Acyltransferase Activity of the Wide Spectrum Amidase of *Brevibacterium* sp. R312

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The wide spectrum amidase from *Brevibacterium* sp. R312, which can hydrolyse many amides to the corresponding acids, was shown to transfer the acyl groups of amides, acids and esters to hydroxylamine. The transfer rates of these reactions in cytoplasmic fractions were measured and compared. The K_m and V_{max} were determined for different substrates in the presence of hydroxylamine. The enzyme was also shown to transfer the acyl group of the amide analogue *N*-methylacetamide to hydroxylamide and that of acetamide to the hydroxylamine analogue methylhydroxylamine.

INTRODUCTION

Brevibacterium sp. R312 is able to transform different nitriles to the corresponding acids, with an intermediary amide step (Jallageas *et al.*, 1978*a*). Two enzymes with broad specificity are involved in this transformation. A nitrile hydratase first hydrates the nitriles to the corresponding amides (Arnaud *et al.*, 1977; Bui *et al.*, 1984*a*). Subsequently an amidase hydrolyses the amides to the corresponding acids (Jallageas *et al.*, 1978; Maestracci *et al.*, 1984).

Besides having amide hydrolysing activity, the amidase of *Brevibacterium* sp. R312, in common with other microbial amidases (Clarke, 1970; Thalenfeld & Grossowicz, 1976; Miller & Gray, 1982; Asano *et al.*, 1982), is also an acyltransferase (Thiéry *et al.*, 1986*b*). It has the following activities: amide hydrolase, amide transferase, acid transferase and ester transferase (Table 1). It seemed interesting to us to study the activity ratios and the affinities of the enzyme for different substrates involving transferase activity.

METHODS

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

Culture conditions. The basal minimum medium (MM) used had the following composition ($g\ l^{-1}$): KH_2PO_4 , 1.2; $Na_2HPO_4 \cdot 12H_2O$, 1.95; K_2HPO_4 , 0.98; $CaCl_2 \cdot 2H_2O$, 0.012; $ZnCl_2$, 0.0012; $FeSO_4 \cdot 7H_2O$, 0.0012; $MnSO_4 \cdot 4H_2O$, 0.0012. This medium was adjusted to pH 7.0 and sterilized by autoclaving. Filter-sterilized solutions of $MgSO_4 \cdot 7H_2O$ and thiamin chlorhydrate (Thiéry *et al.*, 1986*a*) were added to give concentrations of $0.5\ g\ l^{-1}$ and $0.002\ g\ l^{-1}$, respectively. Glucose and $(NH_4)_2SO_4$ were added to give final concentrations of 10 and $5\ g\ l^{-1}$, respectively. The non-substrate inducer *N*-methylacetamide was added to induce the amidase (Maestracci *et al.*, 1984). The cultures were grown aerobically in 5 l Erlenmeyer flasks filled to one-tenth of their volume and incubated at 28 °C with shaking.

Preparation of extracts. Disruption of bacteria by grinding and preparation of extracts were done as described by Jallageas *et al.* (1978*a*). K_m values were determined in supernatants obtained by centrifugation of crude extracts at 180000 *g* for 90 min.

Enzyme assays. Amide hydrolase was assayed using a GLC apparatus to monitor the disappearance of the substrate (amide) as described by Jallageas *et al.* (1978*b*) and Bui *et al.* (1984*b*). Acyltransferase was assayed using the method developed by Brammar & Clarke (1964), based on the colorimetric determination of the resulting hydroxamic acids which yield red-brown complexes with Fe(III). The reaction medium was of the following

Table 1. *Comparison of relative reaction rates of hydrolysis and transfer*

Reaction	Relative specific activity (%)*
Amide hydrolase $\text{CH}_3\text{-CONH}_2 + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + \text{NH}_3$	3.0
Amide transferase $\text{CH}_3\text{-CONH}_2 + \text{NH}_2\text{OH} \rightarrow \text{CH}_3\text{-CONHOH} + \text{NH}_3$	100
Acid transferase $\text{CH}_3\text{-COOH} + \text{NH}_2\text{OH} \rightarrow \text{CH}_3\text{-CONHOH} + \text{H}_2\text{O}$	8.9
Ester transferase $\text{CH}_3\text{-COO-CH}_2\text{CH}_3 + \text{NH}_2\text{OH} \rightarrow \text{CH}_3\text{-CONHOH} + \text{CH}_3\text{CH}_2\text{OH}$	4.4

* The 100% value corresponds to an activity of $35 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

Table 2. *Amide, acid and ester transferase activity: determination of K_m and relative $V_{\max}$ for different substrates*

	Substrate	Relative $V_{\max}$ (%)*	K_m (mM)
Amides	Acetamide	100	31
	Propionamide	28	88
	Acrylamide	49	93
Acids	Acetate	8.9	83
	Propionate	111	628
	Acrylate	3.3	88
Esters	Ethyl acetate	4.4	18
	Ethyl propionate	13.5	16
	Ethyl acrylate	14.7	218

* The 100% value corresponds to an activity of $35 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

composition: 1 ml of an aqueous solution of amide, organic acid (brought to pH 7 by NaOH), or ester; 1 ml hydroxylamine (2 M, in pH 7 buffer); 1 ml phosphate buffer (150 mM, pH 7); 1 ml enzyme extract (diluted in 50 mM-phosphate buffer, pH 7). Samples (0.5 ml) were taken at regular intervals and were immediately assayed by adding 1 ml FeCl_3 solution (21 ml iron perchloride, 5.3 ml 12.5 M-HCl, distilled water to 1 litre). This stops the reaction and yields a coloured complex; the absorbance was measured at 500 nm. The acetohydroxamic acid concentration (Q) was derived from a standard plot: $Q \text{ (mM)} = (9.5969 \times A_{500}) - 0.0413$. The reaction kinetics were of the Michaelis-Menten type; K_m and $V_{\max}$ were determined from a Lineweaver-Burk plot.

Protein assays. Proteins were assayed by the Lowry method using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Comparison of the relative rates of hydrolysis and transfer reactions

Transfer reactions were assayed under conditions of hydroxylamine saturation (500 mM). The transfer of the acyl group of acetamide to hydroxylamine was about 30-fold faster than the hydrolysis of acetamide to ammonium acetate (Table 1), and 10- and 20-fold faster than the transfer of the acyl group of acetate and ethyl acetate. It appears that in the presence of hydroxylamine, the amidase would convert acetamide to acetohydroxamic acid rather than to ammonium acetate.

Amide transferase activity

This study was assayed with acetamide, propionamide and acrylamide (Table 2). The rate of reaction was greatest with acetamide. The order of the relative rates of hydrolysis of these amides (propionamide > acrylamide > acetamide) (Maestracci *et al.*, 1984) was the reverse of the transfer reaction rates of the acyl groups to hydroxylamine, as was also observed for the amidase of *Pseudomonas aeruginosa* (Clarke, 1970). Acetamide was also the substrate for which the enzyme had the highest affinity in the hydrolysis reaction ($K_m = 2.3, 12$ and 43 mM for acetamide, acrylamide and propionamide, respectively).

Acid transferase activity

This was assayed with acetate, propionate and acrylate (sodium salts resulting from the neutralization of the corresponding acids with NaOH) (Table 2). The rate of reaction was greatest with propionate but the amidase had the lowest affinity for this compound. The highest transfer rate with *P. aeruginosa* was with acetate (Clarke, 1970).

Ester transferase activity

This was assayed with ethyl acetate, ethyl propionate and ethyl acrylate (Table 2). The rate of reaction was greatest with ethyl acrylate but the enzyme had the lowest affinity for this substrate. The highest transfer rate with *P. aeruginosa* was with ethyl acetate (Clarke, 1970).

Effect of amide and hydroxylamine analogues on acyltransferase activity

The action of amidase in the presence of hydroxylamine was assayed with the amide analogues thioacetamide, acetamidine, *N*-methylacetamide and *N,N'*-dimethylacetamide. In the case of thioacetamide no conclusion could be reached since spontaneous decomposition occurred. Of the other compounds, only *N*-methylacetamide was converted to acetohydroxamic acid by the amidase. However, the transfer rate of the acyl group of this amide analogue to hydroxylamine was 180-fold slower than for acetamide. Also, the affinity of the enzyme for acetamide ($K_m = 31$ mM) was greater than for *N*-methylacetamide ($K_m = 62$ mM).

Hydrolysis of *N*-methylacetamide by the amidase of *Brevibacterium* sp. R312 was not observed by Maestracci *et al.* (1984). It is thus surprising to find that in the presence of hydroxylamine, this enzyme converts *N*-methylacetamide to acetohydroxamic acid. It is presumed that there was hydrolysis of *N*-methylacetamide, although the reaction might have occurred at such a slow rate that it was not detected by the GLC technique used.

Finally, the enzyme was shown to transfer the acyl group of acetamide to the hydroxylamine analogue methylhydroxylamine. However, the rate of reaction was three-fold slower than with hydroxylamine.

Conclusions

The amidase of *Brevibacterium* sp. R312 is known to hydrolyse many amides (Maestracci *et al.*, 1984). We have now shown that it also has transferase activity. It is able to catalyse the transfer of acyl groups from amides, organic acids and esters to hydroxylamine. However, the non-acyl compound acetamidine was not a substrate. The enzyme is thus an acyltransferase. As the hydrolysis reaction of amides can be considered a transfer reaction of their acyl groups to water, the enzyme should be classed as an acyltransferase (EC 2.3.1) instead of an acylamide amidohydrolase (amidase) (EC 3.5.1.4).

REFERENCES

- ARNAUD, A., GALZY, P. & JALLAGEAS, J. C. (1976a). Remarques sur l'activité nitrilasique de quelques bactéries. *Comptes rendus de l'Académie des Sciences* **283**, 571–573.
- ARNAUD, A., GALZY, P. & JALLAGEAS, J. C. (1976b). Etude de l'activité nitrilasique de quelques bactéries. *Revue des fermentations et des industries alimentaires* **31**, 39–44.
- ARNAUD, A., GALZY, P. & JALLAGEAS, J. C. (1977). Etude de l'acétonitrilase d'une souche de *Brevibacterium*. *Agricultural and Biological Chemistry* **41**, 2183–2191.
- ASANO, Y., TACHIBANA, M., TANI, Y. & YAMADA, H. (1982). Purification and characterization of amidase which participates in nitrile degradation. *Agricultural and Biological Chemistry* **46**, 1175–1181.
- BRAMMAR, W. J. & CLARKE, P. H. (1964). Induction and repression of *Pseudomonas aeruginosa* amidase. *Journal of General Microbiology* **37**, 307–319.
- BUI, K., FRADET, H., ARNAUD, A. & GALZY, P. (1984a). A nitrile hydratase with a wide substrate spectrum produced by a *Brevibacterium* sp. *Journal of General Microbiology* **130**, 89–93.
- BUI, K., FRADET, H., THIÉRY, A., MAESTRACCI, M., ARNAUD, A. & GALZY, P. (1984b). Use of NMR and GLC for the study of microbial nitrile hydratases and amidases. *Analytica chimica acta* **163**, 315–322.
- CLARKE, P. H. (1970). The aliphatic amidases of *Pseudomonas aeruginosa*. *Advances in Microbial Physiology* **4**, 179–222.
- JALLAGEAS, J. C., ARNAUD, A. & GALZY, P. (1978a). Etude de l'acétamidase d'une souche de *Brevibacterium*. *Journal of General and Applied Microbiology* **24**, 103–114.

- JALLAGEAS, J. C., ARNAUD, A. & GALZY, P. (1978*b*). Application de la chromatographie en phase gazeuse à l'étude des nitrilases et des amidases. *Journal of Chromatography* **166**, 181–187.
- MAESTRACCI, M., THIÉRY, A., BUI, K., ARNAUD, A. & GALZY, P. (1984). Activity and regulation of an amidase (acylamide amidohydrolase EC 3.5.1.4) with a wide substrate spectrum from a *Brevibacterium* sp. *Archives of Microbiology* **138**, 315–320.
- MILLER, J. M. & GRAY, D. O. (1982). The utilization and amides by a *Rhodococcus* sp. *Journal of General Microbiology* **128**, 1803–1809.
- THALENFELD, B. & GROSSOWICZ, N. (1976). Regulatory properties of an inducible aliphatic amidase from a thermophilic *Bacillus*. *Journal of General Microbiology* **94**, 131–141.
- THIÉRY, A., MAESTRACCI, M., ARNAUD, A. & GALZY, P. (1986*a*). Nitriles as growth substrates for *Brevibacterium* sp. R 312 and its mutant M2. *Zentralblatt für Mikrobiologie* (in the Press).
- THIÉRY, A., MAESTRACCI, M., ARNAUD, A., GALZY, P. & NICOLAS, M. (1986*b*). Purification and properties of an acylamide amidohydrolase (EC 3.5.1.4) with a wide activity spectrum from *Brevibacterium* sp. R312. *Journal of Basic Microbiology* (in the Press).