Purification and some properties of glutamate dehydrogenase and glutamine synthetase from Paracoccus denitrificans

H. Křemečková, B. Švrčula and V. Mikeš*

Department of Biochemistry, Faculty of Science, Masaryk University, Kotlarska 2, 611 37 Brno, Czechoslovakia

(Received 14 January 1992; revised 20 April 1992; accepted 5 May 1992)

The purification and some properties of NADP-dependent glutamate dehydrogenase (GDH) and glutamine synthetase (GS) from the facultatively anaerobic Gram-negative bacterium Paracoccus denitrificans were investigated. The enzymes were purified to homogeneity using a procedure which involved affinity chromatography on Blue Sepharose CL-6B as the major purification step. The recoveries in the purification of GDH and GS were 28% and 64%, respectively. The specific activity of purified GDH was 183 nkat (mg protein)^{-1} (deaminating reaction). GDH was composed of subunits of molecular mass 47 kDa and the native enzyme was either a tetramer or hexamer. The apparent $K_m$ values for L-glutamate, NADP, 2-oxoglutarate, NADPH and ammonia were 1.5 mM, 5.9 pM, 0.47 mM, 12.5 pM and 14 mM, respectively. The specific activity of purified GS was 1125 nkat (mg protein)^{-1} (transferase reaction). The molecular mass of native GS was 570 kDa; it was composed of 12 subunits of molecular mass 50 kDa. The apparent $K_m$ values for L-glutamine and hydroxylamine in the transferase reaction were 2.1 and 2.4 mM, respectively; those of ammonia, L-glutamate and ATP in the biosynthetic reaction were 0.03, 1 and 0.17 mM, respectively. After the adenylylation of GS, the $K_m$ for L-glutamine and L-glutamate increased and reached the values of 8.0 and 27 mM, respectively. The effects of the changes in GS activity on the ammonia metabolism of Paracoccus denitrificans are discussed.

Introduction

Ammonia transported into the bacterial cell or formed therein as a metabolite is assimilated in bacteria mostly by two pathways. With high concentrations of ammonia as a nitrogen source, L-glutamate is formed via NADP-dependent glutamate dehydrogenase (GDH) (EC 1.4.1.4). The alternative pathway utilized at low ammonia concentrations requires the cooperation of two enzymes. Glutamine synthetase (GS) (EC 6.3.1.2) catalyses the transfer of ammonium to L-glutamate forming L-glutamine, which then reacts with 2-oxoglutarate giving rise to two molecules of L-glutamate in a reaction catalysed by glutamate synthase (GOGAT) (EC 1.4.1.13). GDH has been purified from many Gram-positive and Gram-negative bacteria, including cyanobacteria, thermophilic bacteria, nitrogen-fixing bacteria and methanotrophs, the most extensively studied being the GDH from Escherichia coli (Smith et al., 1975). The mechanism of regulation of GDH synthesis in cells is not known.

* Author for correspondence. Tel. 5 7129405, fax 5 740108.

Abbreviations: GDH, NADP-dependent glutamate dehydrogenase; GS, glutamine synthetase.

The key regulatory enzyme in nitrogen assimilation in many bacteria is GS which is controlled by various mechanisms: feedback inhibition, post-translational modification by the adenylylation of a tyrosine residue in each subunit of the enzyme, activation and inhibition by divalent cations and nucleotides, and repression and derepression by substrates and products of nitrogen metabolism (Stadtman & Ginsburg, 1974). The assimilation of ammonia catalysed by GS is much more efficient than that catalysed by GDH because the former has a tenfold lower $K_m$ for NH$_4^+$.

In Gram-negative bacteria, a drop in nitrogen concentration in the culture medium causes a very rapid deadenylylation of GS and the synthesis of enzyme in the cells. Both processes, i.e. deadenylylation and synthesis of the enzyme in E. coli, are probably controlled by the ratio of 2-oxoglutarate and L-glutamine concentrations in cells (Magasanik, 1988). In Gram-positive bacteria, GS is subjected to feedback inhibition rather than post-translational modification (Deuel & Stadtman, 1970).

Paracoccus denitrificans is a facultatively anaerobic Gram-negative bacterium using ammonia, nitrate (assimilation of nitrate and nitrite; Ferguson, 1987) and methyamine (Hussain & Davidson, 1987) as nitrogen sources.
sources. Because of the similarity of its respiratory chain to that of mammalian mitochondria (Ferguson, 1987) it was used as a model for bioenergetic studies. As reported previously (Míček et al., 1991; Míček, 1991), the assimilation of high concentrations of ammonia in *P. denitrificans* was catalysed by GDH. Alternatively ammonia derived from nitrate was assimilated by means of GS. Under conditions of nitrogen starvation, however, both enzymes were synthesized. GS from *P. denitrificans* is regulated by covalent modification (Míček, 1991).

This paper describes the purification, molecular and kinetic properties of GDH and GS of *P. denitrificans*.

**Methods**

Organisms and growth conditions. *Paracoccus denitrificans* (CCM 982) was grown aerobically at 30°C in synthetic medium containing 17 mM-Na₂HPO₄, 33 mM-KH₂PO₄, 1.2 mM-MgSO₄, 0.03 mM-ferric citrate and 25 mM-sodium succinate, pH 7.3. As a source of nitrogen either 50 mM-NH₄Cl (to obtain a high GDH level) or 10 mM-KNO₃ (to obtain a high deacylated GS level) were added. The cultures were harvested in late exponential phase (12 h). The cells were centrifuged, washed twice with 0.1 M-Potassium phosphate, pH 7.3, suspended in the same buffer and stored at −60°C. In order to prepare highly deacylated GS, 50 mM-NH₄Cl was added, just before harvesting, to the bacteria cultivated in the presence of KNO₃. After 3 min the culture was cooled on ice and centrifuged as described above.

Cell-free extracts were prepared by sonication (Dyntach disintegrator, 10 × 30 s), after which intact cells were removed by centrifugation. To protect the deacylated GS against deacylation, 1 mM-2-oxoglutarate was added to the suspension before sonication.

Enzyme assays. The activity of GDH was estimated spectrophotometrically as the rate of deamination of glutamate or as the rate of amination of 2-oxoglutarate as described previously (Míček et al., 1991). The activity of GS was measured using the transferase reaction (Bender et al., 1977).

The biosynthetic reaction of GS was measured by a direct method based on the decomposition of ATP resulting in the release of protons to a slightly buffered medium (V. Míček, unpublished results):

\[
\text{glu}^{1-} + \text{NH}_2^+ + \text{ATP}^{4-} \rightarrow \text{glu} + \text{ADP}^{3-} + \text{Pi}^{2-} + \text{H}^+ \\
\text{NH}_3 + \text{ATP}^{3-} \rightarrow \text{ADP}^{2-} + \text{Pi}^{1-}
\]

The reaction mixture contained 5 mM-imidazole pH 7.2, 50 mM-NH₄Cl, 2 mM-ATP, 30 mM-MgCl₂ (in the case of the deacylated enzyme) or 1 mM-MnCl₂ (in the case of the deacylated enzyme) and phenol red as a pH indicator. The absorbance of phenol red was measured with a Shimadzu UV 3000 dual wavelength spectrophotometer at a wavelength couple (560 and 600 nm) corresponding to the basic form of the dye. After thorough equilibration at 30°C, the starting value was registered and the reaction started by the addition of enzyme solution. The enzyme activity was expressed in nmol H₂O⁺ produced per min. For calibration, 10–20 μl 20 mM-HCl was added to the mixture containing all components and the deflection of the recorder was measured.

Purification of enzymes. (1) GDH. Protein in a cell-free extract of bacteria cultivated in an excess of ammonium chloride was precipitated with ammonium sulphate. Proteins precipitating between 35 and 67% saturation were dissolved in 0.1 M-potassium phosphate buffer containing 5 mM-EDTA, pH 7.3 (buffer A) and dialysed overnight against the same buffer. The dialysed extract (260 mg protein) was concentrated by ultrafiltration and applied to a column of Blue Sepharose CL-6B (6 × 1.7 cm) equilibrated with 0.1 M-KCl in buffer A. The column was washed with 0.1 M-KCl in buffer A to remove unadsorbed material and the enzyme was eluted with 1 M-KCl in buffer A. The fractions containing GDH were pooled, dialysed against buffer A and concentrated by ultrafiltration. The concentrated enzyme fractions (3 ml) were applied to a HR 5/5 column loaded with Mono Q (Pharmacia) equilibrated with 0.1 M-KCl in buffer A and eluted with a linear gradient of 0-1 M-KCl in buffer A using FPLC (Pharmacia). Fractions containing GDH activity were collected, concentrated by ultrafiltration and applied to a HR 10/30 column loaded with Superose 12 (Pharmacia). The elution was carried out with buffer A.

(2) GS. Cell-free extract of bacteria cultivated in the presence of KNO₃ was fractionated with ammonium sulphate. The proteins precipitated between 0 and 50% saturation were centrifuged and sediment was discarded. The supernatant containing more than 90% activity was dialysed overnight against 20 mM-Tris/HCl, pH 7.4, containing 1 mM-MnCl₂ (buffer B). The dialysed extract was concentrated by ultrafiltration and 250 mg protein was applied to a column of Blue Sepharose CL-6B (see above) equilibrated with buffer B. The unadsorbed material was eluted with 1 M-KCl in buffer B. GS was eluted with 3 column volumes of 3 mM-ADP in buffer B or, more efficiently, with 6 M-urea in buffer B. The fractions containing GS activity were collected, dialysed immediately overnight against buffer B and subjected to ion exchange chromatography on Mono Q (see above) with a gradient of 0-1 M-KCl in buffer B. The pooled fractions containing GS activity were dialysed against buffer B and purified by gel permeation chromatography on Superose 6 (column HR 10/30, Pharmacia). The last step did not prove to be necessary (see below) because of the high purity of the preparation after ion exchange chromatography.

Molecular mass determination. Gel permeation chromatography on a Bio-Sil TSK-250 column (Bio-Rad) was used for determination of molecular mass of GDH and GS. Pure preparations of enzyme were applied to the column and eluted with 0.1 M-potassium phosphate buffer, pH 6.8, using HPLC (Knauer). The calibration of the column was performed with thyroglobulin, IgG, ovalbumin, myoglobin and cytocobalamin (Bio-Rad).

**SDS-PAGE.** SDS-PAGE was performed using a gel gradient of 8–21% run on Protein Double Slab Electrophoresis (Bio-Rad) according to Laemmli (1970). The subunit molecular mass was determined by comparison with the migration of standard protein markers (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and lactalbumin).

Protein determination. Protein was determined by the method of Bradford (1976).

**Results and Discussion**

**Purification of enzymes**

The results of a typical purification procedure of GDH are summarized in Table 1. The most effective purification step was represented by affinity chromatography on Blue Sepharose. The final step specific activities of the enzyme preparation were 182.6 nkat mg⁻¹ (30°C, 0.1 M-Tris/HCl, pH 9-0) when expressed as the rate of glutamate oxidation and 1300 nkat mg⁻¹ (30°C, 0.1 M-
Table 1. Purification of GDH from P. denitrificans

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity* (nkat)</th>
<th>Specific activity* [nkat (mg protein)^-1]</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>822</td>
<td>991</td>
<td>1-2</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>356</td>
<td>670</td>
<td>1-9</td>
<td>67.3</td>
<td>1-6</td>
</tr>
<tr>
<td>Blue Sepharose</td>
<td>43</td>
<td>766</td>
<td>17-8</td>
<td>77.3</td>
<td>14-8</td>
</tr>
<tr>
<td>Mono Q</td>
<td>6-2</td>
<td>470</td>
<td>75-8</td>
<td>47</td>
<td>63-1</td>
</tr>
<tr>
<td>Superose 12</td>
<td>1-5</td>
<td>274</td>
<td>182-6</td>
<td>28</td>
<td>152</td>
</tr>
</tbody>
</table>

* The activity of the enzyme is expressed as the rate of reduction of NAD in the deaminating reaction.

Table 2. Purification of GS from P. denitrificans

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity* (nkat)</th>
<th>Specific activity* [nkat (mg protein)^-1]</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>728</td>
<td>7-431</td>
<td>10-2</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate (50-100%)</td>
<td>223</td>
<td>6-687</td>
<td>30-0</td>
<td>90</td>
<td>3</td>
</tr>
<tr>
<td>Blue Sepharose</td>
<td>8-1</td>
<td>6-340</td>
<td>770-0</td>
<td>84</td>
<td>75-5</td>
</tr>
<tr>
<td>Mono Q</td>
<td>5-9</td>
<td>6-152</td>
<td>1043</td>
<td>83</td>
<td>102</td>
</tr>
<tr>
<td>Superose 6</td>
<td>4-0</td>
<td>4-500</td>
<td>1125</td>
<td>61</td>
<td>110-3</td>
</tr>
</tbody>
</table>

* The activity of the enzyme is expressed as the rate of the transferase reaction.

Tris/HCl, pH 7-8) when measured by the aminating reaction. As judged by SDS-PAGE, the enzyme preparation appeared to be homogeneous (see below).

The results of a typical purification procedure of GS are summarized in Table 2, showing that affinity chromatography is a very efficient step of purification after which the enzyme was purified 75-5-fold. GS was bound very strongly on Blue Sepharose and eluted with 6 M-urea in buffer B, but not with 2 column volumes of 3 mM-ADP as shown for GS from E. coli (Robertson & Jervis, 1985) and Azotobacter (Lepo et al., 1979).

Unadenyllylated GS from P. denitrificans seems to be stable in 6 M-urea. After removal of urea by dialysis, the enzyme regained 98% of its activity and was stable during subsequent operations. This behaviour is not surprising because the unadenyllylated GS from E. coli was not affected by 4-5 M-urea. Electron microscopy showed that individual subunits of GS from E. coli, dissociated after exposure of the enzyme to urea in the absence of Mn^{2+}, reassociated after dialysis to form aggregates similar in size to the native enzyme although, in contrast to GS from P. denitrificans, the activity was not completely restored (Valentine et al., 1968).

GS from P. denitrificans was routinely purified 110-fold and showed a specific activity of 1125 nkat (mg protein)^-1 as measured by the transferase reaction (30 °C, 0-1 M-Tris/HCl, pH 7-4). The preparation after ion exchange chromatography showed only one protein peak, so the last step, i.e. gel permeation chromatography, is not necessary.

Molecular mass and subunit composition

SDS-PAGE of GDH revealed one band which migrated at a distance corresponding to a subunit size of 47 kDa. The molecular mass of the native enzyme was determined after gel permeation chromatography on Superose 12. The fractions containing the active enzyme corresponded to a molecular mass of 210 kDa. However, two lower molecular mass fractions (140 and 55 kDa) without any enzymic activity were detected, which could have resulted from enzyme cleavage of smaller subunits. Similar cleavage was observed if the fractions containing the active enzyme (210 kDa) after the Superose 12 step were collected, concentrated, and chromatography was repeated. Most GDHs isolated from eukaryotes have a hexameric structure and a molecular mass of about 300 kDa. The GDH from Pseudomonas aeruginosa is composed of two subunits (Janssen et al., 1980), whereas that from Pseudomonas sp. strain AM1 has four subunits (Bellion & Tan, 1984) and the enzyme from Bacillus fastidiosus has four or two subunits (Op den Camp et al., 1989). Although the GDH from P. denitrificans appears to be a tetramer, a hexameric structure cannot be
excluded. The electrophoresis of GS revealed a single band with a molecular mass of 50-1 kDa. Gel permeation chromatography on Bio-Sil showed one peak with an apparent molecular mass of 570 kDa. Hence, GS from *P. denitrificans* is composed of 12 identical subunits. The native molecular mass of about 600 kDa and an oligomeric composition of 12 subunits with a molecular mass of about 50 kDa appears to be a common feature of most bacterial GSs, in contrast to octameric mammalian enzymes (Stadtman & Ginsburg, 1974).

**Kinetic properties**

The kinetic properties of GDH were measured with enzyme after the affinity chromatography step. GDH from *P. denitrificans* was strictly NADP(H)-specific. Ammonia was the preferred donor for the aminating reaction. A very low rate (less than 6% of that measured with ammonia) was detected with L-glutamate as the donor. 2-Aminobutyrate, norvaline and 4-aminobutyrate were all substrates for GDH from *P. denitrificans* although utilized at much lower rates than glutamate (10-3, 9-7 and 5-6% of that measured with L-glutamate, respectively). No enzymic activities were detected with valine, aspartate, norleucine and 2-aminobutyrate. The pH optima of the aminating and deaminating reactions determined in imidazole/HCl and Tris/HCl buffers were 7-8 and 9-7, respectively. The values generally correspond to the pH optima of GDH isolated from the other bacteria.

From Lineweaver-Burk plots, apparent *Kₘ* values of 1-5 mM, 5-9 µM, 0-47 mM, 12-5 µM and 14 mM were calculated for L-glutamate, NADP⁺, 2-oxoglutarate, NADPH and ammonia, respectively. The *Kₘ* values of GDH from most bacteria for NADP and NADPH are rather similar whereas the *Kₘ* values for ammonium and glutamate vary widely (Smith *et al.*, 1975). The *Kₘ* value for glutamate found for GDH from *P. denitrificans* (1-5 mM) was similar to that of *E. coli* (Sakamoto *et al.*, 1975), *Sulfolobus* (Schinkinger *et al.*, 1991) and *Methylophilus* (Sokolov & Troitsenko, 1987). Constants of 30-40 mM for glutamate were reported for other groups of bacteria (Op den Camp *et al.*, 1989; LeJohn *et al.*, 1968; Bellion & Tan, 1984; Hemmila & Mantsala, 1978). With respect to the high and relatively stable intracellular concentration of L-glutamate (15 mM) (Mikes, 1991) and the high affinity of GDH from *P. denitrificans* for glutamate and NADP, a possible role of GDH in glutamate deamination in *P. denitrificans* could be suggested. However, GDH in *P. denitrificans* was repressed in the presence of glutamate (Mikes *et al.*, 1991). The apparent *Kₘ* values for 2-oxoglutarate and NADPH are comparable to those reported for the other bacteria (Smith *et al.*, 1975). As reported previously (Mikes *et al.*, 1991), GDH in *P. denitrificans* is involved in the synthesis of glutamate in the presence of high concentrations of ammonia. In this case, the intracellular 2-oxoglutarate concentration was 0-3 mM whereas in nitrogen-starved cells it was 3 mM (Mikes, 1991). With respect to the *Kₘ* values, the activity of GDH in cells grown in an excess of ammonia is regulated at the substrate level by the 2-oxoglutarate and ammonia concentrations. In nitrogen-starved cells, however, GDH is saturated by 2-oxoglutarate and the enzyme activity is regulated only by the ammonia concentration.

As shown previously, GS of *P. denitrificans* is regulated by adenylylation. The pH optima of unadenylated and adenylylated forms are 7-5 and 7-0, respectively (Mikes, 1991). The kinetic measurements were performed with preparations obtained after the ammonium sulphate precipitation step. The adenylylated preparation contained 10% of the adenylylated form whereas the adenylylated preparation contained only 9% of the unadenylated form. The *Kₘ* values for the biosynthetic reaction were measured at a fixed saturated concentration of the two substrates, whilst the concentration of the third was varied. The method we used for measurement of the biosynthetic reaction of GS based on measurement of pH changes was very sensitive and suitable for determination of initial reaction rates. The purified enzyme exhibited Michaelis-Menten kinetics both in biosynthetic and transferase reactions. Table 3 summarizes and compares the *Kₘ* values of adenylylated and unadenylated forms with respect to L-glutamate, ATP, ammonium, L-glutamine and hydroxyamine. Like GS purified from other sources, the enzyme of *P. denitrificans* requires divalent cations as an essential activator. Maximal biosynthetic activity of the unadenylated enzyme was reached with 25 mM-MgCl₂, this cation having a small effect on the adenylylated enzyme stimulated by 1 mM-MnCl₂. The adenylylation of the enzyme resulted in a marked increase in *Kₘ* for L-glutamine and a 27-fold increase in *Kₘ* for L-glutamate (Table 3), whereas the *Kₘ* value for the other substrates remained unchanged. The apparent *Kₘ* values for L-glutamate, L-glutamine, hydroxyamine and ATP of the unadenylated GS from *P. denitrificans* are comparable to those reported in the literature. GS from *P. denitrificans* has a high affinity for ammonium, the *Kₘ* value being ten times lower than that of GS from *E. coli* (Senior, 1975), *Pseudomonas aeruginosa* (Janssen *et al.*, 1980), *Bacillus polymyx* (Kanamori *et al.*, 1987) and *Phormidium laminosum* (Blanco *et al.*, 1989). Similar low values of *Kₘ* for ammonium were reported for GS from *Rhodospirillum rubrum* (Soliman & Nordlung, 1989) and *Hyphomicrobium X* (Duchars & Attwood, 1991). The marked change of enzyme affinity for L-glutamate after the adenylylation can play an important role in regula-
Table 3. Apparent $K_m$ values for substrates of GS from $P$. denitrificans

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ value (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-unadenylylated</td>
<td>GS-adenylylated</td>
</tr>
<tr>
<td>Transferase reaction*</td>
<td></td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>2.4</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>2.1</td>
</tr>
<tr>
<td>Biosynthetic reaction†</td>
<td></td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>1.0</td>
</tr>
<tr>
<td>ATP</td>
<td>0.17</td>
</tr>
<tr>
<td>Ammonia</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Activities of both forms of GS were measured in the presence of 0.3 mM-MnCl$_2$.
† The biosynthetic reaction was measured in presence of 25 mM-MgCl$_2$ (unadenylylated form) and 1 mM-MnCl$_2$ (adenylylated form).

We are grateful to Dr O. Janicek for assistance with electrophoresis and molecular mass determination by GPC.

References


