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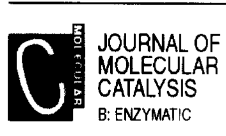

Study of the acyl transfer activity of a recombinant amidase overproduced in an *Escherichia coli* strain. Application for short-chain hydroxamic acid and acid hydrazide synthesis

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Abstract

The study of acyl transfer activity of a wide spectrum amidase from *Rhodococcus sp.* R312, overproduced in an *Escherichia coli* strain, revealed that the 'bi-bi-ping-pong' type reaction was efficient with only four very-short chain (C_2–C_3) aliphatic amides as substrates. The optimum working pH was 7.0 for all neutral amides. Very short-chain aliphatic carboxylic acids were 10–1000-fold less efficient and the corresponding optimum working pH values depended on the acid used. Very polar molecules, such as water, hydroxylamine and hydrazine, were good acyl acceptors. An [acyl donor]/[acyl acceptor] ratio lower than 0.3–0.5 had to be maintained to avoid enzyme inhibition by excess acyl donor. The different acyl–enzyme complexes generally exhibited high affinity for hydroxylamine or hydrazine (except the propionyl–enzyme complex), so that the residual hydrolysis activities were almost totally inhibited at appropriate acyl acceptor concentrations. Molar conversion yields were higher with hydrazine as acyl acceptor (e.g., 97% with acetamide as acyl donor) because of the higher V_{max} values, but in all cases, interesting quantities of short-chain hydroxamic acids (2.9–6.5 g l^{-1}) and acid hydrazides (6.4–7.8 g l^{-1}) could be quickly obtained (10–60 min) with small amounts of enzyme (0.04–0.20 g l^{-1}).
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1. Introduction

Hydroxamic acids (R–CO–NR′–OH) are known to possess high chelating properties [1,2]. Medical applications of such compounds have been extensively investigated, and they are currently known to be potent inhibitors of several matrix metalloproteases, a family of zinc endopeptidases involved in tissue remodeling [3–5]. Indeed, these enzymes are ubiquitous in human diseases, such as arthritis, for example, osteoarthritis and rheumatoid arthritis. These diseases also include corneal ulceration, osteoporosis, periodontitis, tumor growth and metastasis. Some hydroxamic acids have also been investigated as anti-HIV agents, because of their overadditive effects through a combined action with the AZT and DDI drugs [6]. Siderophores (natural or synthetic high molecular weight hydroxamic acids), such as desferrioxamine, fer-

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(1) $R\text{-}CO\text{-}NH_2 + H_2O \leftrightarrow R\text{-}CO\text{-}OH + NH_3$

(2) $R\text{-}CO\text{-}X + NH_2OH \leftrightarrow R\text{-}CO\text{-}NHOH + HX$ with $X = NH_2$, OR' or OH

Fig. 1. Amidase-catalyzed reactions. (1) amide hydrolysis (or acyl transfer on water); (2) acyl transfer on hydroxylamine.

richromes, and others, have also been used as antimalarials due to their action on *Plasmodium falciparum* growth [7]. Acetohydroxamic acid (Lithostat, Mission Pharmacal) has been recommended for treating ureaplasma and anemia [8,9]. And finally, several fatty hydroxamic acids have been studied as inhibitors of cyclooxygenase and 5-lipoxygenase with a potent antiinflammatory activity [10,11].

Apart from their medical applications, hydroxamic acids have received attention in phytochemistry and agronomy. The cyclic hydroxamic acid 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and its methoxy analogue 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) occur as glucosides in *Graminae* species, including maize, wheat and rye, and are implicated in plant resistance to pathogens and insects [12,13] after hydrolysis by an endogenous β-glucosidase.

Homopolymers or copolymers of hydroxamic acids have also been extensively investigated in waste water treatment and in nuclear technology to eliminate contaminating metal ions. These studies gave rise to many publications [14–16] and patents [17–19] over the last 10 years.

Lastly, besides their ability to complex metal ions, long-chain hydroxamic acids are of considerable interest as efficient surfactants in the detergent industry [20,21].

Several methods of hydroxamic acid chemical synthesis have been described [22–26] but, due to the high polarity of the hydroxamate group, reactions between *O*-benzylated hydroxylamine and methyl esters are most frequently used. The resulting *O*-benzylated hydroxamic acid can be extracted with an appropriate solvent and then submitted to catalytic hydrogenation with palladium to release the free hydroxamic acid. These chemical methods have the drawback of requiring many solvents and sometimes high temperatures, nitrogen atmosphere and tricky steps. In producing these molecules, biocatalysts could therefore obviously facilitate some otherwise difficult reactions. Few enzymes, other than those required in natural synthesis of siderophores in microorganisms (fungi, bacteria and yeasts), are known to catalyze hydroxamic acid synthesis [27–29]. For instance, the wide-spectrum amidase (acylamide amidohydrolase, EC 3.5.1.4) from *Rhodococcus sp.* R312 was shown to transfer acyl groups of some short-chain amides, acids or esters to water or hydroxylamine, thus producing the corresponding carboxylic acids or hydroxamic acids (Fig. 1). This was found to involve a 'bi-bi-ping-pong' mechanism [30]. One substrate A (acyl donor) reacts with the enzyme to give an acyl–enzyme complex E*, which then transfers the acyl group to the second substrate B (acyl acceptor) (Fig. 2).

The *amiE* gene from *Rhodococcus sp.* R312, encoding amidase, was previously isolated by genetic complementation [31]. High expression levels were obtained in *Escherichia coli* after substitution of the *amiE* promoter by the *tac* promoter, so that large quantities of amidase could be produced and easily purified in one chromatographic step [32]. This was also due to the absence of other amidases that could be found in the *Rhodococcus sp.* R312 strain. The overproduced amidase was previously immobilized on an anion exchange carrier and was confirmed to be very efficient for acetohydroxamic acid synthesis [32].

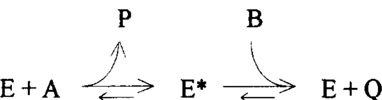


Fig. 2. Enzyme-substituted mechanism. For example, A = amide, B = hydroxylamine, P = ammonia, and Q = hydroxamic acid. E = enzyme and E* = acyl–enzyme complex.

In this study, the recombinant amidase from the modified *Escherichia coli* strain was purified and used to investigate several potential acyl donors (A) and acyl acceptors (B). Some kinetic constants were determined in order to clearly characterize the amidase specificity and produce some short-chain hydroxamic acids and acid hydrazides.

2. Experimental

2.1. Bacterial strain

The genetically modified strain used was *E. coli* XL1-Blue[pASTL-77R] [31]. The plasmid pASTL-77R used for cell transformation was obtained by cloning the *amiE* gene in the shuttle vector pRPCG200 [33]. In the plasmid pASTL-77R, the *amiE* gene was under control of the *tac* promoter which could be derepressed by isopropyl-β-D-thiogalactopyranoside (IPTG).

2.2. Chemicals

Amides, carboxylic acids, hydroxylamine hydrochloride, hydrazine monohydrate, acetic hydrazide and all hydroxamic acids, except propionohydroxamic acid, acrylohydroxamic acid and valerohydroxamic acid, were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Valeramide was the only amide which was purchased from Interchim (Montluçon, France). The non-commercially available propionohydroxamic acid, acrylohydroxamic acid and valerohydroxamic acid were chemically synthesized (data not shown) using the original method of Ando and Tsumaki [22], based on the reaction between the corresponding acid chloride and *N,N,O*-tris(trimethylsilyl)hydroxylamine.

2.3. Methods

2.3.1. Purification of amidase

Amidase was purified according to a previously described method [32].

2.3.2. Hydrolysis activity assay

Unless otherwise specified, the reaction medium was of the following composition: an aqueous solution of amide (2 ml; 400 mM); sodium phosphate (NaH_2PO_4/Na_2HPO_4) buffer pH 7 (4 ml; 100 mM); and enzyme preparation diluted in the same buffer (2 ml). The reaction was performed at 30°C. Samples of 0.5 ml reaction medium were taken at regular intervals and immediately mixed with 0.5 ml of 1 M orthophosphoric acid to stop the reaction. The amounts of carboxylic acid formed and residual amides were determined by analytical HPLC. HPLC was performed on a Waters system consisting of a model 150 pump, a U6K injector (2 ml loop volume), and a model 450 UV detector. The integration-calculation of peak areas was performed with a model 740 integrator. The chromatographic column used was a reverse-phase LiChrosorb 5 μm C_{18} (150 × 4.6 mm i.d.; SFCC-Shandon, Eragny, France) and the analytical conditions were as follows: eluent, 25 mM orthophosphoric acid with 1% (v/v) methanol; flow rate, 1.0 ml min^{-1}; detection wavelength, 210 nm; and sample volume injected, 5 μl.

2.3.3. Acyl transfer activity assay

2.3.3.1. Hydroxylamine as acyl acceptor. Unless otherwise specified, the reaction medium was of the following composition: an aqueous solution of amide or carboxylic acid adjusted to pH 7 with 10 M NaOH (2 ml; 400 mM); hydroxylamine hydrochloride, adjusted to pH 7 with 10 M NaOH (2 ml; 2 M); sodium phosphate buffer, pH 7 (2 ml; 100 mM); and enzyme preparation diluted in the same buffer (2 ml). The reaction was performed at 30°C. The resulting hydroxamic acid was assayed using the method developed by Brammar and Clarke [27], based on colorimetric determination of the red–brown complexes with Fe(III). Samples of 0.5 ml reaction medium were taken at regular intervals and immediately assayed by adding 1 ml of iron chloride solution (21 ml $FeCl_3$ 27.5%

Table 1
Determination of the most efficient amide substrates for the amidase-catalyzed acyl transfer reaction on hydroxylamine. Reactions were performed at pH 7 for 30 min with 500 mM hydroxylamine

Potential substrate and final concentration in reaction medium	Chemical formula	Relative acyl transfer activity (%)	Spontaneous non-enzymatic reaction ($mM\ min^{-1}$)
C1			
Urea (100 mM)	NH_2-CONH_2	< 0.03	
Formamide (100 mM)	$H-CONH_2$	< 0.03	0.50
C2			
Acetamide (100 mM)	CH_3-CONH_2	100[b]	0.05
Glycinamide[a] (100 mM)	$NH_2-CH_2-CONH_2$	6	0.08
C3			
Propionamide (100 mM)	$CH_3-CH_2-CONH_2$	40	0.05
Acrylamide (100 mM)	$CH_2=CH-CONH_2$	34	0.10
D-alaninamide[a] (100 mM)	$NH_2-CH(CH_3)-CONH_2$	0.12	—
L-alaninamide[a] (100 mM)	$NH_2-CH(CH_3)-CONH_2$	0.15	—
DL-lactamide (100 mM)	$CH_3-CH(OH)-CONH_2$	(0.14)	(0.02)
L-serinamide[a] (100 mM)	$HO-CH_2-CH(NH_2)-CONH_2$	< 0.05	—
Malonamide (100 mM)	$H_2NCO-CH_2-CONH_2$	(0.07)	(0.03)
C4			
Methacrylamide (100 mM)	$CH_2=C(CH_3)-CONH_2$	0.28	—
Butyramide (100 mM)	$CH_3-CH_2-CH_2-CONH_2$	1.33	—
Isobutyramide (100 mM)	$CH_3-CH(CH_3)-CONH_2$	0.72	—
D-asparagine (25 mM)	$HOOC-CH(NH_2)-CH_2-CONH_2$	0.04	—
Succinamide (10 mM)	$H_2NCO-CH_2-CH_2-CONH_2$	(< 0.03)	—
C5			
Valeramide (50 mM)	$CH_3-CH_2-CH_2-CH_2-CONH_2$	< 0.03	—
Pivalamide (50 mM)	$(CH_3)_3C-CONH_2$	< 0.03	—
L-prolinamide[a] (100 mM)	NH, $CONH_2$	(< 0.03)	(0.02)
L-valinamide[a] (100 mM)	$(CH_3)_2CH-CH(NH_2)-CONH_2$	< 0.05	—
DL-methioninamide[a] (100 mM)	$CH_3-S-CH_2-CH_2-CH(NH_2)-CONH_2$	< 0.05	—
C6			
L-leucinamide[a] (100 mM)	$(CH_3)_2CH-CH_2-CH(NH_2)-CONH_2$	< 0.05	—
Adipamide (6.25 mM)	$H_2NCO-CH_2-CH_2-CH_2-CH_2-CONH_2$	(< 0.03)	—
Hexanoamide (25 mM)	$CH_3-CH_2-CH_2-CH_2-CH_2-CONH_2$	< 0.03	—
Arylamides			
Salicylamide (2 mM)	$CONH_2$, OH	(< 0.03)	(0.10)
Benzamide (7.5 mM)	$CONH_2$	(< 0.03)	—
Heterocycles			
Nicotinamide (100 mM)	N, $CONH_2$	(< 0.03)	—
Isonicotinamide (100 mM)	N, $CONH_2$	(< 0.03)	(0.02)

(w/v), 5.3 ml 12.5 M HCl, distilled water to 100 ml). This stopped the reaction and yielded a stained complex. Absorbance was measured at 500 nm and the hydroxamic acid concentration was calculated from previously determined molar extinction coefficients (data not shown).

2.3.3.2. Hydrazine as acyl acceptor. Unless otherwise specified, the reaction medium was of the following composition: an aqueous solution of amide (2 ml; 400 mM); hydrazine monohydrate, adjusted to pH 7 with 12.5 M HCl (2 ml; 2 M); sodium phosphate buffer, pH 7 (2 ml; 100 mM); and enzyme preparation diluted in the same buffer (2 ml). The reaction was performed at 30°C. Samples of 0.5 ml reaction medium were taken at regular intervals and immediately mixed with 0.5 ml of 1 M orthophosphoric acid to stop the reaction. The amount of acid hydrazide formed was determined by analytical HPLC, using a C18 reverse-phase Spherisorb column ODS-2 5 μm (250 × 4.6 mm i.d.; Alltech Associates, Deerfield, IL, USA). The analytical conditions were identical to those previously described.

2.3.4. Protein concentration

Protein concentration was determined by Lowry's method [34].

3. Results

After purification, solutions of native recombinant amidase contained 1.6 mg protein/ml 50 mM Tris–HCl buffer (pH 7.4) containing 0.40–0.45 M NaCl. The enzymatic activity used as reference, based on acyl transfer from acetamide to hydroxylamine, was determined after dilution of a stock solution of the enzyme down to a concentration of 17.8 nM (3.2 ng protein/ml) in 100 mM sodium phosphate buffer pH 7. The acetohydroxamic acid formed was assayed every min for 4 min as previously described. The activity used as reference was found to be 787 mmol acetohydroxamic acid/min/g protein.

3.1. Determination of the most efficient amide substrates for acyl transfer activity

Hydroxylamine was used as acyl acceptor. Several potential amide substrate solutions were prepared at a concentration of 400 mM when possible. Hydrophobic amide solutions were prepared at a lower concentration. Each reaction medium was prepared with and without enzyme to detect possible spontaneous non-enzymatic reactions. The absorbance of the resulting stained complex was read at 500 nm. Hydroxamic acid concentrations were calculated from the calibration curves previously obtained from colorimetric assays of five hydroxamic acid families: saturated aliphatic hydroxamic acids, β-unsaturated hydroxamic acids, α-aminohydroxamic acids (except glycinehydroxamic acid), glycinehydroxamic acid, and β-aminohydroxamic acids (data not shown). The other hydroxamic acids were assayed using the calibration curve obtained from saturated hydroxamic acids (numbers in parentheses). Enzymatic syntheses and spontaneous non-enzymatic reactions were thus determined for the different hydroxamic acids formed. Results are shown in Table 1. Spontaneous non-enzymatic reactions were negligible compared to enzymatic syntheses, except for formamide and salicylamide which were not amidase substrates for acyl transfer activity but which spontaneously reacted with hydroxylamine. Only very short-chain amides (1, 2, 3 or 4 carbon atoms) were amidase substrates. Moreover, substitution of hy-

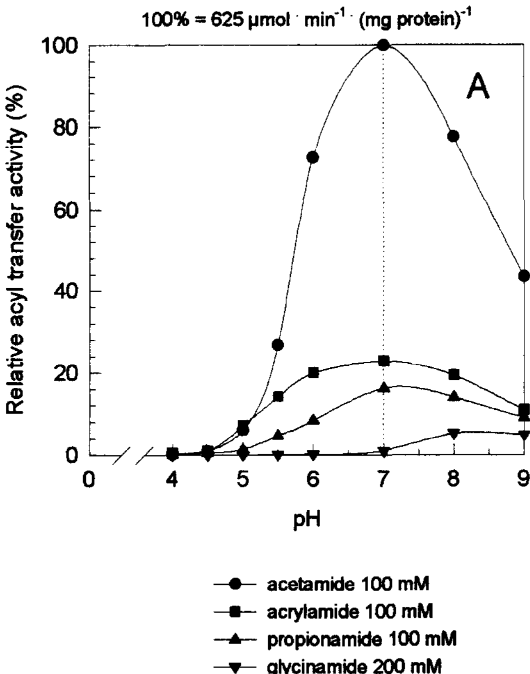
Notes to Table 1:
[a]These products were in chlorhydrate form. They were previously adjusted to pH 7 with 10 M NaOH.
[b]100% = 750 mmol min^{-1} (g $protein)^{-1}$.

drogen atom by a NH_2 or OH group considerably decreased amidase acyl transfer activity. As an example, the acyl transfer activity obtained with glycinamide as substrate represented only 6% of the acyl transfer activity obtained with acetamide as substrate. In conclusion, unsubstituted short-chain aliphatic amides (except formamide) were shown to be the most efficient substrates of the recombinant amidase for acyl transfer on hydroxylamine. This was in accordance with the results of Thiéry et al. [35], indicating that short-chain amides were the most efficient acyl donors for amide hydrolysis (or acyl transfer on water) catalyzed by the amidase of the wild strain *Rhodococcus sp.* R312.

Six amides were found to be interesting for enzymatic production of the corresponding hydroxamic acids: acetamide, acrylamide, propionamide, glycinamide, butyramide, and isobutyramide.

3.2. Comparisons of the most efficient amides with their corresponding carboxylic acids as acyl donors

Comparisons between amides and their corresponding carboxylic acids as amidase substrates were performed as a function of pH. Four acyl groups were studied: acetyl group, acryloyl group, propionyl group, and glycyl group. As shown in Fig. 3A, the optimum working pH of the amidase with amides as substrates was pH 7, except with glycinamide which is the only amide studied to be charged at a such pH value



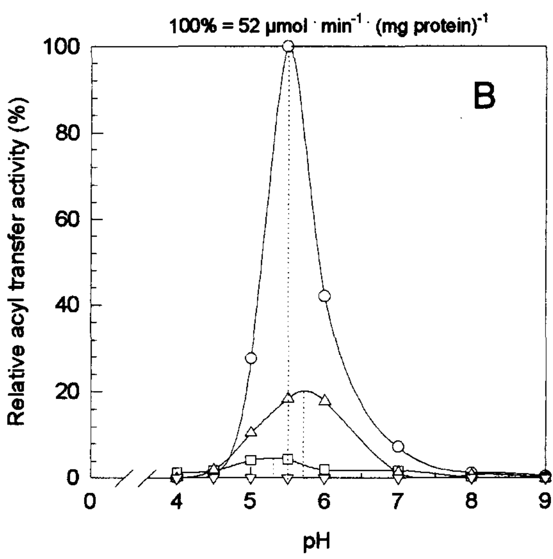


Fig. 3. Influence of pH on recombinant amidase acyl transfer activity. (A) acyl transfer activity from amides to hydroxylamine 500 mM; (B) acyl transfer activity from carboxylic acids to hydroxylamine 500 mM. Buffers used for reactions were 100 mM citrate–phosphate buffer for pH 4–6, 100 mM sodium phosphate buffer for pH 6–8, and 100 mM Tris–HCl buffer for pH 8–9. Aqueous carboxylic acid solutions and aqueous hydroxylamine hydrochloride solutions were previously adjusted to the corresponding pH with 10 M NaOH, and dilution of the enzyme stock solution was performed in the corresponding buffer. The resulting hydroxamic acids were assayed every min for 4 min as described in Section 2.

(NH_2 group protonation). The wide spectrum amidase from the *Rhodococcus sp.* R312 strain was also shown to have optimum activity at pH 7 for amide hydrolysis [36]. Hence, pH 7 was determined to be the actual optimum pH for amidase activity.

The optimum working pH values determined with acids as substrates (Fig. 3B) revealed that a new phenomenon was involved in acyl transfer when substrates were electronically charged. Except with glycine, for which no acyl transfer activity was detected, these apparent optimum working pH values depended on the acid used: the apparent optimum working pH was lower with lower acid pK_A. The carboxylate form of the acid substrates thus seemed to be unsuitable for the acyl transfer activity of the amidase, so that carboxylic acids appeared to be bad amidase substrates for hydroxamic acid synthesis. This might have been due to enzyme–carboxylate ionic interactions impeding the carboxylate from reaching the active site. Indeed, the apparent optimum pH values obtained with the different acids ranged between the pH value of the electronic neutrality of the acid and the actual optimum working pH of the amidase (pH 7). This electronic neutrality required for an efficient enzymatic reaction could explain the higher apparent optimum pH obtained with glycinamide as substrate ($pK_A = 8.1$).

3.3. Kinetic study of acyl transfer activity of the recombinant amidase from amides to hydroxylamine

In enzyme-substituted or double displacement or ‘bi-bi-ping-pong’ mechanisms (Fig. 2), the inverse rate of reaction is given by [37]

$$\frac{1}{v} = \left[1 + \frac{K_A}{[A]} + \frac{K_B}{[B]}\right] \cdot \frac{1}{V_{max}}. \tag{1}$$

When the acyl donor (A) is in saturation conditions for the enzyme, Eq. (1) becomes

$$\frac{1}{v} = \left[1 + \frac{K_B}{[B]}\right] \cdot \frac{1}{V_{max}}. \tag{2}$$

If the rate data are plotted in Lineweaver–Burk form, a straight line is obtained so that K_B and V_{max} constants can be determined by regression analysis. In the same way, K_A and V_{max} constants can be determined when the acyl acceptor (B) is in saturation conditions for the acyl–enzyme complex.

3.3.1. Study with constant hydroxylamine and variable amide concentrations

This study aimed at calculating apparent V_{max} and K_{amide} constants for the six most efficient amide substrates of the recombinant amidase determined previously. The corresponding hydroxamic acids were assayed every min for 4 min at each amide concentration. The different apparent V_{max} and K_{amide} values obtained are shown in Table 2. These values have been corrected because, as we shall see, 500 mM hydroxylamine was not in agreement with the saturation conditions for all acyl–enzyme complexes. For instance, if [hydroxylamine] = 9 · $K_{hydroxylamine}$ for an acyl–enzyme complex, then

$$\frac{1}{V_{max,exp}} = \left[1 + \frac{K_{hydroxylamine}}{9 \cdot K_{hydroxylamine}}\right] \cdot \frac{1}{V_{max}}. \tag{3}$$

Eq. (3) thus becomes

$$V_{max,exp} = 0.9 V_{max}. \tag{4}$$

This means the previously determined experimental $V_{max,exp}$ value represented 90% of the actual V_{max} value.

The apparent turnover number, $k_{cat,app}$, represents the maximum number of hydroxamic acid molecules that could be produced per time unit and per active site (the amidase is constituted of four identical subunits), and has the advantage of being independent beyond the amidase concentration used in the experiments. The $k_{cat,app}/K_{amide,app}$ ratio provides an indication of the enzyme specificity for the amide substrate.

As shown in Table 2, the enzyme affinities for the different amides decreased when their side chain length increased ($K_{acetamide,app} = 9$ mM, $K_{propionamide,app} = 22$ mM, $K_{butyramide,app} =$

Table 2
Determination of the kinetic constants for amidase-catalyzed acyl transfer reaction on hydroxylamine. Reactions with glycinamide were performed at pH 8. Reactions with other amides were performed at pH 7. Numbers in parentheses are corrected values

Acyl donor	$K_{amide,app}$ (mM)	$K_{hydroxylamine,app}$ (mM)	Relative $V_{max,app}$ (%)	$k_{cat,app}$ (s^{-1})	$k_{cat,app}/K_{amide,app}$ (mM^{-1} s^{-1})	$k_{cat,app}/K_{hydroxylamine,app}$ (mM^{-1} s^{-1})
Acetamide	9[a]	96[b]	100[a,d] (100[e])	590[a] (711[a])	66[a] (79[a])	6[a] (7[a])
	lit. 31[h]		100[b,f] (100[g])	547[b] (608[b])	61[b] (68[b])	6[b] (6[b])
			lit. 100[h]			
Acrylamide	34[a]	25[b]	29[a,d] (26[e])	171[a] (185[a])	5[a] (5[a])	7[a] (7[a])
	lit. 93[h]		17[b,f] (20[g])	93[b](122[b])	3[b] (4[b])	4[b] (5[b])
			lit. 49[h]			
Propionamide	22[a]	262[b]	19[a,d] (23[e])	112[a] (164[a])	5[a] (7[a])	0.4[a] (0.6[a])
	lit. 88[h]		20[b,f] (22[g])	110[b] (134[b])	5[b] (6[b])	0.4[b] (0.5[b])
			lit. 28[h]			
Glycinamide (pH 8)	421[a]	415[c]	9.7[a,d] (16[e])	57[a] (114[a])	0.1[a] (0.3[a])	0.1[a] (0.3[a])
			13[c,f] (24[g])	71[c] (146[c])	0.2[c] (0.3[c])	0.2[c] (0.4[c])
Butyramide	620[a]	nd	1.4[a,d]	8[a]	0.01[a]	nd
Isobutyramide	276[a]	nd	0.9[a,d]	5[a]	0.02[a]	nd

Lit., literature; nd, not determined.
[a]Value determined with $[NH_2OH]$ = 500 mM and [amide] = from 16.6 to 200 mM.
[b]Value determined with $[NH_2OH]$ = from 25 to 500 mM and [amide] = 100 mM.
[c]Value determined with $[NH_2OH]$ = from 25 to 500 mM and [amide] = 500 mM.
[d]100% = 787 mmol min^{-1} g^{-1}.
[e]100% = 948 mmol min^{-1} g^{-1}.
[f]100% = 730 mmol min^{-1} g^{-1}.
[g]100% = 811 mmol min^{-1} g^{-1}.
[h]Thiéry et al. ([35]).

620 mM). Moreover, substitution of hydrogen atom by a NH_2 group considerably decreased the amidase affinity ($K_{acetamide,app}$ = 9 mM, $K_{glycinamide,app}$ = 421 mM). The same observations were valid for the different $V_{max,app}$ values: acetamide was the fastest transformed substrate

Table 3
Determination of the kinetic constants for amidase-catalyzed hydrolysis. Reactions were performed at pH 7 and 30°C. Numbers in parentheses are corrected values

Acyl donor	$K_{amide,app}$ (mM)	Relative $V_{max,app}$ (%)	$k_{cat,app}$ (s^{-1})	$k_{cat,app}/K_{amide,app}$ (mM^{-1} s^{-1})
Acetamide	9	29[a] (26[b])	75 (82)	8 (9)
	lit. 2.6[c]	lit. 9[c]		
Acrylamide	34	43[a] (47[b])	111 (148)	3 (4)
	lit. 16[c]	lit. 21[c]		
Propionamide	22	100[a] (100[b])	258 (315)	12 (14)
	lit. 49[c]	lit. 100[c]		
Butyramide	620	nd	nd	nd
	lit. 0.28[c]	lit. 1.4[c]		
Isobutyramide	276	nd	nd	nd
	lit. 0.50[c]	lit. 9[c]		

Lit., literature; nd, not determined.
[a]100% = 344 mmol min^{-1} g^{-1}.
[b]100% = 420 mmol min^{-1} g^{-1}.
[c]Maestracci et al. ([38]).

($V_{max,app}$ = 948 mmol min^{-1} g^{-1}) and butyramide and isobutyramide were the slowest ($V_{max,app}$ = 11 and 7 mmol min^{-1} g^{-1}, respectively). Hence, acetamide was the amide substrate for which the amidase had the highest specificity for acyl transfer activity ($k_{cat,app}/K_{amide,app}$ = 79 mM^{-1} s^{-1}). Specificities for acrylamide and propionamide were almost 15-fold lower (5 mM^{-1} s^{-1} and 7 mM^{-1} s^{-1}, respectively), and the specificity for glycinamide at pH 8 was 300-fold lower (0.3 mM^{-1} s^{-1}). Butyramide and isobutyramide turned out to be relatively poor substrates for acyl transfer activity, since the enzyme specificity was around 5000-fold lower (0.01 mM^{-1} s^{-1} and 0.02 mM^{-1} s^{-1}, respectively) than that of acetamide. This confirmed the amidase specificity for very short-chain amide substrates. In conclusion, with respect to the acyl transfer activity on hydroxylamine, the enzyme specificities for the different amides were, in decreasing order: acetamide, propionamide, acrylamide, glycinamide, butyramide and isobutyramide.

In order to compare the acyl transfer activity on hydroxylamine and on water (or hydrolysis), amidase specificities for some amides were determined for the hydrolysis activity. The results shown in Table 3 were in accordance with those of Maestracci et al. [38], indicating that the highest specificity of the wide spectrum amidase from the *Rhodococcus sp.* R312 strain was for propionamide. Concerning the hydrolysis activity, the recombinant amidase specificities were, in decreasing order: propionamide, acetamide, acrylamide.

3.3.2. Study with constant amide and variable hydroxylamine concentrations

This study was aimed at calculating the V_{max} and $K_{hydroxylamine}$ values of Eq. (2), and then at determining the residual hydrolysis activity throughout the acyl transfer reaction on hydroxylamine. Indeed, both reactions (1) and (2) described in Fig. 1 occurred simultaneously since the enzymatic acyl transfer on hydroxylamine was performed in aqueous medium. The reaction times were 60 min. When possible, amide, carboxylic acid and hydroxamic acid concentrations were determined as previously described every 10 min by HPLC analysis. Initial rates were used for V_{max} and $K_{hydroxylamine}$ determination. These values have been corrected since, as we have seen, 100 mM amide concentration was not in agreement with the enzyme saturation conditions for all amides. As shown in Table 2, the apparent V_{max} values were logically close to those determined previously. Among acetyl–, acryloyl–, and propionyl–enzyme complexes, the acryloyl–enzyme complex exhibited the highest affinity for hydroxylamine ($K_{hydroxylamine}$ = 25 mM), whereas the propionyl–enzyme complex was found to have the lowest affinity ($K_{hydroxylamine}$ = 262 mM). The affinity for hydroxylamine had a direct influence on the residual hydrolysis activity since, as shown in Fig. 4, no residual hydrolysis activity was detected with acrylamide as substrate when

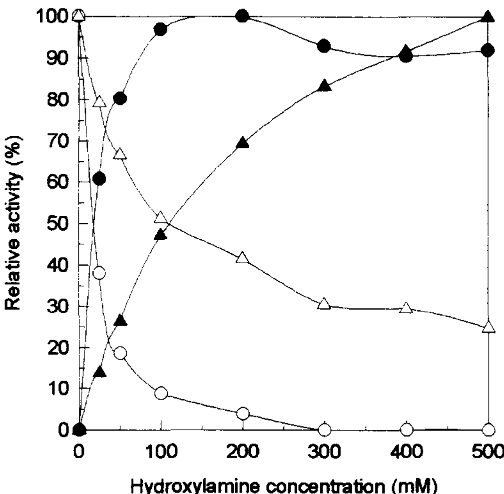


Fig. 4. Influence of hydroxylamine concentration on acyl transfer and residual hydrolysis activities of the recombinant amidase. Reactions were performed at pH 7 and 30°C, with 100 mM acrylamide and propionamide. Decrease of acyl transfer activity from acrylamide with high hydroxylamine concentrations was due to slight acrylamide polymerization, resulting in lower free acrylamide availability for the enzyme.

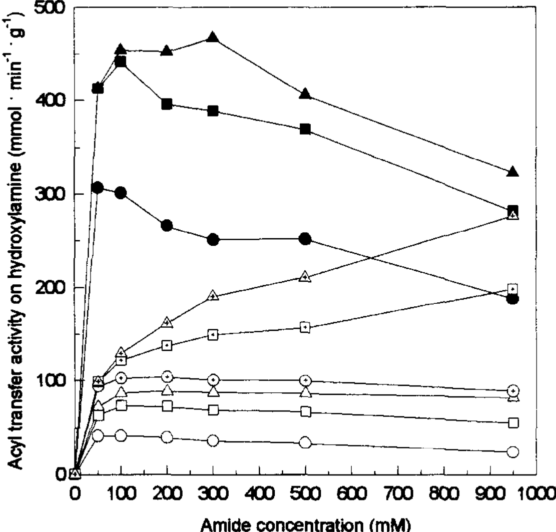


Fig. 5. Inhibition of the amidase acyl transfer reaction by relative amide excess. Black symbols: acetamide as substrate; crosshair symbols: acrylamide as substrate; white symbols: propionamide as substrate; circles: hydroxylamine 100 mM; squares: hydroxylamine 300 mM; triangles: hydroxylamine 500 mM.

the initial hydroxylamine concentration was 300 mM, whereas 30% of the maximum hydrolysis activity remained with propionamide as substrate.

In conclusion, analysis of acyl transfer activity from amides to hydroxylamine revealed that the most efficient results were obtained with acetamide as substrate. Propionamide and acrylamide were also quite good substrates, although propionamide required relatively high hydroxylamine concentrations to correctly reduce the residual hydrolysis activity. Glycinamide could also be used but required both very high amide and hydroxylamine concentrations.

3.4. Influence of the [amide] / [hydroxylamine] ratio

Several acyl transfer reactions were performed by combining six amide concentrations (from 50 to 950 mM) with three hydroxylamine concentrations (from 100 to 500 mM). The results (Fig. 5) showed that slight inhibition of acyl transfer activity occurred with acetamide and propionamide as substrates when the [amide]/[hydroxylamine] ratio was higher than 0.3–0.5. This means that a relative excess of amide inhibited the acyl transfer reaction. With respect to acrylamide, this slight inhibition was detected when the [amide]/[hydroxylamine] ratio was higher than 3. The influence of the [amide]/[hydroxylamine] ratio thus seemed to be related to the type of amide used.

3.5. Search for other acyl acceptors

Water and hydroxylamine were already shown to be quite efficient acyl acceptors for recombinant amidase acyl transfer activity. Four other potential acyl acceptors were tested because of their polarity, and compared to water and hydroxylamine: *N*-methylhydroxylamine (CH_3–NHOH), ethanolamine (NH_2–CH_2–CH_2–OH), hydrazine (NH_2–NH_2) and methyl alcohol (CH_3–OH). The results are shown in Table 4. No acyl transfer activity was detected

Table 4
Potential amidase-catalyzed reactions with acrylamide as acyl donor. The potential acyl acceptors were used at 500 mM in the reaction media. Acrylamide 100 mM was used as acyl donor and the enzyme concentration was 0.04 g l^{-1}. The reactions were performed at 30°C for 5 min and stopped by adding one volume of 1 M orthophosphoric acid. Samples were analyzed by HPLC

Potential acyl acceptor	Potential final product	Acyl transfer activity (mmol final product min^{-1} g^{-1})	Residual hydrolysis (mmol acrylic acid min^{-1} g^{-1})
H_2O	CH_2=CH–COOH	—	148
NH_2OH	CH_2=CH–CONHOH	124	0
CH_3NHOH	CH_2=CH–CON(CH_3)OH	< 5	0
$NH_2CH_2CH_2OH$	CH_2=CH–CONHCH_2CH_2OH	0	94
NH_2NH_2	CH_2=CH–CONHNH_2	194	11
CH_3OH	CH_2=CH–COOCH_3	0	141

with ethanolamine and methyl alcohol as acyl acceptors, but the presence of such compounds slightly reduced the hydrolysis activity of the enzyme. *N*-methylhydroxylamine was a very poor acyl acceptor, but no residual hydrolysis remained. Hydrazine was shown to be the most efficient acyl acceptor tested, with only 5% of residual hydrolysis. We checked whether the final product was actually an acid hydrazide ($RCONHNH_2$) by comparing, by HPLC (Spherisorb ODS-2 5 μm, 250 × 4.6 mm), the retention time of the final product resulting from acyl transfer from acetamide onto hydrazine, and the retention time of a commercially purchased acetic hydrazide solution. The same value was obtained: the retention time for acetic hydrazide was 2.87 min in the previously described HPLC conditions. The kinetic constants of the acyl transfer activity of the recombinant amidase from amides (acetamide, propionamide and acrylamide) to hydrazine were thus determined and compared to those previously determined with hydroxylamine as acyl acceptor. The results are shown in Table 5. As observed for acyl transfer on hydroxylamine, the amidase apparent affinities decreased when the amide side chain lengths increased ($K_{acetamide,app}$ = 56 mM, $K_{propionamide,app}$ = 98 mM), and the highest $V_{max,app}$ value was obtained with acetamide as acyl donor ($V_{max,app}$ = 2659 mmol min^{-1} g^{-1}). Hence, the recombinant amidase exhibited the highest specificity for acetamide ($k_{cat,app}/K_{amide,app}$ = 36 mM^{-1} s^{-1}), whereas the specificities for acrylamide and propionamide were almost 10-fold lower ($k_{cat,app}/K_{amide,app}$ = 5 mM^{-1} s^{-1} and 3 mM^{-1} s^{-1}, respectively). With respect to the desacylation step, the acryloyl–enzyme complex exhibited the highest affinity for hydrazine. Once again, this had a direct influence on the residual hydrolysis activity since, as shown in Fig. 6, no residual hydrolysis activity was detected with acrylamide as substrate when the initial hydrazine concentration was 800 mM, whereas 26% of the maximum hydrolysis activity remained with propionamide as substrate. In every case, the acyl transfer activity on hydrazine was around 2-fold higher than that on hydroxylamine, in spite of the lower affinity of the different acyl–enzyme complexes for hydrazine.

3.6. Applications for short-chain hydroxamic acid and acid hydrazide synthesis

The recombinant amidase was used to catalyze the formation of hydroxamic acids and acid hydrazides corresponding to acetamide, propionamide, acrylamide and glycinamide. The previous results were taken into consideration to determine the different reaction conditions: pH

Table 5
Determination of the kinetic constants for amidase-catalyzed acyl transfer reaction on hydrazine. Reactions were performed at pH 7 and 30°C. Numbers in parentheses are corrected values

Acyl donor	$K_{amide,app}$ (mM)	$K_{hydrazine,app}$ (mM)	Relative $V_{max,app}$ (%)	$k_{cat,app}$ (s^{-1})	$k_{cat,app}/K_{amide,app}$ (mM^{-1} s^{-1})	$k_{cat,app}/K_{hydrazine,app}$ (mM^{-1} s^{-1})
Acetamide	56[a]	620[b]	100[a,d] (100[e])	890[a] (1994[a])	16[a] (36[a])	1[a] (3[a])
			100[b,f] (100[g])	309[b] (1652[b])	6[b] (30[b])	1[b] (2[b])
Acrylamide	86[a]	68[b]	38.4[a,d] (19.5[e])	342[a] (388[a])	4[a] (5[a])	5[a] (6[a])
			16.2[b,f] (19.4[g])	172[b] (320[b])	2[b] (4[b])	3[b] (5[b])
Propionamide	98[a]	697[b]	21.3[a,d] (17.0[e])	189[a] (340[a])	2[a] (3[a])	0.3[a] (0.5[a])
			17.6[b,f] (22.3[g])	186[b] (368[b])	2[b] (4[b])	0.3[b] (0.5[b])

[a] Value determined with $[NH_2NH_2]$ = 500 mM and [amide] = from 25 to 200 mM.
[b] Value determined with $[NH_2NH_2]$ = from 50 to 800 mM and [amide] = 100 mM.
[d] 100% = 1187 mmol min^{-1} g^{-1}.
[e] 100% = 2659 mmol min^{-1} g^{-1}.
[f] 100% = 1413 mmol min^{-1} g^{-1}.
[g] 100% = 2204 mmol min^{-1} g^{-1}.

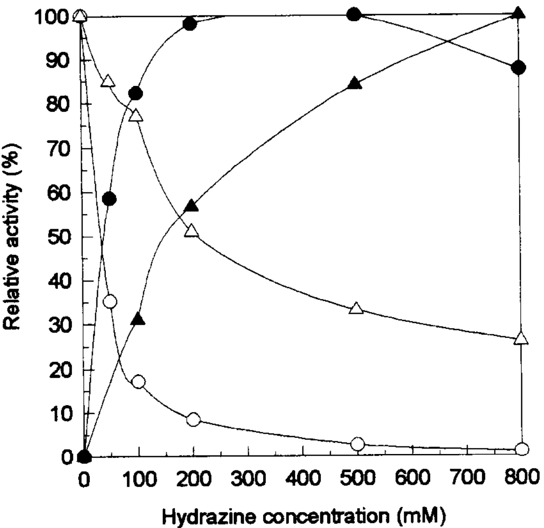


Fig. 6. Influence of hydrazine concentration on acyl transfer and residual hydrolysis activities of the recombinant amidase. Reactions were performed at pH 7 and 30°C, with 100 mM acrylamide and propionamide. Decrease of acyl transfer activity from acrylamide with high hydroxylamine concentrations was due to slight acrylamide polymerization, resulting in lower free acrylamide availability for the enzyme.

8 was used with glycinamide and pH 7 with other amides; hydrazine was prepared at higher concentrations than hydroxylamine because of the lower affinities of the different acyl–enzyme complexes for hydrazine; reactions with hydrazine were generally shorter because of the higher V_{max} values; higher acyl acceptor concentrations were used with propionamide as substrate in order to reduce the residual hydrolysis; the [acyl donor]/[acyl acceptor] ratios were in every case lower than 0.3 to avoid the slight inhibition due to excess amide. The reaction conditions and molar conversion yields are shown in Table 6. Acetamide and acrylamide were the fastest transformed substrates. Propionamide was also rapidly transformed, but around 30% of the initial propionamide was hydrolyzed in propionic acid. This explained the lower molar conversion yields obtained for the synthesis of propionohydroxamic acid and propionic hydrazide.

tTable 6
Amidase-catalyzed synthesis of several short-chain hydroxamic acids and acid hydrazides

Acyl donor	Acyl acceptor	Final product	Enzyme ($g l^{-1}$)	Reaction time (min)	Molar conversion yield (%)	Final product ($g l^{-1}$)	Residual acyl donor ($g l^{-1}$)	Carboxylic acid g (l^{-1})
Acetamide (0.1 M)	hydroxylamine (0.5 M)	acetohydroxamic acid	0.04	30	86	6.5	0.6	0.2
	hydrazine (0.8 M)	acetic hydrazide	0.04	10	97	7.2	0.2	traces
Propionamide (0.1 M)	hydroxylamine (0.8 M)	propionohydroxamic acid	0.08	40	57	5.1	0.5	2.7
	hydrazine (1 M)	propionic hydrazide	0.08	60	72	6.4	0.2	1.9
Acrylamide (0.1 M)	hydroxylamine (0.3 M)	acrylohydroxamic acid	0.08	30	68	5.9	2.1	0.1
	hydrazine (0.5 M)	acrylic hydrazide	0.08	20	90	7.8	0.6	traces
Glycinamide (0.1 M)	hydroxylamine (0.5 M)	glycinehydroxamic acid	0.20	60	32	2.9	nd	nd
	hydrazine (0.5 M)	glycine hydrazide	0.20	nd	nd	nd	nd	nd

nd, not determined.

4. Discussion

Cloning of the wide spectrum amidase gene from *Rhodococcus sp.* R312 [31] and its overexpression in an *E. coli* strain made it possible to use this enzyme for the production of different high value-added molecules. However, the 'bi-bi-ping-pong' mechanism involved was submitted to several different constraints. The amidase-catalyzed reaction was submitted to steric constraints since the reaction rates and affinities decreased when the amide chain length increased. We thus concluded that the active site would be hard to reach. Hence, only very-short chain aliphatic acyl donors (less than four carbon atoms) were efficient substrates. The amidase-catalyzed reaction was also submitted to electronic constraints since, at pH 7, carboxylates and protonated glycinamide were harmful for the acyl transfer reaction. Only acetamide, acrylamide and propionamide at pH 7, and glycinamide at pH 8 could thus be considered as interesting substrates. However, it should be noted that acetamide was the most efficient acyl donor for transfer on hydroxylamine (or hydrazine), whereas propionamide was the most efficient one for transfer on water (i.e. hydrolysis). These results were surprising since the same mechanism was involved for acyl transfer on hydroxylamine (or on hydrazine) and on water. In a first step, the amide substrate reacted with the enzyme to give an acyl–enzyme complex which was then attacked by the nucleophilic agent: hydroxylamine, hydrazine, or water. This might be due to ionic interactions at pH 7 between the enzyme and hydrolysis reaction products (carboxylic acid pK_A from 4 to 5), whereas these interactions could not occur with the acyl transfer reaction products since the hydroxamic acid pK_A ranged from 7 to 9.5 [39]. Moreover, hydroxylamine and hydrazine are more potent nucleophilic agents than water and exhibit different affinities for the acyl–enzyme complexes. For these reasons, desacylation of the acyl–enzyme complex seemed to be the limiting step of the amidase-catalyzed acyl transfer reaction. We have also shown that a relative amide excess could result in enzyme inhibition but that this phenomenon was a function of the [amide]/[hydroxylamine] ratio. This could be explained by competition between amide and hydroxylamine for the acyl–enzyme complex, resulting in an inactive complex when two amide molecules are fixed on the enzyme (Fig. 7). The higher the affinity of the acyl–enzyme complex for hydroxylamine, the lower the inhibition by relative excess amide should be. This was the case when acrylamide was used as substrate.

With respect to the different acyl acceptors tested, only water, hydroxylamine and hydrazine were efficient for the acyl transfer reaction. *N*-methylhydroxylamine was sterically hindered, whereas methyl alcohol and ethanolamine were thermodynamically impossible. Potent small nucleophiles thus seem necessary for the limiting desacylation step.

Hydroxamic acids, as described at the begin-

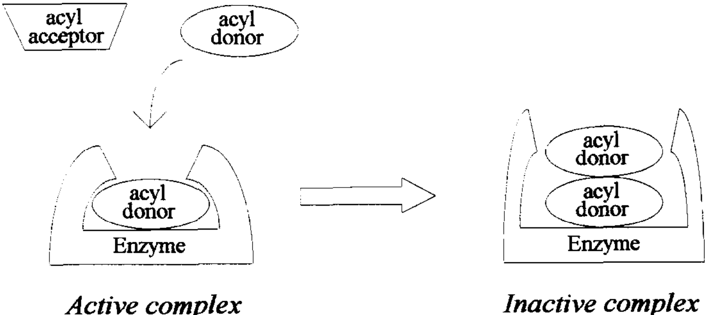


Fig. 7. Proposed mechanism for amidase inhibition by excess acyl donor relative to the acceptor.

ning of the paper, are chelating agents that are useful for many applications. Acid hydrazides are also chelating agents, and they have been extensively investigated. They are known to be tuberculostatic and antileprosy agents [40,41], antibacterial and antifungal compounds [42], and antitumor and anticancer agents [43]. Metal chelates of polyhydrazides have also been studied to determine their utility as thermally stable fibers and films [44]. All of these compounds are generally synthesized from a methyl, ethyl or butyl ester and hydrazine hydrate, either with or without the use of ethanol as additional solvent [40].

In conclusion, the study on the acyl transfer activity of the amidase from *Rhodococcus sp.* R312, overproduced in an *E. coli* strain, highlighted the interest of biological synthesis of hydroxamic acids and acid hydrazides. Reactions occur in aqueous medium and large amounts of final product can be obtained quite rapidly with a few tens of milligrams of enzyme per liter of reaction medium.

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