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Aliphatic Amidase from *Rhodococcus rhodochrous* M8 Is Related to the Nitrilase/Cyanide Hydratase Family

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Abstract—A comparative study of amino acid sequence and physicochemical properties indicates the affiliation of an amidase from *Rhodococcus rhodochrous* M8 (EC 3.5.1.4) to the nitrilase/cyanide hydratase family. Cluster analysis and multiple alignments show that Cys166 is an active site nucleophile. The enzyme has been shown to be a typical aliphatic amidase, being the most active toward short-chain linear amides. Small polar molecules such as hydroxylamine and O-methyl hydroxylamine can serve as effective external nucleophiles in acyl transfer reactions. The kinetics of the industrially important amidase-catalyzed acrylamide hydrolysis has been studied over a wide range of substrate concentrations; inhibition during enzymatic hydrolysis by the substrate and product (acrylic acid) has been observed; an adequate kinetic scheme has been evaluated and the corresponding kinetic parameters have been determined.

Key words: *Rhodococcus rhodochrous* amidase, sequence alignment, amidase classification, substrate specificity, acrylamide hydrolysis

Amidases belong to the hydrolase family, a subclass of acylamide amidohydrolases (EC 3.5.1, 3.5.2). These enzymes catalyze the hydrolysis of carboxylic acid amides to free carboxylic acids and free ammonium [1]. Certain amidases in conjunction with nitrile hydratases are involved in nitrogen exchange of pro- and eukaryotes utilizing nitriles. The reactions, which are catalyzed by these complex enzymes, are of primary interest for large scale production of acrylamide and acrylic acid in industry [2-4]. As amidases are studied, it becomes obvious that they exhibit fairly wide substrate specificity [5, 6], demonstrate stereoselectivity towards amides of α-substituted carboxylic acids [5-7], and catalyze acyl transfer reactions [6, 8, 9]. Enzymes in this group attract attention from scientists in various fields, for instance, in neurobiochemistry [10], industrial microbiology, and chirotechnology [5, 7, 11].

Amidases are still not sufficiently investigated and their classification is not definitely formulated. The classification based on substrate specificity [1] occasionally integrates enzymes with different structural organization, mechanism, and catalytic properties; for instance, the amidases from *R. rhodochrous* J1 [12] and *P. aeruginosa* [13] classified in the EC 3.5.1.4 group. Investigation of amino acid sequences of the enzymes cleaving the C–N bonds distinguishes four families: enzymes containing GGSS motif in the primary structure (family index PF01425, IPR000120), nitrilase/cyanide hydratase (family index PF00795, IPR000132), acyl transferases, and ureases [13, 14]. Along with other enzymes, each of these families contains amidases. For instance, the first family includes bacterial amidases from *R. rhodochrous* J1 [12], *Rhodococcus* sp. R312 [6, 14], *Sulfolobus solfataricus* [15], etc. Their amino acid sequences contain the invariant GGSS motif named amidase consensus (index in Prosite Dictionary PDOC00494; PS00571) [16]. These enzymes have quaternary structure and usually are organized as homodimers and homooctamers [12-14, 16]. The main role in the active site of its typical representative, amidase from *R. rhodochrous* J1, belongs to the invariant amino acid residues Asp191 and Ser195 [12]. These amidases exhibit wide specificity, they are active towards aliphatic and aromatic amides and amides of α-substituted carboxylic acids, and they exhibit stereospecificity [5, 12, 15].

In contrast, the bacterial amidases from the nitrilase/cyanide hydratase family are supposedly sulfhydryl enzymes [13, 17, 18]. These include amidases from *P. aeruginosa* [13], *B. stearothermophilis* [19], *R. erythropolis* [20], *Rhodococcus* sp. R312 [9, 20, 21], and others. The

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characteristic feature of their primary structure is the two conservative amino acid sequences named nitrilase/cyanide hydratase consensus (index in Prosite Dictionary PDOC00712; PS00920/1). Unlike amidases from the first family, the amidases from the nitrilase/cyanide hydratase family form homotetramers and homohexamers. The data available in the literature show that aliphatic amides are the preferred substrates for this enzyme group.

An amidase from *R. rhodochrous* M8 [22] was recently isolated and characterized, and its gene was cloned [23]. The first results of the study demonstrate significant differences between this enzyme and amidase from *R. rhodochrous* J1. The detailed comparison of these two amidases is of great interest: it is necessary to understand the differences in mechanisms of protein induction and production, similarities and differences in their catalytic mechanisms; establish the correlation between structure and function, as well as the patterns of quaternary structure formation and its role in catalysis. In this work a number of potential substrates for the new enzyme are considered, the kinetic principles of hydrolysis and acyl transfer reactions are investigated, and bioinformatic analysis of amino acid sequence of *R. rhodochrous* M8 amidase is performed.

MATERIALS AND METHODS

Analysis of protein amino acid sequences. *A search for homologous sequences* was performed using protein databases and the following software: BLAST and PSI-BLAST [24] (NCBI, www.ncbi.nlm.nih.gov) or FASTA [25] (EMBL, www.ebi.ac.uk).

Alignment of amino acid sequences was performed using CLUSTAL W v. 1.82 [26] (EMBL, www.ebi.ac.uk) software.

A search for conservative regions in the BLOCK database was performed via BLOCK Search software on SWISS-PROT server (http://www.ebi.ac.uk/swissprot) and using MACAW software [27].

Profile construction for the nitrilase/cyanide hydratase family was carried out using LINEUP, PILEUP, and PROFILEMAKE software. Profile specificity was verified by SWISS-PROT database using PROFILESEARCH software. All software is a part of the GCG v. 7.3 package [28].

A dendrogram of related proteins was constructed based on results of multiple alignment of amino acid sequences using CLUSTAL W v. 1.82 software at the EMBL site [26], and further refinement was done with Philip software package and the PromL program.

Protein secondary structure was predicted by hydrophobic cluster analysis [29] and the PredictProtein Server (www.embl-heidelberg.de/predictprotein) [30].

Data about enzymes and proteins were extracted from SWISS-PROT, PIR, and TrEMBL databases (Table 1).

Chemicals. The following reagents were used in this work: acrylamide (Reanal, Hungary); mercaptoethanol, mandelic acid, semicarbazide, O-methyl hydroxylamine hydrochloride, sodium azide (Fluka, Switzerland); phenylglycine (Sigma, USA); methanol, sodium dodecyl sulfate (Merck, Germany); acrylic acid, phenylacetic acid (Aldrich Chemie, Germany); dithiothreitol (Serva, Germany); phenylacetamide, phenoxyacetic acid (Reakhim, Russia); *o*-phthalic aldehyde (Koch Light, UK); amide of phenoxyacetic acid (Acros, Belgium); acetonitrile (Cryochrom, Russia); other reagents and buffer solution components were of domestic origin and chemically pure.

Homogeneous amidase preparation was obtained from E. L. Kotlova (Institute of Genetics and Selection of Industrial Microorganisms, Russia). The protein concentration in the solution was 1 mg/ml by the Bradford method [31]. Sample purity was characterized by using denaturing SDS electrophoresis in polyacrylamide gel by the Laemmli method [32]. Acyl transferase activity of amidase measured using acetyl hydroxamic acid was 2.9 μmol/min per mg protein at 37°C.

HPLC-analysis. The chromatographic system consisted of a Waters M6000 eluent supply module, Luna 5u C-18 column for the reverse-phase chromatography (250 × 4.6 mm, 5 μm) (Phenomenex, USA), Marathon autosampler (Spark Holland, The Netherlands), and LC-235 Diode Array detector (Perkin Elmer, USA). The chromatograms were recorded at 210 nm on a Multichrome software–hardware complex for acquisition and processing of chromatographic data (Ampersand, Russia). Flow rate was 0.5 ml/min. The mobile phase contained (v/v) 40% CH_3CN, 0.9 g/liter SDS, and 0.78 g/liter KH_2PO_4 (pH 3.0). Retention times (min): acrylamide (7.1), acrylic acid (9.0), N-acryloyl hydroxylamine (6.5), N-acryloyl-(O-methyl) hydroxylamine (8.0), phenylglycine (6.5), phenylglycine amide (15), N-acryloyl-phenylglycine amide (13.5), mandelic acid amide (8.0), mandelic acid (10), phenylacetamide (8.1), phenylacetic acid (14.5), phenylacetic acid amide (13), and phenoxyacetic acid (16).

Potentiometry. Potentiometric detection of ammonium ions was performed using an Expert-00-3 ionometer (NPO Ekonix-Expert, Russia) and ammonium selective electrode. The measurements were performed at fixed ionic strength of 0.1 M versus buffer solution containing magnesium sulfate.

Spectrophotometry. The concentration of primary amino groups was determined using modification by *o*-phthalic aldehyde and mercaptoethanol [33] on a UV-1601 spectrophotometer (Shimadzu, Japan).

Protocol for enzymatic reactions. Enzymatic hydrolysis and acyl transfer reactions were performed at 37°C according to the Michaelis–Menten scheme at $[S]_0 >> [E]_0$ in 0.2 M Tris-HCl buffer containing 0.1 M KCl, 1 mM dithiothreitol, and 0.02% NaN_3 (pH 7.5). The

reactions were carried out in a thermostatted cell of 719S Titrino pH-stat (Metrohm, Switzerland); the total volume of the reaction mixture was 3-5 ml. The concentration of the original substrates in the reaction mixture was varied in the range of 0.1-1000 mM; amidase concentration in the range of 0.01-0.3 mg/ml. Aliquots of the reaction mixture were collected after certain periods of time and analyzed by the HPLC-technique, spectrophotometry, and potentiometry.

RESULTS AND DISCUSSION

Study of primary structure. The search for homologous proteins in the databases using the primary structure of *R. rhodochrous* M8 resulted in a number of amino acid sequences (about 100) with identity level 82-28%. The first ten sequences with the highest identity level (82-75%) were amidases from the nitrilase/cyanide hydratase family. Thus, it was already in the first step of our investigation when the similarity between the primary structures of *R. rhodochrous* M8 amidase and proteins of the nitrilase family was found.

Based on a global multiple alignment of protein amino acid sequences listed in Table 1, a dendrogram of amidases and related proteins was constructed (Fig. 1). Nitrilase/cyanide hydratase and GGSS motif-containing amidase families include several groups. Together with other proteins, amidase from *R. rhodochrous* M8 forms the amidase group of the nitrilase family. According to primary structure, the studied enzyme is most similar to amidases from *Brevibacterium* sp. and *R. erythropolis*, with a common ancestor for these enzymes.

Five highly conservative regions were identified as a result of global and local multiple alignment of amino acid sequences of amidases from the nitrilase/cyanide hydratase family (Fig. 2). These include conservative amino acid residues Cys, Glu, Lys involved in a catalytic triad of *P. aeruginosa* active site, a representative of this family [17]. One should note common elements of the secondary structure (Fig. 2) and characteristic structure of nucleophilic region of the active site: C(DY)(DG) for amidases, and C(WA)E for nitrilases. These sulfhydryl elements supposedly act according to the classical "ping-pong" mechanism [8]. An inhibitory effect of specific sulfhydryl group reagents was shown for the studied amidase [22] and related enzymes [8].

Similarity between the primary structure of the studied amidase and amidases from nitrilase family, the existence of characteristic catalytic triad Cys-Glu-Lys, as well as common elements of the secondary structure show that members of this group of enzymes have similar properties.

Substrate specificity. Acrylamide hydrolysis. Large-scale production of acrylic acid by enzymatic hydrolysis of acrylamide is important in polymer industry. *Rhodococcus* strains are presently used for biocatalytic production of acrylamide and acrylic acid [3, 11, 22]. Special interest is aroused by enzymatic conversion at high substrate concentrations. In this connection, enzyme hydrolysis of acrylamide was investigated over a wide range of substrate concentrations (1-1000 mM) (Fig. 3). The maximum reaction rate was achieved already at initial substrate concentrations of 20-30 mM, however further increase in acrylamide concentration resulted in a decrease in hydrolysis rate. Kinetic analysis demonstrates that inhibition of enzyme activity is due to the binding of additional substrate molecules and formation of catalytically inactive ES_3 enzyme–substrate complex (Fig. 4). Such inhibition of enzyme activity was observed earlier in the case of amidohydrolases (EC 3.5.1.11) [34, 35]. Reversible inhibition of amidase activity by acrylamide [19] was recently found for the enzyme from *B. stearothermophilus* (nitrilase family, Table 1). Analysis of integral kinetic curves indicates the inhibition of enzyme hydrolysis by reaction product acrylic acid (the inhibition constant for the product is ~10 mM). Therefore, optimal conditions for acrylamide hydrolysis are determined by the kinetic parameters taking into account the influence of the system components. In general, the enzyme displays relatively high catalytic activity, but it has medium affinity towards acrylamide (15 mM), which is typical for related amidases as well (Table 2), and, obviously, is determined by the structure of the acyl moiety of the substrate. The GGSS motif-containing amidases catalyze acrylamide hydrolysis with lower efficiency.

Hydrolytic and transferase activity. Along with hydrolysis, there is considerable interest in enzymatic transfer of an acyl group on external nucleophile (for instance, hydroxylamine). Production of N-acryloyl-derivatives of amino compounds is one of the steps in production of polyacrylates with modified properties. Considering this, different acyl group donors were investigated as potential substrates for amidase in the reaction of acyl transfer, including aromatic and chiral α-substituted amides: acrylamide, semicarbazide, amides of phenylacetic, phenoxyacetic, (R)- and (S)-mandelic acid, and phenylglycine. The following compounds were investigated as acceptors: hydroxylamine, semicarbazide, methoxyamine, (±)-phenylglycine amide.

Enzymatic reaction of acyl group transfer from acrylamide to hydroxylamine is highly efficient and occurs practically without formation of hydrolysis product. The acyl transfer reaction to methoxyamine is less efficient, whereas phenylglycine amide does not act as an acceptor of acyl group at all. It should be noted that at high concentrations of amine components (more that 100 mM) non-enzymatic side processes were observed (especially in the case of hydroxylamine). Semicarbazide (a urea analog) is not hydrolyzed by amidase, but it inhibits acrylamide hydrolysis as well as that of urea itself [36, 37]. The fact that none of the investigated aromatic

Table 1. Characterization of amidases and related proteins by protein databases

Abbreviation	Enzyme	Source	Number of amino acid residues	EC	Characterization	Number in the database
		Nitrilase/cyanide hydratase family				
AMID_RHOR8	A	*R. rhodochrous* M8	349	3.5.1.4	aliphatic	—
AMIE_RHOER	A	*Bacillus sp.* BR 449	352	#	#	AF257487
AMIE_BACST	A	*B. stearotermophilus*	352	#	#	AF136599
AMIE_BACSP	A	*Brevibacterium sp.*	349	#	#	M76451
ALAM_RHOER	A	*R. erythropolis*	349	#	#	M88614
AMIE_HEPLPY	A	*Helicobacter pylori*	343	#	#	Y12252
NRL1_ARATH	N	*Arabidopsis thaliana*	360	3.5.5		P32961
NRL2_ARATH	N	#	342	#	#	P32962
NRL3_ARATH	N	#	349	#	#	P46010
NRL4_ARATH	N	#	357	#	#	P46011
NRLA_ALCFA	N	*Alcaligenes faecalis*	358	#	#	P20960
NRL1_RHORH	N	*R. rhodochrous*	3390	#	#	Q02068
NRL2_RHORH	N	*R. rhodochrous*	320	#	#	Q03217
CYHY_GLOSO	CH	*Gloeoceocospora sorghi*	361	3.5.9.9		P32964
CYHY_GIBBA	CH	*Fusarum lateritium*	372	#		P32963
1EMS	Protein	*Caenorhabditis elegans*	395	—	—	NP_005591
ALAM_PSEAE	A	*Pseudomonas aeruginosa*	346	3.5.1.4	#	M27612
		Amidase signature family				
AMES_RHOER	A	*Rhodococcus sp.* R312	521	3.5.1.4	enantios.	P22984
RERAG	A	*R. rhodochrous* J1	515	#	#	S38270
AMID_PSECL	A	*Pseudomonas chlororaphis*	506	#	#	P27765
RERAMDANTH	A	*Rhodococcus sp.*	462	#	#	M74531
RERNH	A	*R. erythropolis*	322	#	#	D14454
HYIN_PSESS	IAAH	*Pseudomonas syringae*	455	3.5.1		P06618
HYIN_AGRT3	IAAH	*Agrobacterium tumephaciens*	467	3.5.1		P03868
HYIN_AGRT4	IAAH	*A. tumephaciens*	467	3.5.1		P11922
HYIN_BRAJA	IAAH	*Bradyrhizobium japonicum*	465	3.5.1		Q04557
HYIN_VITIS	IAAH	*A. vitis*	462	3.5.1		Q09102
HYIN_AGRRA	IAAH	*A. rhizogenes*	466	3.5.1		L33866
HYIN_ERWIN	IAAH	*Erwinia herbicola*	460	3.5.1		P13398
NYLA_PSES8	AH	*Pseudomonas sp.* NK87	495	3.5.2.12	nylon	P13397
NYLA_FLASP	AH	*Flavobacterium sp.* K172	493	3.5.2.12	#	P13397
ANAMDS	A	*Aspergillus nidulans*	548	3.5.1.4	aliphatic	P08158
AOAMDS	A	*As. oryzae*	545	3.5.1.4		JS0633
AMD_Y2	A	*Sacharomyces cerevisiae*	464	3.5.1.4		530350
		Urease family				
URE1_MYSTU	U	*Mycobacterium tuberculosis*	577	3.5.1	urea	P50042

Note: A, amidase; CH, nitrile hydratase; N, nitrilase; IAAH, indole acetamido hydrolase; AH, 6-aminohexanoate cyclic dimer hydrolase; U, urease; enantios., enantioselective.

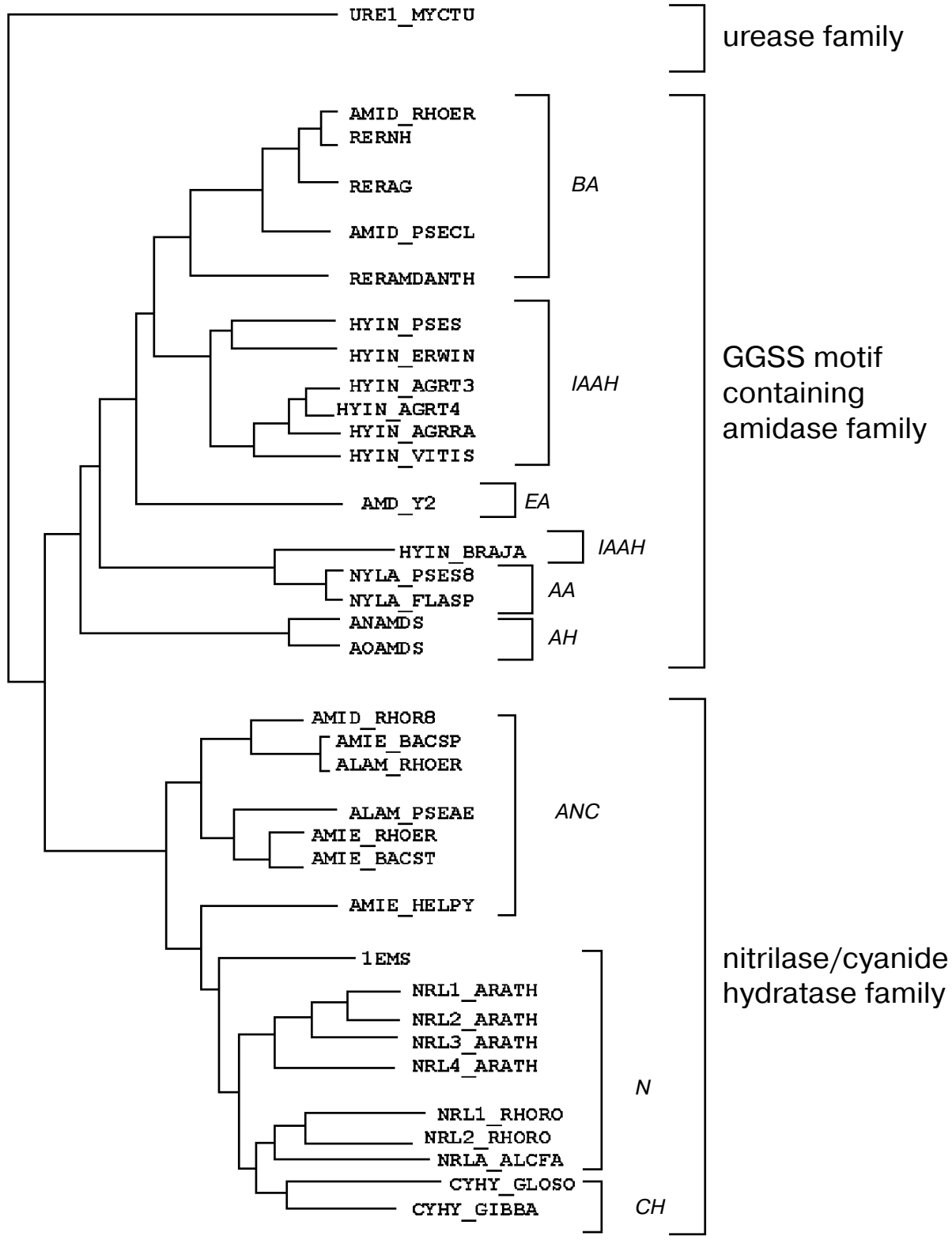

Fig. 1. Dendrogram of amidases and related proteins (see Table 1). Enzyme family names are presented according to the commonly accepted nomenclature [14]. Abbreviations for related enzyme groups: BA, bacterial amidases; IAAH, indole acetamide hydrolase; EA, eukaryotic amidase; AH, nylon oligomer cleaving hydrolases; AA, acetamidases and related eukaryotic amidases; ANC, amidases from the nitrilase/cyanide hydratase family; N, nitrilases; CH, nitrile hydratases.

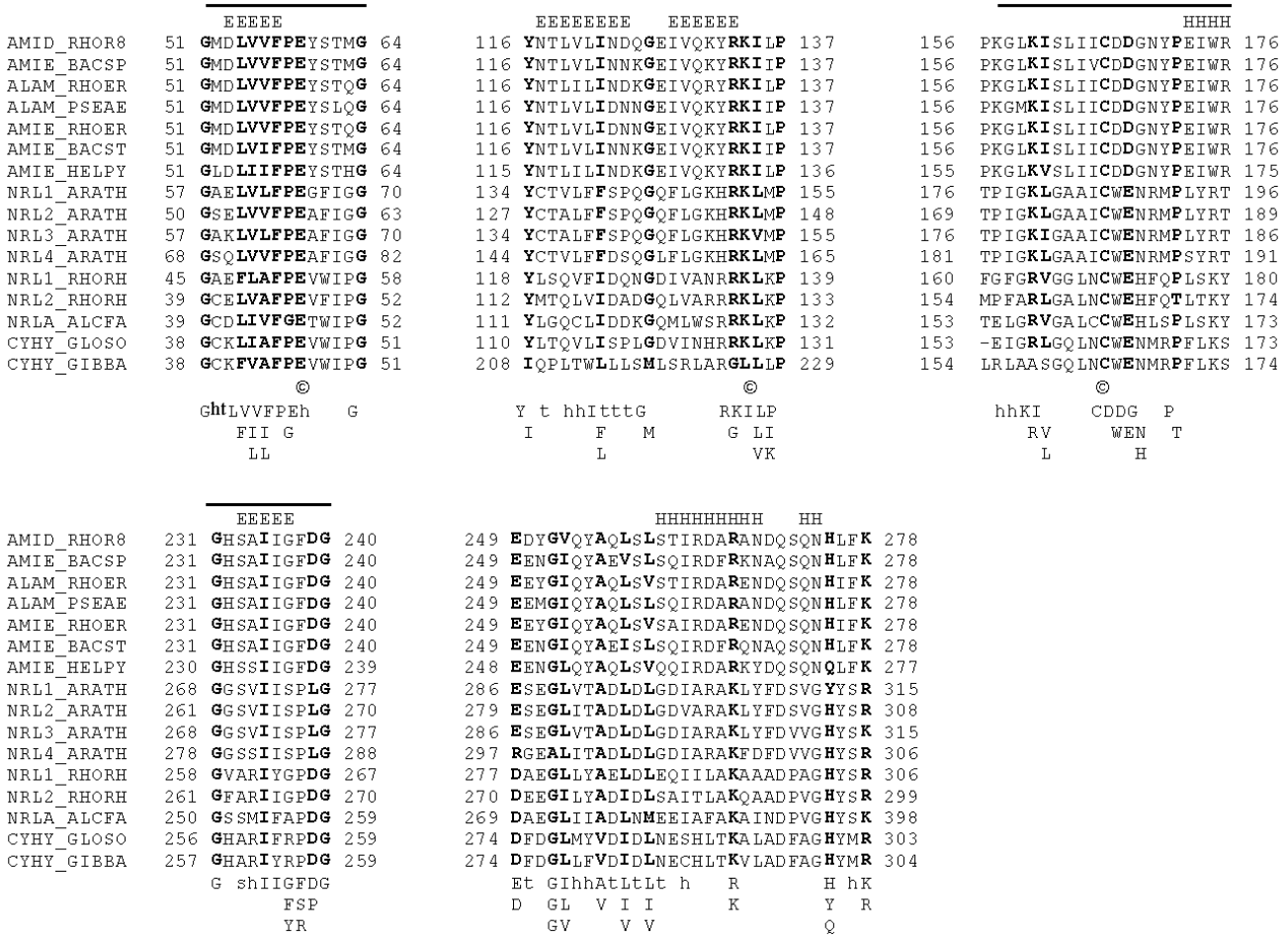

Fig. 2. Multiple alignment of amino acid sequences for amidases from the nitrilase/cyanide hydratase family. Enzyme abbreviations are presented in Table 1. Invariant amino acid residues are marked with bold. © sign indicates the supposed amino acid residues of the active site [17]. Upper line shows the secondary elements: E (β-structures), H (α-helices). Three lower lines show alignment consensuses: one main and three the most frequently encountered amino acid residues (h, hydrophobic residues; t, polar residues; s, small residues). The most conservative residues are marked with the bar above sequences.

amines are donors of an amine group in the reactions catalyzed by amidase should be pointed out.

Summarizing the data accumulated to date, it can be concluded that in contrast to amidases containing the GGSS motif [5, 6, 15], amidases from the nitrilase/cyanide hydratase family [9, 19, 21, 22] efficiently catalyze reactions with aliphatic amides and are not specific towards the substrates with large aromatic acyl moiety.

Table 2. Kinetic parameters of acrylamide hydrolysis catalyzed by amidases of different families

Source of amidase	k_{cat}, $\mu M \cdot sec^{-1} \cdot mg^{-1}$	K_m, mM	$k_{cat}/K_m \times 10^{-6}$, $sec^{-1} \cdot mg^{-1}$	Experimental conditions	Reference
R. rhodochrous M8*	2.9	15	193	pH 7.5, 37°C	—
Rhodococcus sp. R312*	2.5	34	74	pH 7.0, 30°C	[9]
B. stearothermophilus BR388*	0.13	8.4	15	pH 7.0, 50°C	[19]
R. erythropolis MP50**	0.016	—	—	pH 7.5, 30°C	[5]
*S. solfataricus***	0.17	3.0	57	pH 7.5, 70°C	[15]

* Nitrilase/cyanide hydratase family.
** GGSS motif containing amidase family.

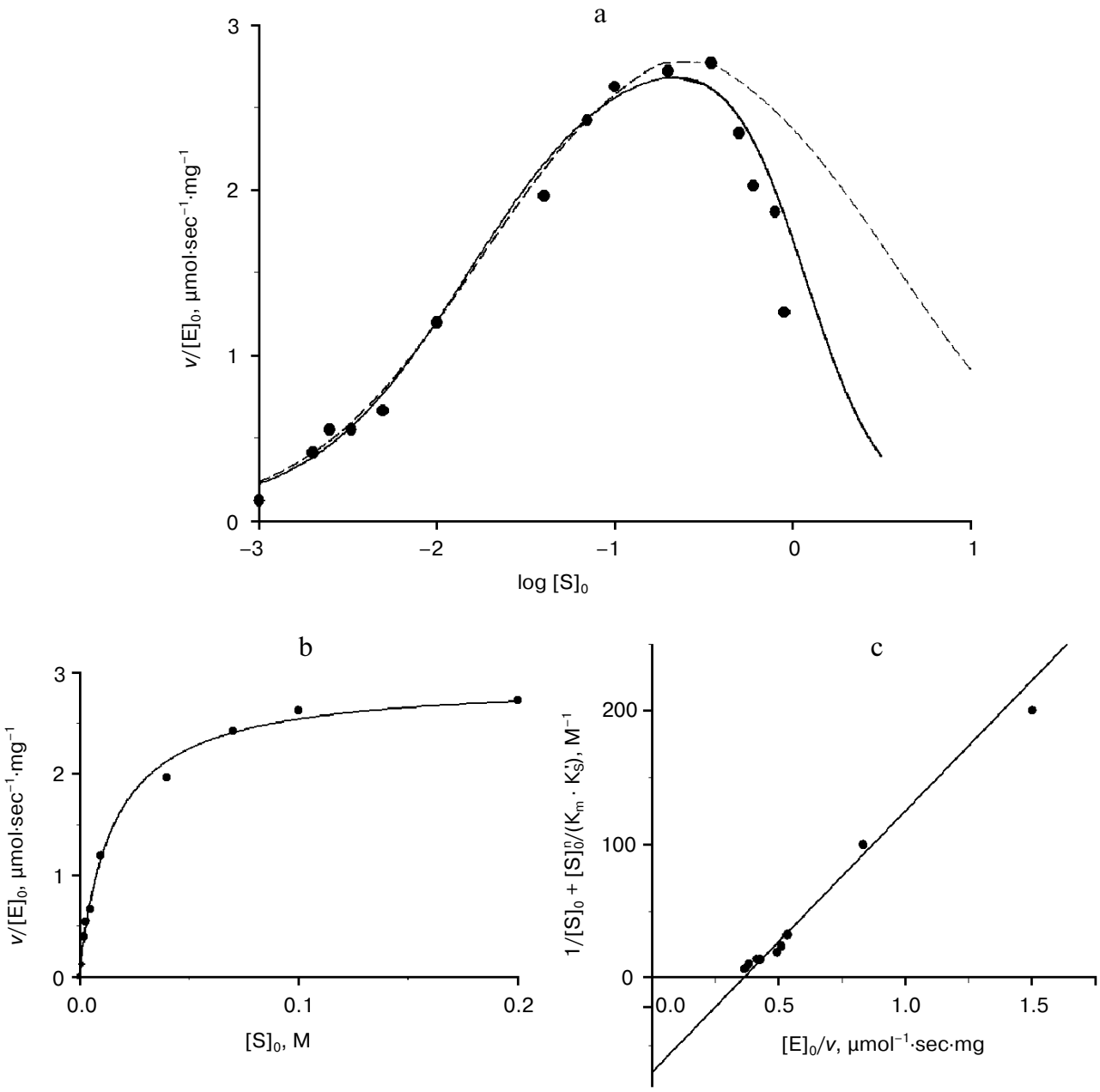

Fig. 3. Dependence of the initial rate of acrylamide hydrolysis catalyzed by amidase from *R. rhodochrous* M8 on initial concentration of the substrate (a). The experimental data are presented with symbols: the line describing the experimental points was calculated in accordance with minimal kinetic scheme (Fig. 4) by the equation: $v = V_{max} \cdot [S]_0/(K_S + [S]_0 + [S]_0^2/K_S')$. The dashed line corresponds to the formation of inactive ES_2 enzyme–substrate complex. The values of K_m = 15 mM and V_{max} = 2.9 μmol/sec per mg were determined from experimental data at low substrate concentrations (b). The value of K_S' = 1.4 M was determined at high substrate concentrations according to the equation: $1/[S]_0 + [S]_0^2/(K_m \cdot K_S') = V_{max}/K_m \cdot v^{-1} - 1/K_m$ (c). Agreement between experimental data and the model is achieved only in the case of formation of ES_3 enzyme–substrate complex. Experimental conditions: pH 7.5, 37°C, ionic strength 0.1 M.

Thereby, specificity towards a nucleophile in acyl transfer reactions catalyzed by both amidase families is obviously limited by small polar molecules such as hydroxylamine and hydrazine. It is surprising that even ethanolamine and methyl hydrazine, having similar structures, do not act as acyl group acceptors [6, 9]. However, the question about the substrate specificity of amidases from the two families towards the acyl group donor as well as acceptor should not be closed. Further investigations of structure-function relationships including three-dimensional enzyme organization are needed.

The performed analysis allows classifying the amidase from *R. rhodochrous* M8 as a member of the nitrilase/cyanide hydratase family and assuming that

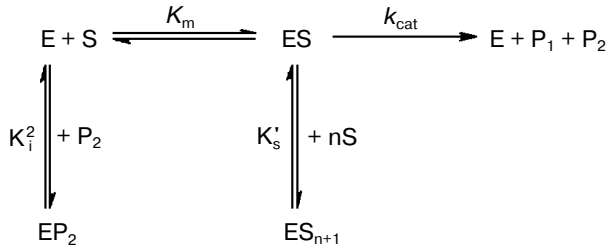

Fig. 4. "Minimal" kinetic scheme for amidase catalyzed acrylamide hydrolysis. E is amidase, S is acrylamide, ES is enzyme–substrate complex, P_1 and P_2 are hydrolysis products (ammonium and acrylic acid, respectively), EP_2 is enzyme complex with acrylic acid, ES_{n+1} is catalytically inactive enzyme complex with additional substrate molecules (ES_3 for amidase from *R. rhodochrous* M8).

Cys166 is the active site nucleophile. Inhibition of activity by specific sulfhydryl-group reagents, specificity towards aliphatic amines, and quaternary structure characteristic for proteins from this family all support this assumption. Effective catalysis of acrylamide hydrolysis as well as acyl transfer is obviously a special feature of aliphatic amidases from the nitrilase/cyanide hydratase family, but the question of the boundaries of substrate specificity of enzymes from this family requires further investigations.

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