and 8.01
$CH_3-S-(CH_2)_2-CH(Z)(OH)$	7	CH	quadruplet at 4.75; 4.80; 4.83 and 4.87	quadruplet at 4.63; 4.67; 4.70 and 4.75

chloric acid, an aliquot volume of solution is injected into the Varian 1200 chromatograph (flame ionization detector, a 10% DEGS and 2% H_3PO_4 column on Chromosorb PAW 80-100, length 1 m, nitrogen as carrier gas). The retention times were 195 s at 120° C for $CH_2=CHCH_2-COOH$ and 120 s at 165°C for $CH_3CH=CHCOOH$.

Gas chromatography was used for confirming the results of NMR and for analyzing the hydrolyzates that could not be analyzed by any of the previous methods. The supernatant was chromatographed on a thin layer of silica gel Merck 60 F-254. After elution, the spots were detected with ninhydrin, generally obtaining a brown spot for the amide (more easily eluted) and a purple one for the acid. Table III summarizes the R_F values for amides and acids in the elution system used and the colour of the corresponding spot.

Extraction was used either for confirming the previous results or for analyzing hydrolyzates that could not be studied otherwise.

Extraction with organic solvents was applied to the supernatant using two 50 ml portions of ether or chloroform. The aqueous phase was then acidified and again extracted with ether and chloroform. The organic phase was dried with anhydrous sodium sulphate and the solvent was evaporated *in vacuo*. The oily residue was refrigerated and crystallized, the melting points of the crystals being 136° C for malonic acid, 145° C for succinic acid, 151° C for adipic acid, 122° C for benzoic acid, 235° C for nicotinic acid, 98° C for homoveratric acid (the anhydrous form) and 290° C for piperidine 2,6-dicarboxylic acid (as hydrochloride).

Extraction by precipitation. For glycine and β-alanine, the supernatant (about 25 ml) was concentrated to 3—4 ml by evaporation *in vacuo*. After cooling to 5—10° C, ammonium chloride was eliminated, the filtrate was combined with 15 ml methanol and left to stand at 4° C overnight. The crystals were filtered and their melting points estimated (225° C for glycine, 205° C for β-alanine). For α-hydroxymethyl-

TABLE III. The R_F values of amides and acids in thin-layer chromatography on silica gel.

Elution system	Amide	R_F	Acid	R_F
n-Butanol- water-CH_3COOH (6-2-2 w/w)	NH_2 O NH	0.30 (yellow)	$NH_2-(CH_2)_4-CH-COOH$ NH_2	0.04 (purple)
96% Ethanol 33% NH_4OH (67/33 v/v)	NH_2 O NH	0.60 (yellow)	$NH_2-(CH_2)_4-CH-COOH$ NH_2	0.10 (purple)
Phenol-water) (94/31 w/w)	N C O NH_2	0.26 (blue)	N COOH	0.51 (blue)
n-Butanol- water-CH_3COOH (6-2-2 w/w)	$/CONH_2$ CH_2 $\backslash NH_2$	0.23 (brown)	$/COOH$ CH_2 $\backslash NH_2$	0.20 (purple)
n-Butanol- water-CH_3COOH (6-2-2 w/w)	$/CONH_2$ CH_3-CH $\backslash NH_2$	0.29 (brown)	$/COOH$ CH_3-CH $\backslash NH_2$	0.26 (purple)
n-Butanol- water-CH_3COOH (6-2-2 w/w)	CH_3 $/CONH_2$ CH_2SCH_2CH $\backslash NH_2$	0.41 (brown)	$/COOH$ $CH_3SCH_2CH_2CH$ $\backslash NH_2$	0.36 (purple)
n-Butanol- water-CH_3COOH (6-2-2 w/w)	$CH_3CH_2CH-CONH_2$ NH_2	0.39 (brown)	$CH_3CH_2CH-COOH$ NH_2	0.34 (purple)
n-Butanol- water-CH_3COOH (6-2-2 w/w)	$/CONH_2$ Φ CH_2CH $\backslash NH_2$	0.51 (brown)	$/COOH$ Φ CH_2CH $\backslash NH_2$	0.45 (purple)
n-Butanol- water-CH_3COOH (6-2-2 w/w)	$/CONH_2$ $(CH_3)_2CHCH$ $\backslash NH_2$	0.38 (brown)	$/COOH$ $(CH_3)_2CHCH$ $\backslash NH_2$	0.33 (purple)
n-Butanol- water-CH_3COOH (6-2-2 w/w)	$NH_2CH_2CH_2CONH_2$	0.31	$NH_2CH_2CH_2COOH$	0.26
n-Butanol- water-CH_3COOH (6-2-2 w/w)	NH =O	—	$NH_2(CH_2)_5COOH$	0.30

TABLE IV. Specificity of bacterial amidases.

Class of compounds	Compound	Hydrolysis at pH 7	Method of analysis[a]
Simple amides	Acetamide	+	NMR-T60
	Isobutyramide	+	NMR-T60
α-Aminoamides	α-Aminopropionamide	++	NMR-T60; CCM
	Glycinamide	++	TLC; E
	α-Amino-γ-methylthiobutyramide	++	TLC
	α-Aminobutyramide	++	TLC
	β-Phenyl-α-aminopropionamide	++	TLC
	α-Amino-methyl-3-butyramide	++	TLC
α-Hydroxyamides	Lactamide	++	NMR-T60
	Hydroxyacetamide	++	NMR-T60
	α-Hydroxy-γ-methylthiobutyronamide	++	NMR-T60; E
β-Aminoamides	Amino-3-propionamide	+	TLC;E
Diamides	Malonamide	+	E
	Succinamide	+	E
	Adipamide	+	E
α-Unsaturated amides	Acrylamide	—	NMRHA 100
	Methacrylamide	—	NMRHA 100
	Crotonamide	+	GLC
β-Unsaturated amides	Allylamide	+	GLC
α-Benzenic amides	Homoveratramide	+	E
	Benzamide	+	E
Heterocyclic amides	Nicotinamide	+	E
	Isonicotinamide	+	NMR-T60
	Piperidine-2-carboxamide	+	TLC
	Piperidine-2,6-dicarboxamide	+	E
Internal amides (lactams)	ε-Caprolactam	—	TLC
	α-Amino-ε-caprolactam	—	TLC
N-Substituted-aminoamides	N-Formyl-α-aminopropionamide	++	NMR-T60
	N-Methyl-α-aminopropionamide	++	NMR-T60

[a] NMR T60, nuclear magnetic resonance on Varian T 60; NMR HA 100, nuclear magnetic resonance on Varian HA 100; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; E, extraction.

thiobutyric acid the supernatant was extracted twice with 30 ml chloroform to eliminate the nonhydrolyzed amine and 1.2 g $Ca(OH)_2$ was added. The precipitate obtained was filtered and its identity was checked by the NMR spectrum.

RESULTS AND DISCUSSION

All the strains tested were found to contain amidase activity and to behave identically. It thus appears that the amidase activity is widely distributed among bacteria, not being specific for any of the groups. The list of compounds tested with the 9 strains is shown in Table IV.

All the experiments were done at pH 7 using 4% amide solutions and incubation done for 4—5 h. Compounds not producing acid after 5 h were designated with a — sign while those completely hydrolyzed in less than 2 h with ++, those hydrolyzed in more than 2 h with +. It should be noted that in this last-named group, the slow reaction can be explained in some cases by a low solubility of the amides. This holds particularly for the diamides, the α-benzyl amides and the heterocyclic ones.

Among the hydrolyzed amides, several cases should be discussed. Internal amides (lactams) are probably not hydrolyzed because there is no enzyme capable of opening the lactam ring. The strain studied do not appear to possess the enzyme system previously described by Fukumura (1966) and by Tosa and Chibata (1965).

On the other hand, several explanations may be offered for the other amides, acrylamide and methacrylamide. It is altogether possible that these two compounds do not penetrate the cell. It is equally possible that the cell contain several amidases but that the specificity for these two amides is lacking but it appeared more likely that there was a single general amidase present which attacks the free amide function but which is inhibited by vinyl-bound amides.

At any rate, the amidase systems are widely distributed among bacteria and deserve a deeper study. From the practical point of view, the spectrum of nitrilase activity is more important than that of amidase activity but the present results are of significance for explaining why some amides are not hydrolyzed by bacteria.

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