Metabolism of Basic Amino Acids in *Pseudomonas putida*

**γ GUANIDINOBUTYRATE AMIDINOHYDROLASE***

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**SUMMARY**

γ-Guanidinobutyrate amidinohydrolase, the third enzyme of L-arginine catabolism in *Pseudomonas putida*, has been purified 6% fold from extracts of cultures grown on L-arginine. Synthesis of the amidinohydrolase is induced by growth on L-arginine or γ-guanidinobutyrate, but not by growth on γ-aminobutyrate or on urea plus D,L-malate.

Hydrolysis of γ-guanidinobutyrate to γ-aminobutyrate and urea proceeds essentially to completion at pH 7 to 11. The purified enzyme specifically requires Mn2+ and exhibits optimum activity at about pH 10 and 50°C. The Km for γ-guanidinobutyrate is 32 mM at pH 10. While 6-guanidinovalerate and e-guanidinocaproate also are hydrolyzed, the value of both Km (δ-guanidinovalerate = 206 mM; ε-guanidinocaproate = 163 mM) and of Vmax (606, 27, and 11 e.u. per mg for γ-guanidinobutyrate, δ-guanidinovaleurate, and ε-guanidinocaproate, respectively) suggest that γ-guanidinobutyrate is the natural substrate. The molecular weight of the purified enzyme was estimated to be 178,000 by gel permeation chromatography and 100,000 by sucrose density gradient centrifugation.

γ-Guanidinobutyrate, formed in marine invertebrates by oxidation of L-arginine by L-amino acid oxidase (L-amino-acid:oxygen oxidoreductase (deaminating), EC 1.4.3.2) (1) and in mammalian tissues by transamidination of γ-aminobutyrate (2, 3), is hydrolyzed to γ-aminobutyrate and urea by amidinohydrolases of animal, plant, and microbial cells. The best characterized γ-guanidinobutyrate amidinohydrolases are those of chicken kidney and liver (4), lizard liver (4), ray liver (5, 6), snail hepatopancreas (7), *Xtreptomyces griseus* (8), and *Penicillium roqueforti* (9, 10). γ-Guanidinobutyrate amidinohydrolase has been purified 85- and 96-fold from extracts of chicken kidney and liver respectively, 42-fold from lizard liver, and is in all three cases distinct from L-arginase (L-arginine amidinohydrolase, EC 3.5.3.1). The Km for γ-guanidinobutyrate in crude extracts of chicken kidney and liver is about 30 mM, and the purified chicken liver enzyme is activated by Mn2+ (4). The molecular weight of the purified chicken liver enzyme is about 252,000 (4). γ-Guanidinobutyrate amidinohydrolase purified 32-fold from ray liver is optimally active toward γ-guanidinobutyrate, but also catalyzes hydrolysis of the next lower and of the two next higher homologs of α-guanidinobutyrate and of their α-hydroxy derivatives (5, 6). The γ-guanidinobutyrate amidinohydrolase present in crude extracts of *S. griseus* catalyzes hydrolysis of the next higher and lower homologs of the substrate, and γ-guanidinobutyramide is not a substrate (8). The corresponding amidinohydrolase of the hepatothecae of the snail *Helix pomatia*, present primarily in the mitochondria, has been purified 200-fold, is optimally active at pH 9.5, is activated by Mn2+ but not by Co2+ or Cd2+ and is heat-stable (7). While the γ-guanidinobutyrate amidinohydrolase of *P. roqueforti* has not been purified, its reported properties (optimum temperature = 40°C; optimum pH = 7.5 to 8.5) (10) differ significantly from those of other γ-guanidinobutyrate amidinohydrolases and from that reported here.

The intermediates formed initially during catabolism of L-arginine by *Pseudomonas putida* are (11):

\[
\begin{align*}
\text{L-Arginine} & \rightarrow \text{γ-ketoarginine} \\
\text{γ-guanidinobutyrate} & \rightarrow \text{γ-aminobutyrate}
\end{align*}
\]

We report here the purification and properties of *P. putida* γ-guanidinobutyrate amidinohydrolase, which catalyzes Reaction 3 of the above pathway.

**MATERIALS AND METHODS**

Reagents—n-Ornithine, crystalline beef heart lactate dehydrogenase (l-lactate:NAD oxidoreductase, EC 1.1.1.27) and crystalline rabbit skeletal muscle pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) were from Sigma. Urea was from Baker Chemical, α-isonitrosopropiophenone from K and K Laboratories, bovine serum albumin from Mann, DEAE-cellulose (Cellex D) from Bio-Rad Laboratories, and Sephadex G-200 from Pharmacia. All other chemicals were from previously listed sources (11).

ε-Guanidinocaproic Acid—ε-Guanidinocaproic acid methyl ester, 180 mg, was refluxed for 45 min in 5.0 ml of 0.45 N NaOH in 95% ethanol, then cooled to 5°C. The white precipitate which formed was collected by filtration, washed with 95% ethanol,
liminary assays for γ-guanidinobutyrate amidinohydrolase activ-
tures were revised to include Mn$^{2+}$. The concentration selected,
bovine serum albumin as a standard.

Determination of γ-Guanidinobutyrate—γ-Guanidinobutyrate
was determined by a general colorimetric procedure for guanido
compounds (13). γ-Aminobutyrate and urea, the products of the
reaction, neither reacted nor interfered with color develop-
ment of γ-guanidinobutyrate standards.

Determination of Urea—Urea was determined by the method of
Archibald (14). γ-Guanidinobutyrate and γ-aminobutyrate
neither reacted nor interfered with color development of urea
standards.

Measurement of Protein Concentration—Protein concentra-
tion was measured according to the method of Lowry et al. (15) with
bovine serum albumin as a standard.

Assay of γ-Guanidinobutyrate Amidinohydrolase Activity—Pre-
liminary assays for γ-guanidinobutyrate amidinohydrolase activity
were conducted at suboptimal concentrations of Mn$^{2+}$. Since
crude extracts were activated by addition of MnSO$_4$, assay mix-
tures were revised to include Mn$^{2+}$. The concentration selected,
0.134 mm MnSO$_4$, gives maximal activity. Standard assay
mixtures contained, in 0.5 ml: 150 mmol of glycine buffer, pH
10.0, 67 mmol of MnSO$_4$, 20 mmol of γ-guanidinobutyrate,
and 0.005 to 0.05 unit of γ-guanidinobutyrate amidi-
nohydrolase. The final pH was 9.7. Incubation at 30° was
terminated after 30 min by adding 0.3 ml of 10% HClO$_4$.
Precipitated protein was removed by centrifugation and the super-
natant liquid was decanted and analyzed for urea. Specific
activity is expressed throughout as micromoles of urea formed per
min per mg of protein. Analogous conditions were employed to
study hydrolysis of substrate analogs by substituting the appro-
priate analog for γ-guanidinobutyrate at the concentrations in-
dicated in table and figure legends.

Detection of Other Enzymic Activities—Lactate dehydrogenase
activity was assayed by observing the pyruvate-dependent rate of
NADH oxidation at 340 nm (16) and pyruvate kinase activity
by the method of Bücher and Pfieiderer (17). The rate of
release of ammonia from t-arginine, a measure of the activity of
the first enzyme of arginine catabolism in Pseudomonas, and the
rate of $^{14}$CO$_2$ release from α-ketoglutaric acid, a measure of the
second enzyme of arginine catabolism in Pseudomonas, were
measured as previously described (11).

Organism and Growth Conditions—P. putida P2 (ATCC 25571)
can grow at the expense of a variety of amino acids including lys-
ine, ornithine, and arginine (11, 18, 19). The amidinohydrolase
activity of cultures grown on l-arginine in Ionic Medium (20)
varied widely from culture to culture. Since arginine and γ-
 guanidinobutyrate amidinohydrolase from other sources were known
to require Mn$^{2+}$, we investigated the effect of additional
MnSO$_4$ on γ-guanidinobutyrate amidinohydrolase activity. Ex-
trates of cultures grown on Ionic Medium were activated when
incubated for 30 min at 25° with excess MnSO$_4$. The Mn$^{2+}$
concentration of Ionic Medium (0.002 mm) is far below that required
for optimal amidinohydrolase activity. The growth medium
was therefore modified to provide adequate Mn$^{2+}$, and "Modified
Ionic Medium" (Ionic Medium containing 0.1 mm MnSO$_4$) was
used for all subsequent studies.

Cultures were grown with vigorous shaking in 2.8 liter Fern-
back flasks containing 500 ml of Modified Ionic Medium con-
taining 25 mm l-arginine as the sole source of carbon and nitro-
gen. Large scale cultures were grown in 12 liters of medium of the
same composition with a New Brunswick model FS 307 fer-
mentor. Growth was followed turbidimetrically with a Klett
photometer with a red (No. 54) filter. Cells harvested in late log
phase growth by centrifugation at 6500 x g for 10 min were
washed once by resuspension in Modified Ionic Medium at 5°,
centrifuged again, and either stored frozen as a cell paste or resus-
pended for immediate use.

Purification of γ-Guanidinobutyrate Amidinohydrolase—All
manipulations were at 0–5° unless otherwise stated. A 3.5%
(w/v) suspension of fresh or frozen cells in Modified Ionic Me-
dium was disrupted by six 30-sec bursts of sonic energy in a Bran-
son model S-125 sonic oscillator (Branson Instruments, Inc.,
Stamford, Conn.) at a power setting of 5. The sonic extract was
centrifuged for 20 min at 10,000 x g and discarded. The supernatant
liquid contained the "crude extract." Crude extract was placed in
a water bath at 62°, stirred until its temperature reached 60°,
maintained at 60 ± 1° for 5 min, and then cooled rapidly in an ice
bath. Precipitated protein was removed by centrifugation for 20 min at 10,000 x g and discarded. The supernatant liq-
uid ("60° fraction") was dialyzed for 24 hours against three
changes of 50 volumes of 10 mM Tris-HCl0.20 mM MnSO$_4$ buffer,
pH 8.0 (Tris-Mn buffer). A portion, 65 ml, was applied to a
column, 2.3 x 27 cm, of DEAE-cellulose equilibrated with Tris-
Mn buffer and washed in with 170 ml of 0.10 M KCl in Tris-Mn
buffer. The column was then eluted with a KCl gradient. The
mixing chamber contained 250 ml of 0.10 M KCl in Tris-Mn
buffer and the reservoir contained 0.60 M KCl in Tris-Mn buffer.
Fractions, 10 ml, were collected at a flow rate of about 70 ml
per hour. Fractions in the region 0.3 to 0.4 M KCl, which to-
gether contained about 70% of the applied activity, were con-
bined, placed in dialysis tubing, and buried in Carbowax 20 M
overnight to reduce the volume. Concentration was continued
until the protein concentration reached at least 10 mg per ml.
Concentrated "DEAE-fraction" was dialyzed overnight against
500 volumes of Tris-Mn buffer and a portion, 1.0 ml, applied to a
column, 2.5 x 50 cm, of Sephadex G-200 equilibrated with Tris-
Mn buffer. The column was eluted with Tris-Mn buffer at a
flow rate of 15 ml per hour. Active fractions, which contained
about 60% of the applied activity, were combined and concent-
trated against Carbowax to give the "Sephadex G-200 fraction." 
Table I summarizes a typical purification.

At protein concentrations of or above 3 mg per ml, all frac-
tions are stable to storage at −20° for at least 1 month.

Acrylamide Gel Electrophoresis—Samples containing 80 μg of
protein in 10 mM Tris-HCl buffer, pH 7.5, applied to 7.5% acry-
amide-0.32% N,N'-methylenebisacrylamide gels at pH 5.3 were
electrophoretically treated at 4 mA per tube until the tracking
dye was 0.6 cm from the bottom of the gel. For visualization of
proteins, gels were stained for 1 hour in 1.0% naphthol blue-
black in 7.5% acetic acid, destained electrophoretically, and
examined in a Gifford model 2410 Linear Transport Scanning spec-
trophotometer. To detect amidinohydrolase activity, slices of
unstained gels were macerated in 1.0 ml of 0.2 mM MnSO$_4$ in 10

1 The amidinohydrolase specific activity of crude extracts of cultures
grown at 2, 20, and 200 μM MnSO$_4$ were 0.44, 1.4, and
2.7, respectively.
butyrate, or urea plus DL-malate were used to inoculate 500 ml of
Ionic Medium containing the substrates listed below at 25
mm Tris-HCl, pH 7.5, incubated at 30° for 1 hour, centrifuged,
and the supernatant liquids were assayed for amidinohydrolase activity.

Sucrose Density Gradient Centrifugation—Sucrose gradients were 5 to 20% (w/v). Tube 1 contained 20 μg of L-lactate dehydrogenase in 0.1 ml of 10 mm Tris-HCl buffer, pH 7.5, tube 2 contained 50 μg of γ-guanidinobutyrate amidinohydrolase in 0.1 ml of the same buffer, and tube 3 contained 0.1 ml of each enzyme solution. Centrifugation in a Spinco SW 39 rotor was for 12 hours at 37,500 rpm. The molecular weight of the amidinohydrolase was calculated according to the method of Martin and Ames (21).

RESULTS

Induction of γ-Guanidinobutyrate Amidinohydrolase Activity—Extracts of cultures grown on L-arginine or γ-guanidinobutyrate, but not of cultures grown on γ-aminobutyrate or on an urea plus DL-malate, catalyze the γ-guanidinobutyrate amidinohydrolase reaction (Table II). Since the specific activity did not vary significantly during the latter stages of growth, cultures were grown on 25 mm L-arginine and harvested during late log phase growth to obtain enzyme for purification.

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\[ \text{γ-Guanidinobutyrate + H}_2\text{O} \rightarrow \text{γ-aminobutyrate + urea} \]

Extent of γ-Guanidinobutyrate Hydrolysis—To ascertain the extent to which γ-guanidinobutyrate is hydrolyzed, a large excess of enzyme was used in an experiment otherwise similar to that of Fig. 1. Incubation mixtures contained, in 0.5 ml: 100 μmole of γ-guanidinobutyrate, 2.0 units of amidinohydrolase, and either 150 μmole of Tris-HCl, pH 7.5, or 150 μmole of glycine-HCl at pH 9, 10, or 11. Analysis of portions removed after 12 hours at 30° for urea and for residual γ-guanidinobutyrate was performed as described in Fig. 1. Recovery of urea ranged from 18.0 to 19.9 μmole (90 to 99% theory) while levels of residual γ-guanidinobutyrate were essentially undetectable (<0.05 μmole). To attempt to show reversibility, incuba-

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total enzyme units (μmol/min/mg)</th>
<th>Total protein (mg)</th>
<th>Specific activity (μmol/min/mg)</th>
<th>Enrichment fold</th>
<th>Recovery of activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>70</td>
<td>4480</td>
<td>1000</td>
<td>4.48</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>00° fraction</td>
<td>65</td>
<td>3250</td>
<td>260</td>
<td>12.5</td>
<td>2.8</td>
<td>72.5</td>
</tr>
<tr>
<td>DEAE-fraction</td>
<td>14</td>
<td>2520</td>
<td>28</td>
<td>90.0</td>
<td>20</td>
<td>56.0</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>10</td>
<td>1670</td>
<td>5.6</td>
<td>303</td>
<td>68</td>
<td>37.0</td>
</tr>
</tbody>
</table>

### Table II

γ-Guanidinobutyrate amidinohydrolase activity of cultures grown on L-arginine, γ-guanidinobutyrate, or γ-aminobutyrate

Cultures grown on L-arginine, γ-guanidinobutyrate, γ-aminobutyrate, or urea plus DL-malate were used to inoculate 500 ml of Ionic Medium containing the substrates listed below at 25 mm initial concentration. The 50,000 × g supernatant liquids from cultures harvested at the indicated bacterial densities were assayed for amidinohydrolase activity.

\[ \text{γ-Guanidinobutyrate + H}_2\text{O} \rightarrow \text{γ-aminobutyrate + urea} \]

Extent of γ-Guanidinobutyrate Hydrolysis—To ascertain the extent to which γ-guanidinobutyrate is hydrolyzed, a large excess of enzyme was used in an experiment otherwise similar to that of Fig. 1. Incubation mixtures contained, in 0.5 ml: 100 μmole of γ-guanidinobutyrate, 2.0 units of amidinohydrolase, and either 150 μmole of Tris-HCl, pH 7.5, or 150 μmole of glycine-HCl at pH 9, 10, or 11. Analysis of portions removed after 12 hours at 30° for urea and for residual γ-guanidinobutyrate was performed as described in Fig. 1. Recovery of urea ranged from 18.0 to 19.9 μmole (90 to 99% theory) while levels of residual γ-guanidinobutyrate were essentially undetectable (<0.05 μmole). To attempt to show reversibility, incuba-

\[ \text{γ-Guanidinobutyrate + H}_2\text{O} \rightarrow \text{γ-aminobutyrate + urea} \]
tions containing, in 1.0 ml: 150 μmoles of glycine buffer, pH 10, 200 μmoles of MnSO₄, 4.1 units of γ-guanidinobutyrate amidinohydrolase, 20 μmoles of γ-aminobutyrate, and 20 μmoles of urea were incubated at 30° for 12 hours. Essentially no formation of γ-guanidinobutyrate was detected (< 0.01 pmole).

Optimum pH and Temperature—γ-Guanidinobutyrate amidinohydrolase is optimally active at about pH 9.9 (Fig. 2) and at about 50°.

Activation by MnSO₄—Since supplementation of the growth medium with MnSO₄ produced improved yields of amidinohydrolase, we attempted to remove bound Mn²⁺ and to establish the concentration of Mn²⁺ required for optimum activity. As shown in Table III, 0.12 mm MnSO₄ gives maximal reactivation.

Substrate Specificity and Kinetic Parameters for Substrates—The ability of various guanidine compounds to serve as substrates at pH 10.0 was tested under conditions designed to detect their hydrolysis at or above 50% of the rate observed with γ-guanidinobutyrate. Incubations contained, in 0.5 ml: 100 μmoles of glycine buffer, pH 10.0, 20 μmoles either of a γ-guanidinobutyrate analog or other guanidino compound, pH 10.0, and 0.027 unit of amidinohydrolase. The urea released was measured after 30 min incubation at 30°. Urea released from δ-guanidinovalerate

![Figure 2: Effect of pH](image2)

**Table III**

| Addition          | γ-Guanidinobutyrate amidinohydrolase activity | Specific activity | Fraction of that present initially %
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MnSO₄, 200 μm</td>
<td></td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>MnSO₄, 0 μm</td>
<td></td>
<td>69</td>
<td>24</td>
</tr>
<tr>
<td>MnSO₄, 20 μm</td>
<td></td>
<td>158</td>
<td>54</td>
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<tr>
<td>MnSO₄, 40 μm</td>
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<td>108</td>
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</tr>
<tr>
<td>MnSO₄, 120 μm</td>
<td></td>
<td>209</td>
<td>72</td>
</tr>
<tr>
<td>MnSO₄, 200 μm</td>
<td></td>
<td>209</td>
<td>72</td>
</tr>
<tr>
<td>MgSO₄, 200 μm</td>
<td></td>
<td>79</td>
<td>27</td>
</tr>
<tr>
<td>Co(NO₃)₂, 200 μm</td>
<td></td>
<td>64</td>
<td>23</td>
</tr>
</tbody>
</table>

* Activity of enzyme dialyzed for an equivalent time versus 10 mM Tris-HCl, pH 8.0, containing 0.2 mM MnSO₄, rather than against EDTA.

![Figure 3: Chromatography](image3)

**Table IV**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH during assay</th>
<th>Kₚₑₜ</th>
<th>Vₗₚₑₜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-Guanidinobutyrate</td>
<td>9</td>
<td>41</td>
<td>320</td>
</tr>
<tr>
<td>γ-Guanidinobutyrate</td>
<td>10</td>
<td>32</td>
<td>606</td>
</tr>
<tr>
<td>γ-Guanidinobutyrate</td>
<td>11</td>
<td>46</td>
<td>358</td>
</tr>
<tr>
<td>δ-Guanidinobutrate</td>
<td>10</td>
<td>206</td>
<td>27</td>
</tr>
<tr>
<td>δ-Guanidinobutrate</td>
<td>10</td>
<td>163</td>
<td>11</td>
</tr>
</tbody>
</table>

![Graph](image4)
and from ε-guanidinocaproylase was 11 and 6% that released from γ-guanidinobutyrate. The kinetic constants for all three substrates are summarized in Table IV. No hydrolysis (less than 2% that observed with γ-guanidinobutyrate) of guanidinocetate, β-guanidinopropionate, L-α-amino-γ-guanidinobutyrate, L- or D-arginine, L-homoarginine, or agmatine was detected. Compounds which failed to serve as substrates were, with a single exception, without detectable effect on hydrolysis of γ-guanidinobutyrate. In an experiment similar to that described above, an 8-fold molar ratio of guanidinocetate, β-guanidopropionate, L-α-amino-γ-guanidinobutyrate, L- or D-arginine, or L-homoarginine did not significantly alter the rate of γ-guanidinobutyrate hydrolysis. Some inhibition (20%) was, however, observed with agmatine.

**Molecular Weight**—A value of 178,000 ± 3,000 was obtained by gel exclusion chromatography on Sephadex G-200 (Fig. 3) and of 190,000 by sucrose density gradient centrifugation.

**DISCUSSION**

The hydrolysis of γ-guanidinobutyrate proceeds essentially to completion at all pH values tested (pH 7 to 11). The purified enzyme has a molecular weight of 178,000 to 190,000 compared to 252,000 reported for the analogous enzyme from lizard liver (4). Unlike the amidinohydrolase from ray liver (5, 6), the purified Pseudomonas amidinohydrolase exhibits a high order of substrate specificity. The minimal structural requirements for a substrate appear to be:

\[
\text{NHN}^+ + \text{H}_2\text{N}\text{C}-\text{N}^2-\text{(CH)}_n\text{C}-\text{COO}^- \\
\]

\[
n = 3-5
\]

Of the substrate analogs tested, only δ-guanidinovalerate and ε-guanidinocaproylase were hydrolyzed, and both the \(K_m\) and \(V_{\text{max}}\) values for these compounds suggest that the natural substrate is γ-guanidinobutyrate. The inability of L-arginine, L-homoarginine, or L-α-amino-γ-guanidinobutyrate to act either as substrates or as inhibitors suggests, furthermore, that the presence of an α amino group may preclude binding at the active site.

As reported for P. rouxii (10), the enzyme hydrolyzes both L- and D-arginine, D-homoarginine, or L-α-amino-γ-guanidinobutyrate to act either as substrates or as inhibitors suggests, furthermore, the presence of an α amino group may preclude binding at the active site.

**REFERENCES**

Metabolism of Basic Amino Acids in *Pseudomonas putida* : γ-GUANIDINOButyrate Amidinohydrolase
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