Purification and Some Properties of Glutaminase from *Pseudomonas nitroreducens* IFO 12694

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Received January 29, 1996

Glutaminase (EC 3.5.1.2) was isolated from *Pseudomonas nitroreducens* IFO 12694 grown on 0.6% sodium glutamate as a nitrogen source (325-fold purification, 13% yield). The molecular weight of the enzyme was estimated to be 40,000 by gel filtration and SDS-gel electrophoresis. The enzyme hydrolyzed glutamine optimally at pH 9, and its *Km* was 6.5 mM. d-Glutamine, γ-glutamyl p-nitroanilide, γ-glutamylmethionylamide, γ-glutamylthiophenylamide (theanine), and glutathione showed relatively 107, 85, 78, 74, and 82% reactivity of glutamine. Zn2+, Ni2+, Cd2+, Co2+, Fe2+, and Cu2+ repressed the enzyme activity strongly.

Glutaminase formed γ-glutamylhydroxamate in the reaction mixture containing glutamine and hydroxylamine (transferring reaction). The optimum pH of the transferring reaction was 7–8, and the *Km* for glutamine and hydroxylamine were 4 mM and 120 mM, respectively. γ-Glutamyl derivatives hydrolyzable by glutaminase showed reactivity for the transferring reaction. Methylamine or ethylamine was replaceable for hydroxylamine with 3 or 8% reactivity. The effect of divalent cations was not so striking as in the hydrolyzing reaction.

Key words: glutaminase; *Pseudomonas nitroreducens*; γ-glutamyl transferring reaction

Glutamine and glutamic acid play pivotal roles in the metabolism of nitrogenous compounds: (1) ammonia is converted to glutamine and glutamic acid by many microorganisms and plants through a combined action of glutamine synthetase and glutamate synthase. (2) the α-amino group of most amino acids come from glutamic acid, and (3) glutamine contributes its side-chain nitrogen in the first step of the biosynthesis of a wide range of physiologically important intermediates.

We have investigated metabolic activities of ammonia, glutamine, and glutamic acid in some fermentation bacteria, because the activities relate to their practical use by influencing their growth and probably other metabolic activities. A study from a similar viewpoint might be necessary for denitrifying bacteria, which take an important part in the natural nitrogen cycle. However, the understanding of the organisms is scanty compared to that of their reducing activity of nitrogen oxides.6–10 In this paper, as a part of such investigation, we describe a glutaminase (1-glutamine amidohydrolase, EC 3.5.1.2) of a denitrifier, *Pseudomonas nitroreducens* IFO 12694.

Glutaminase, which hydrolyzes glutamine to form glutamic acid and ammonia, is distributed widely in life. Animal and some bacterial enzymes have been investigated from a physiological or medical point of view.11–27 Glutaminase is also interesting in its reactivity, because not a few glutaminases transfer the γ-glutamyl moiety of glutamine to an acceptor as does glutamine glutamyltransferase (EC 2.3.2.1) or γ-glutamyltransferase (EC 2.3.2.2). Comparative studies of their transferring reaction were described with *Escherichia coli* glutaminase as a representative.28,29 but information on the transferring reaction of glutaminase from other sources has been insufficient. Though the physiological role of the transferring reaction is obscure, Yan6o et al.30 and Tomita et al.31 suggested, in their works on *Aspergillus oryzae* glutaminase, the significance of the reaction in soy sauce fermentation.32,33

Materials and Methods

*Microorganisms, cultivation, and preparation of crude enzyme. Pseudomonas nitroreducens* IFO 12694 was grown in a medium3 containing 1% glucose, 0.6% nitrogen source, 0.1% yeast extract, 0.05% KH2PO4, 0.05% K2HPO4, 0.07% MgSO4·7H2O, and 0.01% EDTA·Fe·7H2O·7H2O. Cultivation was done reciprocally at 30 °C with 1 L of the medium in a 2-L Sakaguchi flask. The nitrogen source was changed in the kind and or the concentration to examine the effects on the enzyme formation. The washed cells were suspended in 0.01 M potassium phosphate buffer (pH 7.0) and disrupted for 10 min by an ultrasonic oscillator (Nissei US-300, 20 Kc, 0.15 C). The supernatant obtained by centrifugation at 12,000 × g for 20 min was dialyzed against 0.01 M potassium phosphate buffer (pH 7.0), and used as a crude enzyme preparation.

**Assay of enzyme activity.** (1) Hydrolyzing reaction of glutaminase: The activity was measured by formation of p-nitroanilide in a reaction mixture containing 2.5 mM γ-glutamyl p-nitroanilide and 100 mM imidazole HCl buffer (pH 9.0). p-Nitroanilide was estimated from the absorbance at 410 nm after adding 2 volumes of 1.5 N acetic acid to the reaction mixture (molar extinction coefficient, 8300).21 One unit of the enzyme was defined as the amount which forms 1 μmol of p-nitroaniline per min at 30 °C.

With the mixture with glutamine as a substrate, the reaction was stopped by immersing the tubes in boiling water for 3 min, and glutamic acid formed was determined.

(2) Transferring reaction of glutaminase: Glutaminase activity was also assayed by estimating the formation of γ-glutamylhydroxamate in another mixture with 35 mM glutamine, 150 mM hydroxylamine and 100 mM

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1. Amino acids are γ-isomers unless otherwise stated.
2. Corresponding author.
Glutaminase from *P. nitroreducens*

imidazole HCl buffer (pH 9.0) at 30 C. The reaction was stopped by adding 2 volumes of the acid mixture (10% FeCl₃, 24% trichloroacetic acid:6 N HCl:water = 8:2:1:13), and γ-glutamylhydroxamate was measured from the absorbance at 540 nm.  

(3) Glutaminase synthetase. The activity was estimated by measuring γ-glutamylhydroxamate synthesized at 30 C in a reaction mixture consisting of 30 mM sodium glutamate, 15 mM hydroxylamine, 7.5 mM ATP, 30 mM MgCl₂, and 100 mM imidazole HCl buffer (pH 8.0).  

The procedures for stopping the reaction and measuring the product were the same as those described for the transfer reacting glutaminase. One unit of glutaminase synthetase was defined as the amount forming 1 μmol of γ-glutamylhydroxamate per min.

Analysis. Amino acids and γ-glutamyl derivatives in the reaction mixture were measured by ninhydrin colorimetry after paper chromatography.  

Protein was estimated by the method of Lowry et al., with egg albumin as a standard. During column chromatography, it was followed by the absorbance at 280 nm.

Chemicals. Molecular weight standard proteins were purchased from Seikagaku Kogyo Co., Ltd. and Boehringer Mannheim GmbH. γ-Glutamyl γ-nitroanilide from Nakarai Tesque Co., Ltd. γ-Glutamylthylester, γ-glutamylphenylalanine and γ-glutamylmethyamide were prepared according to the method described previously. Other reagents were the highest grade commercial products.

Purification of glutaminase. All operations were done at 0 to 15 C.  

Step 1. Cell-free extract. *P. nitroreducens* was grown in the medium containing 0.69% sodium glutamate, which has been used in our previous study on the enzymes involved in glutamine metabolism of *Brevibacterium florum*.  

The 16-h grown cells from 20 L culture broth were suspended in 2 L of 0.01 M potassium phosphate buffer (pH 7.0), and disrupted for 30 min by an ultrasonic oscillator. Cell debris was removed by centrifugation at 12,000 × g for 20 min.

Step 2. Ammonium sulfate fractionation. To the crude extract was added solid ammonium sulfate to 35% saturation, adjusting to pH 7 with 7% ammonia. The precipitate was centrifuged out, and ammonium sulfate was added to the supernatant to 90% saturation. After it was left overnight, the precipitate was collected by centrifugation, dissolved in 0.01 M potassium phosphate buffer (pH 7.5), and dialyzed against the same buffer for 48 h.

Step 3. DEAE-cellulose column chromatography. The dialyzed enzyme solution was put on a DEAE-cellulose column (48 × 46 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.5). After it was washed with the buffer, the column was developed with the buffer containing 0.1 M NaCl. The enzyme activity was found mainly in the 0.01 M NaCl eluate. Ammonium sulfate was added to the combined active fractions (90% saturation), and the precipitate was collected.

The result of this step varied according to slight changes of buffer pH, column size, charge amount, etc.; the activity was detected in the adsorbed fraction in one case, in the adsorbed fraction in another case, or both in the adsorbed and adsorbed fractions in the third case.

Step 4. CM-cellulose column chromatography. The precipitate obtained in Step 3 was dissolved in and dialyzed against 0.01 M acetate buffer (pH 5.5). The dialyzed solution was put on a CM-cellulose column (35 × 20 cm) equilibrated with 0.01 M acetate buffer (pH 5.5), and the enzyme was adsorbed to the column, indicating that isoelectric point of the enzyme protein was greater than pH 5.5.

After it was washed, the column was eluted with buffer containing 0.05, 0.1, and 0.15 M NaCl. The enzyme activity was eluted with the buffer containing 0.05 M NaCl, and the active fractions were concentrated by ultrafiltration with an Amicon PM 30 membrane.

Step 5. Sephacryl S-200 column chromatography. The concentrated enzyme solution was filtered through a Sephacryl S-200 column (3.1 × 149 cm) with 0.01 M potassium phosphate buffer (pH 7.0).

Step 6. Hydroxyapatite column chromatography. The enzyme solution was put on a hydroxyapatite column (1.4 × 3 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.0), and developed by increasing the concentration of the buffer. The active fractions eluted with 0.05 M buffer were concentrated by ultrafiltration.

Step 7. Butyl-Toyopearl 650M column chromatography. To the concentrated enzyme solution, was added ammonium sulfate to 30% saturation. The enzyme solution was filtered to remove impurities through a column of Butyl-Toyopearl 650M (2 × 5 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.0) saturated with 30% ammonium sulfate, and then dialyzed against 0.01 M potassium phosphate buffer (pH 7.0).

Results and Discussion  

Effects of culture conditions on glutaminase activity  

Table I summarizes the activities of glutaminase in the cells grown under various conditions, together with those of glutaminase synthetase (EC 6.3.1.2), which relates metabolically to glutaminase.

Exp. A indicates that (1) sodium glutamate was the most effective nitrogen source for higher glutaminase activity but the activities in ammonium chloride- and sodium nitrate-grown cells were extremely low, (2) glutamate did not have a specific stimulating effect on glutaminase formation, and (3) the activity increased with decreases in the concentration of sodium glutamate or glutamine in the medium.

Sodium glutamate was also effective for the bacterial growth, and the cell yield increased responding to its concentration (OD at 610 nm in the 10 mM sodium glutamate-medium, 1.9; 20 mM, 3.1; 40 mM, 5.2).

Exp. B shows that ammonium chloride had a repressive effect on the enzyme formation when added to the sodium glutamate-medium, and suggested that the lower activity in the cells grown at higher concentrations of sodium glutamate or glutamine might be caused by ammonia liberated from the excess amino acids.

The change of glutaminase synthetase activity, which might be necessary for comprehensive study for nitrogen metabolism of the organism, was different from that of glutaminase in respect to the effects of sodium nitrate or glutamine as a nitrogen source. The findings of the feeble activity in ammonium chloride- or glutamine-grown cells and of the higher activity with lower concentrations of...
nitrogen source were similar to those in many bacteria, and suggested at least that the glutamine synthetase of Ps. nitreducens is regulated by a repression-depression system where glutamine plays a certain role.\textsuperscript{33, 34}

**Purification and some properties of glutaminase**

Glutaminase was purified 325-fold with an overall yield of 13% (Table II). The final preparation was homogeneous on disc gel electrophoresis (Fig. 1). The long electrophoresis period under acidic conditions (2-6 h, pH 4.5) was not inconsistent with the behavior of the enzyme in ion exchange column chromatographies, which are described in Materials and Methods (enzyme purification, Steps 3 and 4).

The molecular weight of glutaminase was calculated to be about 40,000 by gel filtration on a Sepharose CL-6B column. SDS-polyacrylamide gel electrophoresis gave one protein band (M.W. 40,000), indicating that the enzyme consists of a single protein. The molecular weight was rather closer to those of the enzymes from Ps. aeruginosa (enzyme B, 67,000)\textsuperscript{25, 26} and Pseudomonas sp. (25,000)\textsuperscript{23} than those from Ps. aeruginosa (enzyme A, 137,000),\textsuperscript{25, 26} E. coli (110,000),\textsuperscript{19} Aspergillus oryzae (113,000–123,000),\textsuperscript{29} and pig renal cortex (Tris-soluble form, 140,000–160,000; phosphate form, 250,000–290,000).\textsuperscript{13}

The enzyme was stable in the pH range of 5.5 to 8.0, and retained its initial activity after 10 min of incubation at 50 C and pH 6.0. Full activity was maintained for several months at 5 C in 90% ammonium sulfate solution.

**Hydrolyzing reaction**

The results (figures and tables) are shown compared with those of the transferring reaction in the next section.

The optimum pH of the hydrolyzing reaction was 9.0 with glutamine (Fig. 2A) or γ-glutamyl p-nitroanilide (data not shown) as a substrate similarly to those of the enzymes from other microorganisms except for E. coli.\textsuperscript{19}

As shown in Table IIIA, glutaminase hydrolyzed various γ-glutamyl derivatives including D-glutamate but not asparagine, indicating that the Ps. nitreducens enzyme was similar to glutaminase B of Ps. aeruginosa in respect to the substrate specificity.\textsuperscript{25, 26} The $K_m$ were 6.5 mM for glutamine and 0.03 mM for γ-glutamyl p-nitroanilide.

The finding that an asparagine-hydrolyzing activity was observed in the cell-free extract (data not shown) suggested occurrence of another enzyme species like glutaminase A (asparaginase) in Ps. aeruginosa.\textsuperscript{25, 26}

Table IVA, summarizing the effects of various substances on the hydrolyzing reaction shows, that the influence of thiol compounds and p-chloromercuribenzoate was not remarkable whereas iodoacetate and N-ethylmaleimide were inhibitory to some extent. Zn\textsuperscript{2+}, Ni\textsuperscript{2+}, Cd\textsuperscript{2+}, Co\textsuperscript{2+}, Fe\textsuperscript{3+}, and Cu\textsuperscript{2+} strikingly repressed the glutaminase reaction. These results were almost the same as those of other microbial glutaminases.\textsuperscript{19, 25, 26} Addition of potassium phosphate buffer to the reaction mixture brought about a slight increase of the activity (120% activity was found with 0.1 M addition, 144% with 0.2 M), which was not so great as in case of the phosphate-dependent enzyme from animal source.\textsuperscript{17} Among metabolites tested, 5'-AMP, 5'-ADP, 5'-ATP and histidine (5 mM each) inhibited the activity (10-15% inhibition, data not shown).

### Table II. Purification of Glutaminase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg protein)</th>
<th>Total activity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>11,000</td>
<td>0.189</td>
<td>2,080</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>7,900</td>
<td>0.266</td>
<td>2,100</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>790</td>
<td>1.66</td>
<td>1,310</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>44</td>
<td>17.0</td>
<td>750</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>7.1</td>
<td>56.3</td>
<td>400</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>6.5</td>
<td>58.5</td>
<td>380</td>
</tr>
<tr>
<td>Butyl-Toyopearl 650M</td>
<td>4.4</td>
<td>61.4</td>
<td>270</td>
</tr>
</tbody>
</table>

**Fig. 1.** Polycrylamide Gel Electrophoresis of Glutaminase.

The enzyme preparation (12μg) was electrophoresed on 7.5% acrylamide gel (pH 4.3) for 2, 4, and 6 h at a current of 2 mA per tube. β-Alanine-acetic acid buffer (pH 4.5) was used.

**Fig. 2.** Effects of pH on Glutaminase-hydrolyzing (A) and Transferring (B) Reactions.

The enzyme activity was measured in buffers of various pHs, and shown relative to the maximum activity. Symbols: ○, imidazole buffer; ●, borate buffer.
Table III. Substrate Specificity for Hydrolyzing (A) and Transferring (B) Reactions

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
<th>Hydrolyzing reaction (A)</th>
<th>Transferring reaction (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td>100</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>α-Glutamine</td>
<td>107</td>
<td>95</td>
<td>82</td>
</tr>
<tr>
<td>γ-Glutamyl p-nitroanilide</td>
<td>85</td>
<td>108</td>
<td>82</td>
</tr>
<tr>
<td>γ-Glutamylmethylamide</td>
<td>78</td>
<td>62</td>
<td>74</td>
</tr>
<tr>
<td>Theanine</td>
<td>74</td>
<td>83</td>
<td>82</td>
</tr>
<tr>
<td>Glutathione</td>
<td>82</td>
<td>83</td>
<td>0</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>α-Asparagine</td>
<td>0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>0</td>
<td>--</td>
<td>100</td>
</tr>
<tr>
<td>Methylamine</td>
<td>3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Ethylamine</td>
<td>8</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

* Glutamic acid (or aspartic acid) formed was measured by ninhydrin colorimetry, and the activity was expressed relative to that with glutamine.

* γ-Glutamylhydroxamate was measured. In case of α- and γ-asparagine, formation of the hydroxamate was not observed. The activity was expressed relative to that with glutamine.

* The product in the mixture with methylamine or ethylamine was measured as γ-glutamylmethylamino or theaminine by ninhydrin colorimetry after paper chromatography. The activity was expressed relative to that with hydroxylamine.

Transferring reaction

As does glutaminase from other sources, the *Ps. nitroreducens* enzyme formed γ-glutamylhydroxamate in the mixture containing glutamine and hydroxylamine. The maximum activity of the transferring reaction was found in the pH range of 7 to 8 (Fig. 2B), and K_m were 4 mm for glutamine and 120 mm for hydroxylamine. Different pH-dependence of the transferring reaction from that of the hydrolyzing reaction have been reported with rat kidney glutaminase, and with several γ-glutamyltransferases that catalyze the hydrolyzing reaction.

Table IIIIB shows that γ-glutamyl derivatives hydrolyzable by the glutaminase (see Table IIIA) were active in the transferring reaction. Table IIIIB also indicates that methylamine or ethylamine had some reactivity, and the formation of a new ninhydrin-positive substance was confirmed, which corresponded to γ-glutamylmethylamino or theaminine.

As shown in Table IVB, the effects of various substances on the transferring reaction were different from those on the hydrolyzing reaction (Table IVA): the effects of iodoacetic acid, N-ethylmaleimide, Ni^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, and Cu^{2+} were obscure, and Pb^{2+} became more inhibitory. Their effects on the transferring reaction catalyzed by glutaminase have been scarcely described, and the finding in Table IV might be notable for understanding the glutaminase reactions.

This paper described the basic properties of glutaminase of *Ps. nitroreducens* as a first part of studies on metabolic aspects of ammonia, glutamine, and glutamic acid in denitrifying bacteria. Further investigation is necessary for this together with such enzymes as glutamine synthetase, glutamate synthase, etc. and their regulatory systems. The results of the enzyme synthesis (Table I) and of metabolite effect on the activity (data not shown) could be considered as preliminary observations. As for *Ps. nitroreducens* glutaminase, more precise characterization is indispensable, especially on the transferring reaction for discussing its reactivity and its relation to the other enzymes catalyzing similar reactions.

These experiments are now undertaken, and the results will be reported in next papers.

References