Chapter - IV

Glutamate dehydrogenase of *A. brasilense* RG; purification and properties
The enzyme glutamate dehydrogenase (GDH) is an extensively studied enzyme and has been purified from a large variety of organisms both prokaryotes (97-107) and eukaryotes (108-110). Although studies with the nitrogen-fixing enteric bacteria Klebsiella pneumoniae (7, 111) indicate that the synthesis of GDH is strongly regulated by the availability of ammonia in the growth medium, the properties of this enzyme have not been investigated with purified enzyme preparation from any nitrogen fixing bacterium. In Azospirillum brasilense, it has been found that the level of GDH is regulated by the nitrogen source in the growth medium; excess ammonia induced high levels of GDH synthesis whereas limiting ammonia repressed the synthesis of the enzyme. Moreover, in some mutants having a Nif" phenotype which is believed to be caused by some defect in general nitrogen regulation, the control of GDH synthesis seems to be lost. This suggests that GDH might play a significant role in general nitrogen regulation in A. brasilense. Studies with GDH of enteric bacteria like E. coli, S. typhimurium (3) have shown that the enzyme has a high $K_m$ value ($> 1$ mM) for ammonia; whether this is also the case in the free-living nitrogen fixing bacteria like K. pneumoniae has not been investigated. The GDH in a free-living nitrogen fixing bacterium may have different properties than the same enzyme from other free-living bacteria. In order to characterize the enzymatic properties of glutamate dehydrogenase in a nitrogen fixing bacterium,
the enzyme has been purified from *A. brasilense* RG and its properties studied. GDH of *A. brasilense* indeed turned out to be a very unusual enzyme.

4.1. **Results**

4.1.1. **Purification of glutamate dehydrogenase from *A. brasilense* RG**

The enzyme glutamate dehydrogenase had been purified to homogeneity from the crude extracts of *Azospirillum brasilense* RG. The steps included - i) removal of DNA and DNA binding proteins by precipitation with streptomycin sulphate, ii) concentration and precipitation of the protein by 

\[(\text{NH}_4)_2\text{SO}_4\], iii) chromatography on Sephadex G-200 column, iv) affinity chromatography on Red Sepharose CL-6B and v) finally an ion exchange chromatography on DEAE-cellulose column. The enzyme was purified 220-fold and the yield of the enzyme after the final step of purification was 15% of the crude extract. Since this enzyme turned out to be cold-labile, all the steps of purification were carried out at room temperature (25°C-28°C). However, during the disruption of cells by sonic oscillations, the temperature of the cell suspension was kept at 4°C. After sonication, glycerol was added to the cellular extracts at a concentration of 10% (v/v) and the ultracentrifugation step was carried out at 4°C. The enzyme at this stage was found to be reasonably stable against cold exposure for a short time.
Table - 4.1: Purification of GDH from *A. brasilense* RG.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>U</td>
<td>U/mg</td>
<td>-fold</td>
<td>%</td>
</tr>
<tr>
<td>1. Crude extracts</td>
<td>186.0</td>
<td>1613.0</td>
<td>311.0</td>
<td>0.19</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>2. Streptomycin supernatant</td>
<td>194.0</td>
<td>956.4</td>
<td>305.0</td>
<td>0.32</td>
<td>1.7</td>
<td>98.1</td>
</tr>
<tr>
<td>3. (NH₄)₂SO₄</td>
<td>8.0</td>
<td>288.0</td>
<td>198.0</td>
<td>0.69</td>
<td>3.6</td>
<td>63.7</td>
</tr>
<tr>
<td>4. Sephadex G-200</td>
<td>20.0</td>
<td>71.4</td>
<td>161.0</td>
<td>2.25</td>
<td>11.8</td>
<td>51.8</td>
</tr>
<tr>
<td>5. Red Sepharose</td>
<td>20.0</td>
<td>2.2</td>
<td>82.0</td>
<td>37.27</td>
<td>196.1</td>
<td>26.4</td>
</tr>
<tr>
<td>6. DEAE-Cellulose</td>
<td>14.0</td>
<td>1.1</td>
<td>46.1</td>
<td>41.90</td>
<td>220.5</td>
<td>15.0</td>
</tr>
</tbody>
</table>

GDH (NADPH-dependent) activity was assayed according to the method described in Section 2.2.4.
Fig. 4.1. Gel filtration on Sephadex G-200.

The ammonium sulphate fraction was loaded on a Sephadex G-200 column (42 cm x 2.5 cm) pre-equilibrated with buffer A + 20% glycerol and the same buffer was passed through the column at a rate of 9-10 ml/hr. Volume of each fraction was 2.5 ml. Protein concentration was monitored by checking the absorbance of the samples at 280 nm. Total activity in μmol/min of each fraction (2.5 ml) has been shown in the ordinate.
Fig. 4.2. Ion exchange chromatography on DEAE-cellulose.

A DEAE-cellulose column (1.5 cm x 2.0 cm) was equilibrated with buffer A + 20% glycerol and the Red Sepharose fraction was allowed to pass through the column. GDH was eluted with a linear gradient of 0.0 - 0.5 M KCl in buffer A + 20% glycerol, after washing the column with the DEAE-cellulose equilibration buffer.
From step 4 onwards (i.e. the Sephadex G-200 column chromatography) 20% glycerol (v/v) was used in the buffer solution to keep the enzyme stable. In Red Sepharose chromatography step 10% glycerol (v/v) was used with buffer A as the presence of 20% glycerol (v/v) in the buffer was found to interfere with the quantitative adsorption of the enzyme in the Red Sepharose column. The Red Sepharose column chromatography is the key step of this purification method - in this step the specific activity of GDH was increased by 16.5 fold. However, it still failed to eliminate one contaminating protein band, which was removed by DEAE-cellulose chromatography. Electrophoresis on SDS-polyacrylamide gel indicated that the enzyme obtained at this stage was homogeneous. There was a single band of proteins on the SDS-polyacrylamide gel which showed a molecular mass of 48,000 ± 2,000. The elution profiles of GDH in the Sephadex G-200 column chromatography step and in the DEAE-cellulose chromatography step have been shown in Fig. 4.1 and 4.2. Table 4.1 shows the different steps of purification of GDH.

4.1.2. Properties of glutamate dehydrogenase

4.1.2.1. Relative molecular mass

The relative molecular mass of the native glutamate dehydrogenase was determined by gel filtration using Sephadex G-200 column (Fig. 4.3). Standard proteins of known molecular weights and GDH were run simultaneously
Fig. 4.3. Estimation of $M_r$ values of native and denatured GDH by Sephadex G-200 column chromatography and SDS-polyacrylamide gel electrophoresis (inset) respectively.

Elution volumes of the standard proteins, blue dextran and GDH were determined using a Sephadex G-200 column (42 cm x 2.5 cm) having a flow rate of 6 ml/hr; the volume of each fraction was 0.8 ml. SDS-polyacrylamide gel electrophoresis of GDH and marker proteins was carried out as described in Fig. 4.4.
Fig. 4.4. SDS-polyacrylamide gel electrophoresis of GDH at various stages of purification.

Electrophoresis was carried out according to the method of Laemmli on a 10% polyacrylamide slab gel. The gel was stained with Coomassie Brilliant blue R. Marker proteins used were bovine serum albumin (67,000), ovalbumin (45,000) and trypsinogen (24,000) and $\beta$-lactoglobulin (18,400). Lane 1 – marker proteins; lane 2 – crude extracts; lane 3 – streptomycin supernatant fraction; lane 4 – (NH$_4$)$_2$SO$_4$ fraction; lane 5 – Sephadex G-200 fraction; lane 6 – Red Sepharose fraction; lane 7 – DEAE-cellulose fraction.
on a Sephadex G-200 column and fractions of 0.8 ml each were collected. The standard proteins used were ferritin (Mr 440,000), catalase (Mr 210,000), aldolase (Mr 158,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 45,000), cytochrome C (Mr 13,000). The relative molecular mass of the native enzyme determined in this way was found to be 285,000 ± 20,000.

The relative molecular mass of the denatured enzyme was determined by SDS polyacrylamide gel electrophoresis (Fig. 4.4). The standard proteins used as molecular mass markers were ovalbumin (45,000), trypsinogen (24,000), β-lactoglobulin (18,400) and bovine serum albumin (67,000). The molecular mass of denatured GDH determined from the plot was 48,000 ± 2,000. This suggests that the enzyme in its native form is composed of six identical subunits.

4.1.2.2. Effect of pH on enzyme activity

The optimum pH of GDH (NADPH-dependent) for the reductive amination reaction in 50 mM Tris-HCl buffer was between 8.5 and 8.6. The rate of the same reaction in 100 mM potassium-phosphate buffer showed a plateau from 8.4 - 10.0 (Fig. 4.5). In the pH range 8.0 - 9.0 the reaction rate of GDH in potassium-phosphate buffer was found to be comparatively higher than the same in Tris-HCl buffer; but the latter buffer was used routinely for GDH assay because of its biological inactivity. GDH activity
Fig. 4.5. Effect of pH on the rate of reductive amination reaction catalysed by GDH (NADPH-dependent).
Assays were performed with the standard assay mixture using either 50 mM Tris-HCl (•-•-•) or 100 mM potassium phosphate (0-0-0) buffer at various pH values.
was also determined with potassium-phosphate buffer at pHs above 8.0 (where its buffering capacity is low) because the inhibitory effect of amino acids on GDH activity was quite pronounced at high pH. The GDH activity drops considerably after pH 9.0 in Tris-HCl buffer whereas the activity remains unaltered in potassium-phosphate between pH 8.5-10.0. The significance of a bell-shaped curve and a lower GDH activity in Tris-HCl buffer and a plateau with higher GDH activity in potassium-phosphate buffer is not clear. However, these pH-activity curves helped us to choose appropriate buffer systems for various kinetic studies made with this enzyme.

4.1.2.3. Stability of the enzyme at different pH values

The enzyme appears to be highly stable at room temperature in potassium-phosphate buffer between pH 7.0-9.0 in the presence of 20% glycerol. The enzyme stability at different pHs was also determined in the presence of only 10% glycerol; the enzyme lost its activity in potassium-phosphate and Tris-buffer at the same rate (25% of the initial activity was lost in 48 hours) between pH 7.0-9.0 when kept at room temperature.

4.1.2.4. Substrate specificity

The activity of the purified GDH (NADPH-dependent) was assayed with a number of possible alternative substrates. The enzyme showed very high specificity for
Table 4.2: Substrate specificity of GDH (NADPH-dependent) from *A. brasilense* RG.

<table>
<thead>
<tr>
<th>Assay mixture</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reductive amination reaction</strong></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>100.0</td>
</tr>
<tr>
<td>- 2-oxoglutarate + pyruvate (1 mM or 10 mM)(^b)</td>
<td>0.0</td>
</tr>
<tr>
<td>- 2-oxoglutarate + oxaloacetate (1 mM or 10 mM)</td>
<td>0.0</td>
</tr>
<tr>
<td>- NH(_4)Cl + glutamine (5 mM)</td>
<td>0.0</td>
</tr>
<tr>
<td>- NH(_4)Cl + NH(_2)OH (50 mM)</td>
<td>2.0</td>
</tr>
<tr>
<td>- NADPH + NADH (1 mM) + 2-oxoglutarate (9 mM)</td>
<td>91.0</td>
</tr>
<tr>
<td><strong>Oxidative deamination reaction</strong></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>100.0</td>
</tr>
<tr>
<td>- glutamate + alanine (25 mM)</td>
<td>0.0</td>
</tr>
<tr>
<td>- glutamate + lysine (25 mM)</td>
<td>0.0</td>
</tr>
<tr>
<td>- glutamate + glutamine (25 mM)</td>
<td>0.0</td>
</tr>
<tr>
<td>- glutamate + aspartate (25 mM)</td>
<td>0.0</td>
</tr>
<tr>
<td>- NADP(^+) + NAD(^+) (3 mM)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\) Assay conditions for reductive amination reaction and oxidative deamination reaction are given in Section 2.2.4.

\(^b\) Figures in parentheses indicate the concentrations of the substrate.

2-oxoglutarate; other keto acids namely pyruvate and oxaloacetate were found to be completely inactive as substrate. No NADPH oxidation was observed when glutamine replaced ammonia even when very high concentration of the enzyme was used in the reaction mixture; this proves that
the purified GDH (DEAE-cellulose fraction) was free from glutamate synthase. Hydroxylamine was able to replace \( \text{NH}_3 \) in the reaction mixture, but this substitution reduced the reaction rate by about 50-fold. Studies with purified GDH also showed that NADH could replace NADPH as reductant in the enzyme catalysed reaction.

In the reverse reaction, the enzyme showed high specificity for glutamate; the amino acids alanine, lysine, histidine, aspartate and glutamine were found to be completely inactive as substrate in the oxidative deamination reaction. In the oxidative deamination of glutamate, NADP\(^+\) was the preferred co-factor for this enzyme. Presence of NAD\(^+\) reduced the reaction rate to almost zero.

4.1.2.5. Cold-inactivation

The glutamate dehydrogenase of *A. brasilense* RG is a cold-sensitive enzyme (Fig. 4.6). The rate of cold-inactivation at 0°C was determined using the Sephadex G-200 fraction. The kinetics of the enzyme inactivation at 0°C showed an exponential pattern, a loss of about 80% of the initial activity occurred within one hour and a total loss of activity was observed in about 5 hr. Kinetics of inactivation was also carried out between 0°C-20°C. The results show that the enzyme was extremely unstable below 5°C but moderately stable above 15°C. The presence of 20% glycerol (v/v) in the enzyme solution was found to protect the enzyme against cold inactivation. Inactivation of the enzyme in the absence of glycerol could be arrested at any
Fig. 4.6. Cold sensitivity of GDH.

Cold sensitivity of GDH, inactivation of GDH was measured with the Sephadex G-200 fraction (0.17 mg/ml) in buffer A + 1% glycerol (v/v) after incubation at various temperatures from 0°C to 20°C (A) or at 0°C (B). Aliquots were taken out at the indicated times, quickly warmed up to 30°C and assayed for NADPH-dependent GDH activity. Aliquots from cold-shocked GDH (B, ●), taken out at different intervals and further incubated at 30°C were also assayed for enzyme activity (B, 0). The Sephadex G-200 fraction containing 20% glycerol was incubated at 0°C and assayed for GDH activity at different intervals (B, □). DEAE-cellulose fraction (5.6 μg/ml) in buffer A + 10% glycerol was incubated at 0°C and at different time intervals aliquots were taken out and assayed for NADPH-dependent (C, 0) and NADH-dependent (C, ●) activities.
stage during cold exposure by elevating the temperature of enzyme solution to 25°C. The cold inactivation was essentially irreversible - the lost activity could not be restored on warming the enzyme solution. The cold inactivation experiments using homogeneous preparation of GDH (DEAE-cellulose fraction) showed that the NADH-dependent activity was as cold sensitive as the NADH-dependent activity; both the activities decayed at the same rate at 0°C.

4.1.2.6. Dual coenzyme specificity

Glutamate dehydrogenase of *Azospirillum brasilense* was found to possess dual coenzyme specificity. The enzyme could catalyse the reductive amination of 2-oxoglutarate in the presence of both NADPH and NADH though the kinetic parameters for these two reactions were different; the $K_m$ values of this enzyme for NADH and NADPH were $5.0 \times 10^{-4}$ M and $1.3 \times 10^{-5}$ M. The ratio of NADH:NADPH-dependent activities in the homogeneous preparation of the enzyme (DEAE-cellulose fraction) was 0.91. The ratio of two activities remained fairly constant throughout the purification step. The ratio of NADH:NADPH-dependent activities in various enzyme fractions under optimum conditions of their assay, were as follows: $(NH_4)_2SO_4$ fraction 0.81 Sephadex G-200 1.04 and the Red Sepharose fraction 0.99. Since the ratio of these two activities remained reasonably constant during purification, it can be assumed that both the activities are associated
with the same enzyme. The dual coenzyme specificity of GDH is further supported by the fact, that during cold inactivation, the ratio of NADH: NADPH activities of the purified enzyme remained fairly constant (Fig. 4.6C). Although the $V_{\text{max}}$ of this enzyme was nearly the same for both NADH and NADPH-dependent reaction, the $K_m$ values for these two substrates indicate that the enzyme has higher affinity for NADPH.

4.1.2.7. Determination of $K_m$ values of glutamate dehydrogenase for its various substrates

Table-4.3: Michaelis constants for different substrates of GDH from *A. brasilense* RG and the molecular activities of the enzyme.

<table>
<thead>
<tr>
<th>Reaction catalysed</th>
<th>Substrate</th>
<th>$K_m$</th>
<th>Molecular activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>min$^{-1}$</td>
</tr>
<tr>
<td>NADPH-dependent</td>
<td>NADPH</td>
<td>$1.3 \times 10^{-5}$</td>
<td>12000</td>
</tr>
<tr>
<td></td>
<td>2-oxoglutarate</td>
<td>$2.5 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\text{NH}_4\text{Cl}$</td>
<td>$1.0 \times 10^{-1}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$3.8 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>NADH-dependent</td>
<td>NADH</td>
<td>$5.0 \times 10^{-4}$</td>
<td>11000</td>
</tr>
<tr>
<td></td>
<td>2-oxoglutarate</td>
<td>$5.0 \times 10^{-3}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\text{NH}_4\text{Cl}$</td>
<td>$6.6 \times 10^{-2}$</td>
<td></td>
</tr>
<tr>
<td>NAD$^+$-dependent</td>
<td>glutamate</td>
<td>$1.0 \times 10^{-2}$</td>
<td>3660</td>
</tr>
<tr>
<td></td>
<td>NADP$^+$</td>
<td>$4.0 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>NAD$^+$-dependent</td>
<td>glutamate</td>
<td>ND</td>
<td>$&lt; 200$</td>
</tr>
<tr>
<td></td>
<td>NAD$^+$</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Michaelis constants were calculated from the Lineweaver-Burk plots shown in Fig. 4.7.

ND = Not determined.
Fig. 4.7. Determination of the Michaelis constants of the different substrates of GDH from the Lineweaver-Burk plots.

GDH activity (either NADPH-dependent or NADH-dependent) was measured under the standard assay conditions as described in Section 2.2.4. and the concentration of a particular substrate was varied in each case.

A) Lineweaver-Burk plot for NADPH in the NADPH-dependent reductive amination reaction.

B) Lineweaver-Burk plot for 2-oxoglutarate in the NADPH-dependent reductive amination reaction.

C) Lineweaver-Burk plot for NH₄Cl in the NADPH-dependent reductive amination reaction.

D) Lineweaver-Burk plot for glutamate in the NADP⁺-dependent oxidative deamination reaction.

E) Lineweaver-Burk plot for NADP⁺ in the NADP⁺-dependent oxidative deamination reaction.

F) Lineweaver-Burk plot for NADH in the NADH-dependent reductive amination reaction.

G) Lineweaver-Burk plot for 2-oxoglutarate in the NADH-dependent reductive amination reaction.

H) Lineweaver-Burk plot for NH₄Cl in the NADH-dependent reductive amination reaction.
NADPH-dependent reaction

NADPH

A

250 200 150 100 50

\( \frac{V}{[S]} \) (mM/min)

\( \frac{V}{M} \) (mM)

50

25

0
NADPH-dependent reaction

2-oxoglutarate

\[ \frac{1}{v} \left( \text{min}^{-1} \mu\text{moles}^{-1} \right) \]

\[ \frac{1}{[S]} \left( \text{mM}^{-1} \right) \]
NADPH-dependent reaction

NH₄Cl

\[
\frac{\text{mM}}{\text{min}} \times \frac{\text{moles}}{\text{min}}
\]

\[
\frac{1}{[S]} \text{ (mM)}
\]

\[
\frac{1}{[S]} \times \frac{1}{[S]}
\]

500 400 300 200 100

0.5 1.0 1.5 2.0
NADP⁺-dependent reaction

\[ \frac{1}{V} \left( \frac{\text{min}}{\mu\text{mol}} \right) \]

vs

\[ \frac{1}{[S]} \left( \frac{1}{\text{mM}} \right) \]

Graph showing the relationship between the reciprocal of reaction velocity (V) and the reciprocal of substrate concentration ([S]).
NADH-dependent reaction

NADH

\[ \frac{1}{[S]} \text{mM} \]

\[ \frac{1}{\text{mol/min}} \]
The kinetic constants of glutamate dehydrogenase (both NADPH-dependent and NADH-dependent reactions) were determined from the Lineweaver Burk plots (Fig. 4.7). The plot for NADPH show that although Michaelis Menten equation was followed at lower substrate concentrations (upto 0.064 mM), there was a considerable inhibition of reaction rates at higher substrate concentration. Very strong substrate inhibition was also observed in the case of 2-oxoglutarate in the NADPH-dependent reaction where substrate concentrations above 1 mM were found to be inhibitory. In the case of NH₄Cl in the NADPH-dependent reaction, the Lineweaver Burk plot was biphasic in nature; there were two $K_m$ values for this substrate (100 mM and 0.38 mM) - thus this enzyme has both a high affinity site and a low affinity site for NH₄Cl. The double reciprocal plots for glutamate and NADP⁺ i.e. the substrates taking part in the oxidative deamination reaction catalyzed by the enzyme were found to be linear.

For the NADH-dependent reductive amination reaction, the Lineweaver Burk plot for NADH was found to be linear but the $K_m$ value was very high (0.5 mM). The plots for NH₄Cl and 2-oxoglutarate showed substrate inhibition in the NADH-dependent reactions. The $K_m$ values for 2-oxoglutarate and NH₄Cl in the case of NADH-dependent reaction were very much different from those estimated in the case of NADPH-dependent reaction.
Fig. 4.8. Inhibition of GDH activity by alanine and lysine.

The DEAE-cellulose fraction was assayed for GDH (NADPH-dependent) activity spectrophotometrically in an incubation mixture containing 100 mM potassium phosphate pH 10.0, 50 mM NH₄Cl, 0.1 mM NADPH and varying concentrations of 2-oxoglutarate in the presence or absence of alanine (A) or lysine (B).
4.1.2.8. Inhibition by amino acids

It has already been shown that high concentration of amino acids in the growth medium repressed GDH synthesis in the cells at least by 10-fold. Amino acids were also found to have inhibitory effect on the activity of this enzyme. At the optimum pH 8.5, in potassium phosphate buffer, addition of amino acids in the standard assay mixture showed a weak inhibitory effect on NADPH-dependent reductive amination reaction by GDH; only the inhibitory effect of glutamate was quite significant. However, when the same reactions were carried out at higher pH (> 9.5) in potassium phosphate buffer, all the amino acids acted as strong inhibitors of GDH activity. To find the nature of inhibition, the initial velocity of the reaction was measured at varying concentrations of 2-oxoglutarate with several fixed concentrations of alanine and lysine. The plots (Fig. 4.8) clearly show that both alanine and lysine act as competitive inhibitors of the substrate 2-oxoglutarate. The $K_i$ values of alanine, lysine, glutamate and histidine have been also determined at pH 8.5 and pH 10.0 in potassium phosphate buffer by the method of Dixon (112) (Figs. 4.10 and 4.11) and given in Table 4.4. The NADPH-dependent enzyme activity was measured in the presence of varying concentrations of amino acids and fixed concentration of 2-oxoglutarate (1 mM) in the standard assay mixture. The $K_i$ value of a particular amino acid was calculated from the plot shown in Figs. 4.10 and 4.11 with the help of following equation:
Fig. 4.9. Lineweaver Burk plots for 2-oxoglutarate in 100 mM potassium phosphate buffer.

GDH (NADPH-dependent) activity was measured under the standard assay conditions except that 100 mM potassium phosphate buffers (at pH 8.5 and pH 10.0) were used instead of Tris-HCl.
Lineweaver Burk plots for 2-oxoglutarate in phosphate buffer

pH 10.0

pH 8.5
Fig. 4.10. Dixon plots for the determination of the $K_i$ values of glutamate, alanine, lysine and histidine at pH 8.5.

GDH (NADPH-dependent) activity was measured under the standard assay conditions except that 100 mM potassium phosphate buffer (pH 8.5) was used instead of Tris-HCl and varying concentrations of a particular amino acid was present.
Fig. 4.11. Dixon plots for the determination of the $K_i$ values of glutamate, alanine, lysine and histidine at pH 10.0.

GDH (NADPH-dependent) activity was measured under the standard assay conditions except that 100 mM potassium phosphate buffer (pH 10.0) was used instead of Tris-HCl and varying concentrations of a particular amino acid was present.
Dixon plot (pH 10.0)

Dixon plot (pH 10.0)
Value of the intercept on x-axis = \( K_i \left( \frac{[S]}{K_m} + 1 \right) \)

The \( K_m \) value of 2-oxoglutarate at pH 8.5 and pH 10.0 in 100 mM potassium phosphate buffer was found to be 0.55 mM (Fig. 4.9).

Table 4.4: Inhibitor constants for amino acid - GDH complexes.

Inhibitor constants were determined using standard assay mixture except that Tris-HCl was replaced by 100 mM potassium phosphate. The \( K_i \) values were calculated from the Dixon plots (Figs. 4.10 and 4.11) in all cases. \( K_i \) values of alanine and lysine were also calculated from the double reciprocal plots shown in Fig. 4.8.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>( K_i ) pH 8.5</th>
<th>( K_i ) pH 10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>( 23.2 \times 10^{-3} ) M</td>
<td>( 6.4 \times 10^{-3} ) M</td>
</tr>
<tr>
<td>Lysine</td>
<td>( 9.1 \times 10^{-3} ) M</td>
<td>( 4.2 \times 10^{-3} ) M</td>
</tr>
<tr>
<td>Histidine</td>
<td>( &gt; 40.0 \times 10^{-3} )</td>
<td>( 6.4 \times 10^{-3} )</td>
</tr>
<tr>
<td>Glutamate</td>
<td>( 4.1 \times 10^{-3} )</td>
<td>( 1.1 \times 10^{-3} )</td>
</tr>
</tbody>
</table>

\( K_i \) values for alanine and lysine at pH 10.0 calculated from Fig. 4.8 were found to be \( 6.1 \times 10^{-3} \) M and \( 3.7 \times 10^{-3} \) M respectively.

Like the NADPH-dependent reaction, the NADH-dependent reductive amination reaction is also strongly inhibited by amino acids especially glutamate and lysine; the enzyme activity is reduced by 50% in the presence of 25 mM glutamate and lysine. The kinetics of inhibition have not been studied in detail in the case of NADH-dependent reaction.
4.1.2.9. GDH of *A. brasilense* sp.7

Previous investigators had reported the presence of a NADH-dependent GDH in the crude extracts of *A. brasilense* sp.7. Since the results presented here are different from earlier reports, the GDH activity was measured from the crude extracts of *A. brasilense* sp.7 (obtained from Dr. N.R. Kreig). Crude extracts preparation was carried out according to the method described in Section 2.2.5.1. The NADH-dependent activity and the cold sensitivity of GDH of *A. brasilense* sp.7 were measured with the partially purified enzyme ((NH₄)₂SO₄ fraction).

Table 4.5: Comparison of the properties of GDH from *A. brasilense* sp.7 and *A. brasilense* RG.

<table>
<thead>
<tr>
<th></th>
<th><em>A. brasilense</em> sp.7</th>
<th><em>A. brasilense</em> RG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Specific activity of GDH (NADPH-dependent) in the crude extract</td>
<td>0.26</td>
<td>0.22</td>
</tr>
<tr>
<td>2. NADH:NADPH-dependent GDH activity in the (NH₄)₂SO₄ fraction</td>
<td>0.86</td>
<td>0.81</td>
</tr>
<tr>
<td>3. % of initial activity left after incubation of the enzyme sample at 0°C for 1 hr.</td>
<td>5.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

GDH activity (NADH and NADPH-dependent) was assayed according to the method described in Section 2.2.4.

It is evident from the results shown above that the GDH of *A. brasilense* sp.7 and *A. brasilense* RG have identical properties.
4.2. Discussion

The enzyme glutamate dehydrogenase has been characterized after its isolation and purification from a variety of prokaryotic organisms. So far, no detailed investigation has been made with GDH of any N$_2$-fixing soil bacterium, in spite of its importance in our understanding of N-metabolism in such organism. Previous studies established that NADP$^+$-dependent GDH of bacteria plays an ammonia assimilatory role, particularly when ammonia is present in the culture medium at high concentrations. As bacterial GDHs generally show a high $K_m$ for ammonia ($>1$ mM) it cannot function efficiently when ammonia is present in growth limiting concentration, such as during N$_2$-fixation by a bacterium. In such situations glutamine synthetase (which usually shows a much lower $K_m$ for ammonia) and glutamate synthase play a critical role in the assimilation of ammonia.

In *A. brasilense*, the regulation of synthesis of these enzymes by various N-sources have been already studied and described in the previous chapter. The synthesis of the enzyme GDH is very strongly regulated by the nitrogen source in the growth medium. Moreover, the purified enzyme was found to have a number of unusual and interesting properties. The GDH of *A. brasilense* is specific for both NADH and NADPH for its reductive amination reaction. No GDH of bacterial origin is known to use both NADH and NADPH as cofactors. The kinetics of both NADH-dependent and NADPH-dependent reactions
of GDH indicate that this enzyme is primarily designed to catalyse the reductive amination of 2-oxoglutarate, in spite of its dual coenzyme specificity. The $K_m$ of GDH (NADPH-linked) for glutamate is two orders of magnitude higher than the $K_m$ for 2-oxoglutarate and no NAD$^+$-dependent oxidative deamination of glutamate could be demonstrated with this enzyme. In addition to this, the molecular activity of this enzyme (NADPH-dependent reaction) is 3.3 times higher in the forward direction (12000) than in the reverse direction (3600). These properties of GDH of *A. brasilense* are very different from the properties of GDHs from animal sources (108-110) which also show dual coenzyme specificity. The kinetic parameters of animal GDHs, indicate that the enzyme can support biosynthesis as well as degradation of glutamate under physiological conditions.

The amino acids in general are found to be inhibitory for the GDH activity; the amino acids compete with 2-oxoglutarate for the substrate binding site on the enzyme although they cannot substitute for glutamate as the substrate for the oxidative deamination reaction. The inhibitory effect of the amino acids is more pronounced at higher pH values (> 9.5); thus it seems that the $RCH_2(NH_2)COO^-$ form is the active species that compete with 2-oxoglutarate. The inhibitory effect of amino acids on GDH activity has not been reported earlier. Observation of this phenomenon in GDH of *A. brasilense* RG suggests that glutamate synthesis in the bacterium can be shut off when NH$_3$ assimilation becomes unnecessary because of higher amino acid pool in the cell.
The Lineweaver-Burk plots for 2-oxoglutarate in both NADH-dependent and NADPH-dependent reactions show substrate inhibition. The same phenomenon is observed in the case of NADPH but not of NADH. The Lineweaver-Burk plot for ammonia in the NADPH-dependent reaction is biphasic in nature; so far only one NADP+-linked GDH from *Nitrobacter agilis* (106) is reported to have such a kinetic property. This biphasic plot could be because of the presence of a low affinity and a high affinity site for NH$_3$ on the enzyme. In the case of GDH of *A. brasilense*, the high affinity site seems to operate when the concentration of NH$_4^+$ is below 13 mM; at higher concentration ( $>$ 13 mM NH$_4^+$) the low affinity site can become effective for reductive amination. Thus this enzyme is quite versatile in the sense that it is capable of ammonia assimilation from a very low to a high concentration. Cold sensitivity of GDH of *A. brasilense* is an unusual property. So far cold labile GDH has not been encountered in prokaryotes or in eukaryotes except in the case of a mutant of *Neurospora crassa* (113) and a strain of *Candida utilis* (114). The unusual properties of GDH like cold sensitivity and dual coenzyme specificity observed in the case of *A. brasilense* RG are also present in *A. brasilense* sp.7. From the results shown in Table 4.5 it appears that GDH from *A. brasilense* RG and *A. brasilense* sp.7 have identical properties.

Glutamate dehydrogenase of *A. brasilense* thus appears to be quite an unusual enzyme. Careful investigation of the role of glutamate dehydrogenase in nitrogen
assimilation in other nitrogen fixing bacteria in general might throw a new light on the current concepts of the nitrogen regulation system.