Guanidinoacetate Amidinohydrolase from a *Flavobacterium* Purification, Crystallization and Properties

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Guanidinoacetate amidinohydrolase (glycocyaninase, EC 3.5.3.2) was purified 148-fold and crystallized from *Flavobacterium* sp. GE-1 grown in a medium supplemented with guanidinoacetate. The molecular weight of the enzyme was estimated to be about 281,000 daltons by a gel electrophoretic procedure. SDS-polyacrylamide electrophoresis showed a single component of subunit with an estimated molecular weight of 70,000 daltons, suggesting that the enzyme is composed of four subunits identical in molecular weight. These values are significantly distinct from those of an analogous enzyme from a *Pseudomonas* reported by us previously. The native enzyme was active without added divalent metals. Incubation of the enzyme with 1,10-phenanthroline at an elevated temperature (50°C) resulted in almost complete inactivation. The activity of the inactivated enzyme was restored by incubation with Zn^{2+} or Co^{2+} at 50°C; the former cation was more effective. The enzyme was optimally active at pH 8.0~8.5. The apparent K_m value for guanidinoacetate was 15 mM. 3-Guanidinopropionate and 4-guanidinobutyrate were hydrolyzed 19% and 9%, respectively, as fast as guanidinoacetate. PCMB was a potent inhibitor of the enzyme. The properties of the *Flavobacterium* enzyme were compared with those of the analogous *Pseudomonas* enzyme.

Guanidinoacetate amidinohydrolase (glycocyaninase, EC 3.5.3.2) catalyzes the hydrolytic cleavage of guanidinoacetate to glycine and urea. We have purified two distinct guanidinoacetate amidinohydrolase from bacteria. In previous papers^{1,2)} we reported the properties of crystalline guanidinoacetate amidinohydrolase from *Pseudomonas* sp. ATCC 14676, which required Mn^{2+} for the activity. The present paper describes the crystallization and properties of the other amidinohydrolase produced by a strain of *Flavobacterium*. The native enzyme did not require any divalent cations for the activity.

MATERIALS AND METHODS

Organism. *Flavobacterium* sp. GE-1 was isolated from garden soil with a medium which contained guanidinoacetate as the sole nitrogen source. The bacterial

strain was tentatively assigned to the genus *Flavobacterium* on the basis of the following observations: cells gram-negative, short rods without flagella and non-motile; colonies translucent, smooth, light yellow water-insoluble pigmentation, and no swarming on agar media; strictly aerobic and growth temperature optimum below 30°C; nitrate reducing, catalase and oxidase positive.

Chemicals. 3-Guanidinopropionic acid was purchased from Calbiochem, 4-guanidinobutyrate from Sigma, creatine and agmatine from Merck, and guanyl-urea and N-amidinoalanine from Eastman. DEAE-cellulose was the product of Whatman (DE-23). Sephadex G-25 and Sephadex G-200 were from Pharmacia. Horse heart cytochrome C, bovine pancreas chymotrypsinogen A, beef liver catalase and ferritin were from Boehringer Mannheim. Ovalbumin and bovine serum albumin were from Sigma. Other chemicals were of reagent grade and purchased from Nakarai Chemicals.

Growth of cells. The basal medium for cultivation of *Flavobacterium* GE-1 contained 0.5% glycerol, 0.2% NaCl, 0.1% K_2HPO_4 , 0.02% $MgSO_4 \cdot 7H_2O$ and 0.05% yeast extract (pH 7.5). The inoculum culture was grown at 30°C for 14 hr in 2-liter shaking flasks which contained 500 ml of basal medium supplemented with 0.4%

Abbreviations: TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo) tetraacetate; DTNB, 5,5'-dithiobis(*p*-nitrobenzoate); PCMB, *p*-(chloromercuri)benzoate.

peptone. For cultivation of cells used in enzyme preparation, two inoculum cultures were prepared and transferred into 15 liters of basal medium supplemented with 0.04% guanidinoacetic acid. Incubation was carried out at 30°C for 6~10 hr under aeration with a Hitachi SH-5S air compressor (40 liters per min) through a sterilized air filter. When guanidinoacetate disappeared from the medium the cells were harvested by continuous centrifugation at $12,000 \times g$ and washed twice with 0.85% NaCl.

Enzyme assay. As the native guanidinoacetate amidohydrolase from *Flavobacterium* GE-1 did not require divalent cations for maximal activity, the enzyme was assayed without adding divalent metal salts. The assay solution contained, in 1.0 ml, 0.01~0.1 unit of the amidohydrolase, 100 mM Tris-HCl buffer, pH 8.0, and 25 mM guanidinoacetic acid. After incubation at 30°C for 30 min, 1.0 ml of 12.5% TCA was added into the solution to terminate the reaction. The precipitate was centrifuged off and the supernatant was applied to urea determination.

Analytical methods. Guanidinoacetate and other guanidino compounds were determined by a colorimetric procedure using α -naphthol and diacetyl.³⁾ Urea was determined by a simplified procedure⁴⁾ of Archibald.⁵⁾ Protein concentration in crude or slightly purified extract was measured according to the method of Lowry *et al.*⁶⁾ with bovine serum albumin as standard, using an extinction coefficient $E(1\%)$ at 280nm of 6.7. With more extensively purified preparation, protein concentration was estimated from absorbance at 280 nm using $E(1\%)$ values of 10 for enzyme of 10 to 100 units/mg and 8.4 for crystalline preparations. The latter value was determined by counting Rayleigh fringes observed with interference optics on centrifugation of crystalline enzyme in a synthetic boundary cell.⁷⁾ A value of specific refractive increment for protein of 1.874 dl/g⁸⁾ was used for the calculation.

RESULTS

Purification of enzyme

All manipulations were at 0°~5°C unless stated otherwise. Frozen cell paste (500 g wet weight) was thawed in 2 liters of 20 mM potassium phosphate buffer, pH 8.0. The suspension was sonically treated in 300-ml batches for 90 min at 0°~10°C with Kaijo Denki 19 kHz sonic disintegrator, maintaining the pH at about 8.0 with 3 N ammonium hydroxide. The sonicate was centrifuged for 30 min at $10,000 \times g$. The supernatant (1400 ml) retained as the crude extract was brought to 30% saturation

by adding 293 g of solid ammonium sulfate with mechanical stirring; the pH was maintained at about 8.0 with 3 N ammonium hydroxide. The mixture was stored overnight at 4°C and the precipitate formed was removed by centrifugation. To the supernatant (1460 ml) was added 189 g of ammonium sulfate to 55% saturation. After 2 hr of stirring, the precipitate was collected by centrifugation and dissolved in 100 ml of 10 mM potassium phosphate buffer, pH 8.0. The enzyme was dialyzed against three changes of 2 liters each of 10 mM potassium phosphate buffer, pH 7.2, and then applied to a DEAE-cellulose column (4×29 cm) equilibrated with the dialysis buffer. The column was washed with 1000 ml of 10 mM potassium phosphate buffer, pH 7.2, supplemented with 0.3 M KCl; then the enzyme was eluted with the buffer containing 0.4 M KCl at a flow rate of 65 ml per hr. Active fractions were pooled (300 ml) and concentrated by adding 142 g of ammonium sulfate to 70% saturation, maintaining the pH at 7.5~8.0 with 3 N ammonium hydroxide. The precipitate formed during overnight storage at 4°C was collected by centrifugation and dissolved in 5 ml of 10 mM potassium phosphate buffer, pH 8.0 (DEAE-cellulose

FIG. 1. Chromatography of the Enzyme on a Sephadex G-200 Column.

The experimental conditions are given in the text. Absorbance at 280 nm (●); amidohydrolase activity (○).

FIG. 2. Crystalline Guanidinoacetate Amidohydrolase from *Flavobacterium* GE-1. $\times 600$.

fraction). The fraction was applied to a column of Sephadex G-200 (2×93 cm) which had been equilibrated with 10 mM potassium phosphate buffer, pH 8.0. The enzyme was eluted with the same buffer at hydrodynamic pressure of 10 cm. The elution pattern is shown in Fig. 1. Fractions 34, 35 and 36 were pooled (Fraction A) and other active fractions of lower specific activities (fractions 32, 33, 37 and 38) were also pooled (Fraction B). Both fractions were concentrated in a collodion bag to about 5 ml. To Fraction A was added ammonium sulfate until a faint turbidity was obtained. The crystalline enzyme precipitated after the solution was stored overnight in a refrigerator. A smaller amount of crystalline enzyme was obtained from Fraction B by adding ammonium sulfate and a bit of the

crystals from Fraction A. The crystals were colorless, thin plates as shown in Fig. 2. The purification procedure is summarized in Table I.

Purity and molecular weight

Polyacrylamide gel electrophoresis of the enzyme was conducted by use of gels containing 6, 7, 8 and 9% polyacrylamide and the Tris-glycine pH 8.3 buffer system of Hedrick and Smith.⁹⁾ As shown in Fig. 3, the crystalline

FIG. 3. Polyacrylamide Gel Electrophoresis of the Purified Enzyme.

Polyacrylamide gels at the indicated concentrations with a Bis: acrylamide monomer ratio of 1: 30 were prepared according to Hedrick and Smith⁹⁾ using the pH 8.3 Tris-glycine buffer system. The sample consisted of 40 μ l of 10 mM potassium phosphate buffer, pH 8.0, containing 60 μ g of the crystalline enzyme, 20 μ l of 50% glycerol, 20 μ l of 0.01% bromophenol blue and 20 μ l of electrode buffer to a final volume of 100 μ l. Aliquots (50 μ l) of the sample were directly applied to the small pore gels, and electrophoresis was conducted at a current of 2 mA per gel. Protein bands were stained with Amido Black.

enzyme showed a single protein band when stained with Amido Black. Two molecular weight determinations by the procedure of Hedrick and Smith⁹⁾ gave values of 280,000 and 282,000 daltons; the average value was 281,000 daltons. The second experiment is summarized in Fig. 4.

Subunit structure

Subunit structure of the enzyme was examined by polyacrylamide gel electrophoresis with SDS according to Weber and Osborn.¹⁰⁾ The gels contained 10% polyacrylamide and 0.1% SDS. The enzyme (40 μ g) was incubated

TABLE I. SUMMARY OF ENZYME PURIFICATION

Fraction	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	16,900 ^a	21,600	1.28	100
Ammonium sulfate (35~55%)	2,860 ^a	17,200	6.00	80
DEAE-cellulose	273 ^b	12,200	44.7	56
Sephadex G-200	28 ^b	4,430	158	21
Crystallization	19 ^c	3,610	190	17

^a Determined by the method of Lowry *et al.*⁸⁾

^b Determined from A_{280} and $E(1\%) = 10.0$

^c Determined from A_{280} and $E(1\%) = 8.4$.

FIG. 4. Molecular Weight Determination by the Method of Hedrick and Smith.⁹⁾

Electrophoresis was performed by the same manner described in Fig. 3. The reference proteins, ferritin (F), beef liver catalase (CA), bovine serum albumin (B), and bovine pancreas chymotrypsinogen A (CH) were treated similarly. E, guanidinoacetate amidinohydrolase; M. W., molecular weight; R_m , relative mobility.

overnight at 25°C in 0.2 ml of 10 mM potassium phosphate buffer, pH 7.2, which contained 25% glycerol, 1.0% SDS and 5% 2-mercaptoethanol. The solution was then mixed with the same volume of 0.05% bromophenol blue

FIG. 5. Subunit Size Determination by SDS-polyacrylamide Disc Electrophoresis.

Electrophoresis was performed according to Weber and Osborn¹⁰⁾ with 10% polyacrylamide gels. The conditions are given in the text. The reference proteins were horse heart cytochrome C (CY), bovine pancreas chymotrypsinogen A (CH), ovalbumin (OA), beef liver catalase (CA) and bovine serum albumin (B). E, guanidinoacetate amidinohydrolase; M. W., molecular weight.

before application to gels. Marker proteins were treated in the same manner. Aliquots (10~40 μ l) of the samples were applied on top of the gels and electrophoresis was conducted at 8 mA per tube. The electrophoretograms of the enzyme showed a single protein band when stained in 0.5% Amido Black dissolved in 20% acetic acid, and destained electrophoretically. The molecular weight of the dissociated enzyme was estimated to be 70,000 daltons (Fig. 5). These results suggest that the enzyme consists of four subunits identical in molecular weight.

Absorption spectrum of enzyme

The absorption spectrum was taken with a solution of the purified enzyme (167 μ g per ml) in 10 mM potassium phosphate buffer, pH 8.0. The enzyme exhibited a typical protein spectrum with an absorption maximum at 279 nm and a A_{279}/A_{248} ratio of 1.56.

Stoichiometry and irreversibility of reaction

The stoichiometry of the amidinohydrolase reaction was examined by use of a 10-ml reaction mixture. The component were the same as those of the standard assay system. The mixture containing 3.0 units of the enzyme (106 units/mg) was incubated at 30°C for 180 min. Aliquots of 1.0 ml were withdrawn at 30 min intervals and mixed with 1.0 ml of 12.5% TCA, and then the solution was analyzed for guanidinoacetate, urea and glycine. Glycine was determined with ninhydrin by the procedure of Moore and Stein.¹¹⁾ Approximately equimolar amounts of urea and glycine were detected in each sample. The sum of the concentrations of guanidinoacetate and urea or glycine equaled 25.9 ± 1.0 mM at each time point studied.

The reaction mixture for studies on reversibility of the reaction contained 5.3 units of the enzyme (106 units/mg), 100 mM potassium phosphate buffer, pH 8.0, 25 mM glycine and 25 mM urea in 4.0 ml. The mixture was incubated at 30°C. Aliquots of 1.0 ml were withdrawn at 0, 30, 60 and 100 min after initiation of reaction and mixed with 1.0 ml each

of 12.5% TCA, followed by colorimetry for guanidino compounds. Although the enzyme level was about 10 times greater than routine assays, neither guanidinoacetate nor other guanidino compounds were detected at each time point studied. These results indicate that the reaction is apparently irreversible under the conditions applied.

Stability of enzyme on heat treatment

Heat treatment was performed by incubating the enzyme at 55°C for 15 min in various buffer; the pH values ranged from 4.0 to 11.0. Figure 6 shows that the enzyme was stable at pH from 5.5 to 10.0. A slight activation was observed in potassium phosphate buffer, pH 5.5~6.5, and in Tris-HCl buffer, pH 8.0~8.5.

FIG. 6. Effects of pH and Buffers on the Stability of the Enzyme.

The incubation mixture contained, in 0.5 ml, 40 mM concentration of the indicated buffer and 1.0 μ g of the enzyme (134 units/mg). After incubation at 55°C for 15 min, a 0.2-ml aliquot of the mixture was analyzed for amidinohydrolase activity.

Effect of pH on reaction rate

Figure 7 shows that the enzyme is active at neutral to alkaline pH; optimally active at pH 8.0~8.5. The activity at pH 7.0 was about 70% of the maximal activity.

Effect of metal chelators on enzyme

Most amidinohydrolases require divalent metals for maximal activity. Although guanidinoacetate amidinohydrolase of *Flavobacterium* GE-1 did not require any divalent

FIG. 7. Effects of pH and Buffers on the Enzyme Activity.

The reaction mixture contained 100 mM concentration of the indicated buffer and 0.8 μ g of the enzyme (134 units/mg). Other conditions were the same as in the standard assay.

FIG. 8. Inactivation of the Enzyme with 1,10-Phenanthroline.

The incubation mixture contained, in 2.0 ml, 10 mM potassium phosphate buffer, pH 8.0, various concentrations of 1, 10-phenanthroline and 5.3 μ g of the enzyme (106 units/mg). The mixture was incubated at 50°C for the time indicated, and then, a 0.2-ml aliquot was assayed for amidinohydrolase activity. *o*-Phe, 1, 10-phenanthroline.

metals, it seemed possible that the enzyme contained tightly bound cations.

The enzyme was incubated with various metal chelators for 30 min at 30°C in 10 mM potassium phosphate buffer, pH 8.0. With 4.0 mM 1,10-phenanthroline, the activity decreased by about 30% during incubation but no decrease in activity was observed with EDTA or 2,2'-dipyridyl. Incubation with 1,10 phenanthroline in Tris-HCl buffer, pH 9.0, or in potassium phosphate buffer, pH 6.0, gave

similar results. Complete inactivation was accomplished by incubating the enzyme at 50°C for 30 min in 10 mM Tris-HCl buffer, pH 8.0, in the presence of 5.0 mM 1,10-phenanthroline (Fig. 8). No inactivation was observed with EDTA, 2,2'-dipyridyl or 8-hydroxyquinoline-5-sulfonate even at elevated temperature.

Resolution of enzyme and effect of divalent metals on apoenzyme

The apoenzyme was prepared as described in Fig. 9. When EDTA was omitted from the elution buffer used on Sephadex G-25 chromatography, the activity of the eluted enzyme was below 10% of the original activity. During storage for 8 days at 0°C without addition of divalent metals, the enzyme was reactivated to above 90% of original activity. Reactivation was also observed after incubation of the enzyme at 50°C for 90 min. No reactivation was observed, however, after incubation at 50°C in the presence of 1.0 mM EDTA. Tests with diluted EDTA showed that the presence

FIG. 9. Effect of ZnCl_2 and CoCl_2 on the 1,10-Phenanthroline-treated Enzyme.

The preincubation mixture contained 640 μg of the enzyme (150 units/mg), 10 mM Tris-HCl buffer, pH 8.0, and 5.0 mM 1,10-phenanthroline in 4.0 ml. After incubation at 50°C for 30 min, the mixture was applied to a Sephadex G-25 column (1.2 $\times$ 25 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 1.0 mM EDTA. The enzyme was eluted with the equilibration buffer, and the resultant enzyme was retained as 1,10-phenanthroline-treated enzyme. The assay mixture contained 50 mM Tris-HCl buffer, pH 8.0, 1.0 μM EDTA, various concentrations of ZnCl_2 or CoCl_2 , 0.17 μg of the treated enzyme (3.0 units/mg) and 25 mM guanidinoacetate in 1.0 ml. Other conditions were the same as in the standard assay system.

of 10 μM EDTA was sufficient to prevent apparent spontaneous reactivation. These results indicate that the apoenzyme of the aminohydrolase was formed by incubation with 1,10-phenanthroline and suggest that restoration of the activity of 1,10-phenanthroline-treated enzyme in the absence of EDTA was due to the presence of unknown metals which activated the apoenzyme.

The effect of divalent cations on the apoenzyme was examined by adding various metal salts into the standard assay mixture at a concentration of 0.5 mM. ZnCl_2 and CoCl_2 were effective in activating the enzyme. The following metal salts had no activating effect: CaCl_2 , CdCl_2 , CuSO_4 , FeCl_3 , HgCl_2 , MgSO_4 , MnCl_2 and NiCl_2 . Figure 9 shows the activity of the enzyme assayed immediately after the addition of various concentrations of ZnCl_2 or CoCl_2 . The activity was restored to about 65% and 16% of original activity with 10 μM ZnCl_2 and 20 μM CoCl_2 , respectively. An almost complete reactivation was observed after incubation of the apoenzyme at 50°C for 10 min with 8.0 μM ZnCl_2 (Fig. 10). The highest activity at-

FIG. 10. Reactivation of the 1,10-Phenanthroline-treated Enzyme with ZnCl_2 or CoCl_2 .

The preincubation mixture contained, in 0.5 ml, 100 mM Tris-HCl buffer, pH 8.0, 2.0 μM EDTA, 0.17 μg of the 1,10-phenanthroline treated enzyme (3.0 units/mg, original activity 150 units/mg) and metal salt; 8.0 μM ZnCl_2 (A), 4.0 μM ZnCl_2 (B), 20 μM CoCl_2 (C) or 8.0 μM CoCl_2 (D). The mixture was incubated at 50°C for the time indicated. After cooling, 0.5 ml of 50 mM guanidinoacetate was added to the mixture to initiate the enzyme reaction. Other conditions were the same as in the standard assay system.

tained with CoCl_2 was 66%, which was obtained after incubation at 50°C for 20 min in the presence of 8.0 μM of the salt.

Substrate specificity

The ability of the enzyme to hydrolyze various guanidino compounds was examined under conditions designed to detect the formation of urea at or above 1% of the rate observed with guanidinoacetate. Urea released from 3-guanidinopropionate and from 4-guanidinobutyrate was 19% and 9%, respectively, of that released from guanidinoacetate. No urea was formed from N-amidinoalanine, L-arginine, agmatine or creatine.

Km values

The apparent K_m value of the enzyme for guanidinoacetate, determined under the standard conditions, except for the concentration of the substrate, was 15.0 ± 2.0 mM. The K_m values for Zn^{2+} and Co^{2+} could not be determined because conditions for maximal activation of the apoenzyme with various cation concentrations were complex, as described earlier, e.g., in Fig. 10. The data shown in Fig. 9 and Fig. 10 suggest that the K_m values for Zn^{2+} and Co^{2+} are below 5 μM .

TABLE II. EFFECT OF VARIOUS COMPOUNDS ON ENZYME ACTIVITY

The indicated compound was added into the standard assay mixture (1.0 ml) which contained 0.32 μg of the enzyme (132 units/mg). Before addition of the substrate solution (0.5 ml), the mixture was preincubated at 30°C for 3 min.

Compound	Concentration (mM)	Inhibition (%)
Creatine	5.0	3
N-Amidinoalanine	5.0	4
L-Arginine	5.0	21
Agmatine	5.0	21
Guanylsurea	5.0	5
Taurine	10.0	8
β -Alanine	10.0	10
4-Aminobutyrate	10.0	19
5-Aminovalerate	10.0	20
6-Aminocaproate	10.0	16
Monoiodoacetate	1.0	16
N-Ethylmaleimide	1.0	11
DTNB	1.0	25
PCMB	1.0	93

Inhibitors

The effect of various compounds upon the hydrolysis rate of guanidinoacetate was tested and is shown in Table II. Creatine and N-amidinoalanine, their structures are closely related to substrate guanidinoacetate, failed to affect the reaction rate. No appreciable influence was found with various ω -amino acids tested. Among sulfhydryl reagents tested, PCMB was a potent inhibitor of the enzyme, while monoiodoacetic acid and N-ethylmaleimide exhibited almost no effect on the enzyme. DTNB inhibited the reaction slightly.

DISCUSSION

Guanidinoacetate amidinohydrolase was purified to homogeneity from *Flavobacterium* sp. GE-1 and crystallized. The native form of the enzyme was fully active without the addition of any divalent cation, in contrast to an analogous enzyme from a *Pseudomonas*.¹⁾ Incubation of the *Flavobacterium* enzyme with 1,10-phenanthroline at 50°C resulted in a complete loss of activity though the chelator affected the enzyme very weakly at 30°C. The activity lost was restored not only by incubation with Zn^{2+} or Co^{2+} but also by incubation without the addition of divalent cations. If the binding of 1,10-phenanthroline to the enzyme produced inactivation and if reactivation was due to dissociation of the reagent from the enzyme, reactivation must be observed not only with Zn^{2+} or Co^{2+} but also with Fe^{3+} or other cations which can form complexes with 1,10-phenanthroline. As described above, the 1,10-phenanthroline-treated enzyme was specifically reactivated with Zn^{2+} or Co^{2+} . The fact that the apparently spontaneous reactivation was prevented by the addition of 10 μM EDTA suggests the presence of active cations which contaminated the enzyme solution. It seems difficult to assume that EDTA specifically prevents the dissociation of the enzyme-bound 1,10-phenanthroline. Thus, we believe that the 1,10-phenanthroline-treated enzyme was the apoenzyme of guanidinoacetate amidinohydrolase.

In the previous paper¹⁾ we reported on the properties of crystalline guanidinoacetate amidinohydrolase from *Pseudomonas* sp. ATCC 14676. The molecular weight of the *Flavobacterium* enzyme was estimated to be 271,000 compared to 163,000, the value for the *Pseudomonas* enzyme. Both enzymes are composed of four subunits of identical molecular weights. The *Flavobacterium* enzyme can hydrolyze 3-guanidinopropionate and 4-guanidinobutyrate at lower rates, while the *Pseudomonas* enzyme is highly specific for guanidinoacetate. Unlike the Mn^{2+} dependent *Pseudomonas* enzyme, the apoenzyme of *Flavobacterium* guanidinoacetate amidinohydrolase was activated with Zn^{2+} or Co^{2+} . Guanidinosuccinate amidinohydrolase from *Pseudomonas chlororaphis*¹²⁾ and *Bacillus* L-arginase¹³⁾ were reported to be activated with Co^{2+} , while Zn^{2+} has not been reported as a specific activator of amidinohydrolases. The guanidinoacetate amidinohydrolase from *Flavobacterium* shares features in common with the analogous *Pseudomonas* enzyme: a relatively high value of K_m for substrate, an alkaline optimal pH and strong inhibition by PCMB.

Both *Pseudomonas* and *Flavobacterium* guanidinoacetate amidinohydrolases are induced by guanidinoacetate. We have observed that 4-guanidinobutyrate induces 4-guanidinobutyrate amidinohydrolase in *Flavobacterium* GE-1, as well as in *Pseudomonas* sp. ATCC 14676. Although guanidinoacetate amidinohydrolase from the strain of *Flavobacterium* exhibits weak activity toward 4-guanidinobutyrate, a crude preparation of 4-guanidinobutyrate amidinohydrolase from the same organism does

not exhibit guanidinoacetate amidinohydrolase activity (unpublished observation). Although these observations suggest that the two guanidinoacetate amidinohydrolases play the same role in both organisms, this paper demonstrated significant differences in molecular weight, subunit size and substrate specificity. Thus, the two enzymes may be an example of biochemical diversity among analogous enzymes.

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