NAD\(^+\) - and NADP\(^+\)-Dependent Glutamate Dehydrogenases in *Nitrobacter agilis*

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Two isoenzymes of glutamate dehydrogenase located in the cytoplasmic fraction of *Nitrobacter agilis* were specific for NAD\(^+\) and NADP\(^+\), respectively. The NAD\(^+\)-dependent enzyme functioned in both directions, i.e. amination and deamination, whereas the NADP\(^+\) enzyme was primarily for the amination of 2-oxoglutarate to glutamate. The NADP\(^+\) enzyme was purified 52-fold (free of the NAD\(^+\) enzyme) by affinity chromatography on 2',5'-ADP Sepharose 4B, and some of its properties studied. Substrate activation of the amination reaction of the NADP\(^+\) enzyme was observed with NH\(_4\)\(^+\) and NADPH. A comparison is made of the properties of the purified NADP\(^+\) enzyme from *Nitrobacter agilis* and *Nitrosomonas europaea*. The possible roles of two isoenzymes of glutamate dehydrogenase in *Nitrobacter agilis* are discussed.

INTRODUCTION

Glutamate dehydrogenases [L-glutamate : NAD(P) oxidoreductase] catalyse the oxidative amination of 2-oxoglutarate to glutamate as well as the reductive deamination of glutamate (Smith *et al.*, 1975). Bacterial glutamate dehydrogenases can utilize either NAD\(^+\) (EC 1.4.1.2: NAD\(^+\)-GDH) or NADP\(^+\) (EC 1.4.1.4: NADP\(^+\)-GDH) or both (EC 1.4.1.3) (Smith *et al.*, 1975). We have shown that the NO\(_3\)-oxidizing chemolithotroph *Nitrobacter agilis* has two distinct pathways for assimilating ammonia, either involving glutamate dehydrogenase (GDH) or, alternatively, glutamine synthetase and glutamate synthase (Kumar & Nicholas, 1982). The GDH route for ammonia assimilation predominates when this bacterium is grown in cultures with NO\(_3\) or NO\(_2\) + NH\(_3\). As a nitrogen source. Under these conditions glutamate synthase has a relatively low activity (Kumar & Nicholas, 1982) and glutamine synthetase is largely adenylylated (i.e. biosynthetically inactive) (Kumar & Nicholas, 1984) thus restricting ammonia assimilation via this route. Unlike the GDH from the ammonia-oxidizing bacterium *Nitrosomonas europaea*, which is specific for NADP\(^+\) (Hooper *et al.*, 1967), the cell-free extracts of *Nitrobacter agilis* contain NAD\(^+\)- and NADP\(^+\)-dependent GDH activities (Wallace & Nicholas, 1968; Kumar & Nicholas, 1982). It was not clear whether there were two separate enzymes for NAD\(^+\) and NADP\(^+\) in *Nitrobacter agilis* or a single enzyme utilizing both nucleotides. We now report that there are two separate GDH enzymes in *Nitrobacter agilis* specific for either NAD\(^+\) or NADP\(^+\). The NADP\(^+\)-specific GDH from *Nitrosomonas europaea* (Hooper *et al.*, 1967) is also considered in this comparative study.

METHODS

*Bacteria and growth conditions. *Nitrobacter agilis* ATCC 14123 was grown in 40 l batches at 28 °C for 5 d with vigorous aeration in an inorganic medium as described by Aleem (1968) and Wallace & Nicholas (1969). Cells were harvested at 4 °C and 30000 g in a Sorvall RC-2B centrifuge fitted with a continuous-flow rotor, with a flow.
rate of 12 h⁻¹ (Kumar & Nicholas, 1981). Cells were washed several times with 50 mM-Tris/HCl buffer (pH 7-5) and finally suspended in 50 mM-Tris/HCl, 1 mM-β-mercaptoethanol (pH 7-5). A culture of *Nitrosomonas europaea* was kindly supplied by Dr Jane Meiklejohn of Rothamsted Experimental Station, Harpenden, UK. Cultures were grown in either 8 or 40 l batches at 28 °C with vigorous aeration for 3 d in a medium described by Bhandari & Nicholas (1979). The pH was maintained at 7-8 throughout growth by titrating the medium with sterile 20% (w/v) K₂CO₃, using an automatic pH-stat unit (Radiometer). Cells were harvested and washed as for *Nitrobacter agilis*.

Preparation of cell extracts. Washed suspensions of *Nitrobacter agilis* and *Nitrosomonas europaea* in 50 mM-Tris/HCl, 1 mM-β-mercaptoethanol (pH 7-5) were disrupted with an ultrasonic probe (20 kHz) with 2 min bursts over a period of 30 min at 4 °C in an ice bath. Sonicated extracts were centrifuged at 30000 g (average) for 20 min at 4 °C in a Beckman L8-70 using a 50 Ti rotor. The supernatant fraction (S₃₀) was used in these studies.

Purification of NAD⁺-linked GDH. A similar procedure was used to purify glutamate dehydrogenase from both *Nitrobacter agilis* and *Nitrosomonas europaea*. These experiments were done in a cold room at 4 °C. The S₃₀ fraction was further centrifuged at 110000 g (average) for 1 h at 4 °C and the supernatant (S₁₁₀) was loaded onto a 2',5'-ADP Sepharose 4B column (0-8 × 11 cm) pre-equilibrated with buffer (50 mM-Tris/HCl, 1 mM-β-mercaptoethanol, pH 7-5). The column was then washed with buffer (flow rate 40 ml h⁻¹) until the absorbance at 280 nm of the effluent was close to zero. The enzyme was then eluted with 2 mM-NADPH in the buffer and the fractions containing enzyme activity were pooled and dialysed overnight against fresh buffer. The enzyme was stable for at least 2 weeks when stored at -20 °C in the Tris/β-mercaptoethanol buffer.

**GDH assay.** Activity of GDH was determined as described by Hooper et al. (1967) either from the rate of oxidation of NAD(P)(H) (amination reaction) or that of NAD(P) reduction (deamination reaction) at 340 nm at 30 °C. For the amination reaction the assay mixture in a total volume of 3 ml contained (mM): 2-oxoglutarate, 20; NH₄Cl, 240; NAD(P)H, 0.33; Tris/HCl buffer (pH 8.8), 50; and an appropriate quantity of the enzyme preparation. For the deamination reaction, the assay mixture in a final volume of 3 ml contained (mM): K₂CO₃, 240; NAD(P)H, 0.33; Tris/HCl buffer (pH 9.0), 50; and an appropriate quantity of the enzyme preparation. Both reactions were carried out at 30 °C. For the amination reaction the assay mixture in a total volume of 3 ml contained (mM): 2-oxoglutarate, 20; glutamate, 17; NAD(P), 0.33; Tris/HCl buffer (pH 7-8), 50; and an appropriate quantity of the enzyme preparation. After the bands had appeared, the effluent was collected for 10 min period, were corrected for endogenous oxidation/reduction of NAD(P)H/NAD(P).

**Polyacrylamide gel electrophoresis.** Discontinuous, non-denaturing PAGE was carried out in 5% (w/v) polyacrylamide tube gels (Davis, 1964). The stacking gel was 3% (w/v) polyacrylamide in 125 mM-Tris/HCl buffer (pH 6.8) and the running gel 5% (w/v) polyacrylamide in 375 mM-Tris/HCl buffer (pH 8.8). Electrophoresis was carried out at 2 mA per gel, constant current in 12.5 mM-Tris, 96 mM-glycine (pH 8.4) as electrode buffer. Specific staining for GDH activity using either NAD⁺ or NADP⁺ also had two distinct bands. It is known that *Nitrobacter agilis* (S₃₀) was carried out in 5% (w/v) polyacrylamide gels as described in Methods. The gels were stained for GDH activity using either NAD⁺ or NADP⁺ or both (Fig. 1). The activity bands stained in gels A (NAD⁺) and B (NADP⁺) had different electrophoretic mobilities and those in gel C (NAD⁺ + NADP⁺) also had two distinct bands. It is known that 2',5'-ADP Sepharose 4B binds enzymes that require NADP⁺. When crude extracts of *Nitrobacter agilis* were loaded onto a 2',5'-ADP Sepharose 4B column and washed with buffer in Methods, the effluent contained NAD⁺-linked GDH only (gel D). When the affinity column was eluted with 2 mM-NADPH, the eluate did not contain any NAD⁺-associated GDH (gel E) but NADP⁺ activity was detected (gels F and G). There were also two

**RESULTS**

**Evidence for two isoenzymes of GDH in Nitrobacter agilis**

Crude cell extracts of *Nitrobacter agilis* contain GDH which utilizes either NADH or NADPH for its amination reaction (Wallace & Nicholas, 1968; Kumar & Nicholas, 1982). To check whether the two activities are associated with two distinct proteins, electrophoresis of the crude extract (S₃₀) was carried out in 5% (w/v) polyacrylamide gels as described in Methods. The gels were stained for GDH activity using either NAD⁺ or NADP⁺ or both (Fig. 1). The activity bands stained in gels A (NAD⁺) and B (NADP⁺) had different electrophoretic mobilities and those in gel C (NAD⁺ + NADP⁺) also had two distinct bands. It is known that 2',5'-ADP Sepharose 4B binds enzymes that require NADP⁺. When crude extracts of *Nitrobacter agilis* were loaded onto a 2',5'-ADP Sepharose 4B column and washed with buffer in Methods, the effluent contained NAD⁺-linked GDH only (gel D). When the affinity column was eluted with 2 mM-NADPH, the eluate did not contain any NAD⁺-associated GDH (gel E) but NADP⁺ activity was detected (gels F and G). There were also two
Fig. 1. Detection of NAD⁺- and NADP⁺-dependent GDH activities from Nitrobacter agilis. To separate the two enzyme activities, the S₃₀ fraction was loaded onto a 2',5'-ADP Sepharose 4B column (0.8 × 11 cm) and washed with 50 mM-Tris/HCl (pH 7.5), then eluted with 2 mM-NADPH in the buffer. Samples of S₃₀ 2',5'-ADP Sepharose 4B buffer washings and NADPH-eluted proteins were dialysed overnight at 4°C against the same buffer and then loaded onto 5% (w/v) polyacrylamide tubes. Electrophoresis and GDH-specific staining were carried out as described in Methods. Gels A to D were loaded with about 250 µg protein and gels E to G, with about 5 µg protein. Gel A, (S₃₀ + NAD⁺); gel B, (S₃₀ + NADP⁺); gel C, (S₃₀ + NAD⁺ + NADP⁺); gel D, (2',5'-ADP Sepharose 4B buffer washings + NAD⁺ + NADP⁺); gel E, (2',5'-ADP Sepharose 4B, NADPH eluate + NAD⁺); gel F, (2',5'-ADP Sepharose 4B, NADPH eluate + NADP⁺); gel G, (2',5'-ADP Sepharose 4B eluate + NAD⁺ + NADP⁺).

minor bands in gels F and G which may have been either aggregates or active dissociated subunits of NADP⁺-dependent GDH (see Smith et al., 1975). Only the NADP⁺-specific enzyme was detected in crude extracts (S₃₀) of Nitrosomonas europaea analysed in polyacrylamide gels. The results indicate that *Nitrobacter agilis* has two distinct isoenzymes of GDH, one dependent on NAD⁺ and the other on NADP⁺, while in *Nitrosomonas europaea* one single NADP⁺-dependent GDH is present.

**Purification of NADP⁺-dependent GDH**

The NADP⁺-associated GDH from *Nitrobacter agilis* was partially purified by affinity chromatography (Table 1a). The purified enzyme for *Nitrosomonas europaea*, reported to be dependent on NADP⁺ only (Hooper et al., 1967), was also prepared (Table 1b). The crude extracts (S₃₀) of *Nitrobacter agilis* contained both NAD⁺- and NADP⁺-dependent activities (Table 1a) while those of *Nitrosomonas europaea* had NADP⁺-linked GDH with detectable but slight NAD⁺-dependent activity (Table 1b). In S₁₁₀ fractions the specific activity of the NADP⁺-requiring GDH of *Nitrosomonas europaea* was about 13-fold greater than that of the NADP⁺-dependent enzyme from *Nitrobacter agilis*. The NADP⁺ enzymes from *Nitrobacter agilis* and *Nitrosomonas europaea* were purified 52-fold and 142-fold by affinity chromatography on 2',5'-ADP Sepharose 4B. Both purified preparations were free of detectable NAD⁺-dependent GDH activity.

Attempts to purify NAD⁺-linked GDH of *Nitrobacter agilis* by affinity chromatography on Blue Sepharose CL-6B were unsuccessful. Low activity of the NAD⁺ enzyme as well as minimal cell yields of *Nitrobacter agilis* made it difficult to purify adequate amounts of the enzyme to study its properties and regulation.
Table 1. Purification of NADP⁺-GDH from Nitrobacter agilis and Nitrosomonas europaea

Enzyme purification and assay of enzyme activity were as described in Methods. Activity was determined by following the oxidation of either NADH or NADPH at 340 nm at 30 °C. One enzyme unit is defined as nmol NAD(P)H oxidized min⁻¹ and specific activity as units (mg protein)⁻¹ at 30 °C.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Purification (fold)</th>
<th>Percentage recovery</th>
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<tr>
<td></td>
<td>NADP⁺</td>
<td>NAD⁺</td>
<td>NADP⁺</td>
<td>NAD⁺</td>
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<tr>
<td>(a) Nitrobacter agilis</td>
<td></td>
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<td></td>
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<tr>
<td>S₃₀</td>
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<td>3111</td>
<td>2133</td>
<td>26</td>
<td>18</td>
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<tr>
<td>S₁₁₀</td>
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<td>2609</td>
<td>1649</td>
<td>44</td>
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<tr>
<td>Pooled 2',5'-ADP eluate of Sepharose 4B column</td>
<td>1</td>
<td>1353</td>
<td>3</td>
<td>1353</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(b) Nitrosomonas europaea</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>S₃₀</td>
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<td>448000</td>
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<td>330</td>
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<tr>
<td>Pooled 2',5'-ADP eluate of Sepharose 4B column</td>
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<td>0</td>
<td>46670</td>
<td>0</td>
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Table 2. Amination and deamination reaction rates in extracts (S₁₁₀) of Nitrobacter agilis

Amination and deamination reactions were determined in the S₁₁₀ fraction as described in Methods. Specific activity is expressed as nmol NAD(P)H oxidized or NAD(P)+ reduced min⁻¹ (mg protein)⁻¹ at 30 °C.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amination: NADPH → NADP⁺</td>
<td>48.6</td>
</tr>
<tr>
<td>NADH → NAD⁺</td>
<td>23.0</td>
</tr>
</tbody>
</table>

| Deamination: NADP⁺ → NADPH | ≤ 2.0 |
|                           |      |
| NAD⁺ → NADH                | 22.5 |

Amination and deamination reactions in the S₁₁₀ fraction

The NADP⁺-requiring GDH from Nitrosomonas europaea functions in either direction, i.e. amination of 2-oxoglutarate to form glutamate and deamination of glutamate to 2-oxoglutarate (Hooper et al., 1967). It is clear from Table 1 that the amination activity of NADP⁺-requiring GDH from Nitrobacter agilis was about twice that of NAD⁺-dependent GDH at the pH optimum of 8. On the other hand, the intensity of the enzyme activity bands in Fig. 1 indicates that NAD⁺-associated GDH activity was much higher than that observed for NADP⁺-dependent GDH. Since the gels were stained following deamination reaction, it is clear that the NADP⁺-linked GDH of Nitrobacter agilis predominantly operates in the direction of glutamate production. That this is indeed the case is supported by the data in Table 2. In the S₁₁₀ fraction of Nitrobacter agilis the amination and deamination activities of the NAD⁺-requiring enzyme were approximately equal; however, the deamination activity of the NADP⁺-associated GDH was only about 4% of the amination activity (Table 2).

Properties of NADP⁺-dependent GDH from Nitrobacter agilis

Since the NADP⁺-linked GDH from Nitrosomonas europaea has been studied in detail (Hooper et al., 1967), this section will deal with some properties of partially purified (52-fold) NADP⁺-associated GDH from Nitrobacter agilis. The amination reaction had a sharp pH optimum at 8, whereas the deamination reaction was less specific, but with an optimum at 9. The rate of NADPH oxidation (amination reaction) was maximal with 10 mM-2-oxoglutarate. A double reciprocal plot of the rate of NADPH oxidation against substrate concentration gave an apparent $K_m$ value of 3.57 for 2-oxoglutarate. The apparent $K_m$ for 2-oxoglutarate did not
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Fig. 2. Effects of NH$_4^+$ on the amination reaction of NADP$^+$-GDH from *Nitrobacter agilis*. The amination reaction of partially purified NADP$^+$-GDH (Table 1) was determined as described in Methods except that NH$_4$Cl concentration was varied over the range 0–200 mM. The data are plotted as the reciprocal of reaction velocity ($1/v$, (nmol NADPH oxidized min$^{-1}$)$^{-1}$) against the reciprocal of NH$_4$Cl concentration ($1/[S]$, mM$^{-1}$).

Fig. 3. Effects of NADPH on the amination reaction of NADP$^+$-GDH from *Nitrobacter agilis*. The amination reaction of partially purified NADP$^+$-GDH (Table 1) was determined as described in Methods except that NADPH concentration was varied over the range of 0–200 μM. The data are plotted as the reciprocal of reaction velocity ($1/v$, (nmol NADPH oxidized min$^{-1}$)$^{-1}$) against the reciprocal of NADPH concentration ($1/[S]$, mM$^{-1}$).

change with increasing concentrations of NH$_4$Cl. The NADPH oxidation rate increased with increasing concentrations of NH$_4$Cl (0–100 mM); it increased rapidly up to 20 mM-NH$_4$Cl and slowly thereafter so that the system appeared to be biphasic. Double reciprocal plots of the data (Fig. 2) produced two distinct apparent $K_m$ values of 33 mM above 20 mM-NH$_4^+$ and 6.3 mM below 20 mM-NH$_4^+$. This change in apparent $K_m$ for NH$_4$Cl was not a salt effect since NaCl did not affect the value. A similar type of substrate stimulation of enzyme activity (Cleland, 1970) was observed with NADPH (Fig. 3). Two apparent $K_m$ values of approximately 100 μM and 7 μM were recorded for NADPH concentrations above 50 μM and below 50 μM, respectively.

The amination reaction of NADP$^+$-associated GDH from *Nitrosomonas europaea* is known to be inhibited by carboxylic acids and nicotinamide adenine nucleotides (Hooper et al., 1967). The effects of some of these compounds on the amination reaction of NADP$^+$-linked GDH of *Nitrobacter agilis* were studied as described in Methods. The organic acids used were first adjusted to pH 8.0 before addition to the standard reaction mixture. Thus fumaric acid inhibited the amination reaction appreciably (45% at 20 mM), while malic and pyruvic acids (20 mM) and 2-oxoglutarate (100 mM) were without effect. cis-Oxaloacetic acid slightly stimulated the reaction (18% at 5 mM), but this effect was not enhanced by increasing the concentration of oxaloacetic acid to 20 mM. None of the nucleotides (NAD+, NADP+, NADH, NADPH, and ATP) affected enzyme activity.

**DISCUSSION**

The results reported in this paper clearly show that the NAD$^+$ and NADP$^+$-dependent GDH activities in *Nitrobacter agilis* are associated with two distinct protein fractions which differ in their electrophoretic mobilities. These data are similar to those reported for *Thiobacillus novellus* (Le'John et al., 1968), *Hydrogenomonas H16* (Kramer, 1970) and *Micrococcus aerogenes* (Kew & Woolfolk, 1970; Johnson & Westlake, 1972) where NAD$^+$- and NADP$^+$-associated GDH are also associated with separate proteins.

The NAD$^+$-dependent GDH of *Nitrobacter agilis* appears to be capable of functioning in either direction, i.e. amination of 2-oxoglutarate to glutamate and deamination of glutamate to 2-oxoglutarate, whereas the NADP$^+$-specific GDH functions mainly in the direction of
glutamate (ammonia assimilation). It is of interest that the amination reaction of NADP+-
linked GDH from *Nitrobacter agilis* was stimulated by NH₄⁺ and NADPH (substrate stimu-
lation: Cleland, 1970) so that two distinct apparent *Kₘ* values were obtained for either substrate.

Studies of the kinetics of purified bovine GDH have been complicated by the polymerization–
depolymerization phenomena involving the disaggregation or unfolding of the crystalline
enzyme in dilute solutions (Smith *et al.*, 1975). This may also be an explanation of the
appearance of two major electrophoretic bands and one minor one associated with NADP+-
dependent GDH activities in purified preparations from *Nitrobacter agilis* compared with a
single band in crude extracts (Fig. 1). The biphasic kinetics of the purified NADP+-linked
GDH (Fig. 2) together with the substrate stimulation effects (Figs 2 and 3) are more likely to be
associated with changes in aggregation of the same enzyme (Smith *et al.*, 1975) modified in some
way during purification rather than with the presence of two distinct NADP+-dependent enzymes.

*Nitrobacter agilis* invariably contained high concentrations of NH₄⁺ (approx. 30 mM) and this
may explain why (a) glutamine synthetase is highly adenylylated (Kumar & Nicholas, 1984) and
(b) NADP+-dependent GDH (*Kₘ* NH₄⁺, 6-3 to 33 mM) operates in the direction of glutamate
production even when no exogenous NH₄⁺ is available to the bacterium. Although the NADP+-
associated GDH of *Nitrosomonas europaea* can function in either direction, i.e. amination and
deamination, it is likely to be unidirectional since the deamination reaction was almost
completely inhibited (80 to 90%) by 10 mM-NH₄Cl (Hooper *et al.*, 1967). No such regulation of
NADP+-linked GDH from *Nitrobacter agilis* seems necessary because the deamination reaction
of the enzyme is only about 4% of the amination reaction. Unlike the NADP+-dependent GDH
of *Nitrosomonas europaea* (Hooper *et al.*, 1967) the amination reaction of NADP+-linked GDH
from *Nitrobacter agilis* was unaffected by high concentrations (100 mM) of 2-oxoglutarate and
nucleotides (NADPH, NADP+); in fact, NADPH stimulated the activity, as shown in Fig. 3.

The high intracellular NH₄⁺ concentrations observed under laboratory growth conditions
support the view that GDH is the main pathway for ammonia assimilation in *Nitrobacter agilis.*
Accumulation of NH₄⁺ via the assimilatory NO₂⁻ reductase (Wallace & Nicholas, 1968) appears
to be under minimal regulatory control. Under natural growth conditions in the soil the avail-
bility of N source (NO₂⁻ and/or NH₄⁺) for *Nitrobacter agilis* can vary greatly. When NO₂⁻ rate
limiting, the nitrite oxidase for generating ATP would predominate, resulting in a minimal
reduction of NO₂⁻ to NH₄⁺. Under these conditions glutamine synthetase would be
unadenylylated (active form) and would readily assimilate the small amounts of NH₄⁺ available
(Kumar & Nicholas, 1984). On the other hand, under conditions where an ample supply of NO₂⁻
is available (e.g. culture growth conditions), NO₂⁻ reductase would probably produce sufficient
amounts of NH₄⁺ that would result in adenylylation of glutamine synthetase. The NH₄⁺ thus
produced would then be predominantly assimilated by the NADP+-linked GDH enzyme. The
NAD+-associated GDH may be important for 2-oxoglutarate production required primarily for
transamination reactions.

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