CHAPTER IV

DISCUSSION

4.1 Stability of GS

Before attempting to purify and characterize glutamine synthetase from Klebsiella R15, buffer and condition to preserve the enzyme activity were studied. Although imidazole-HCl buffer was good for the activities of GS and GOGAT in B.subtilis (50) and A.brasilense (45), fluctuation of the GS activity occurred when the same buffer was used for the enzyme preparation from Klebsiella R15. The result was improved when imidazole-HCl was replaced by TME buffer (fig.3). Moreover, storing the cells in frozen state could keep the enzyme stable for up to 4 days (fig.4). These buffer and storage condition were thus always used throughout this work.

4.2 Production of GS in NF medium

It has been mentioned in previous report (2), that most microorganisms derepress GS in ammonium-limited medium. To obtain high activity of Klebsiella R15 GS for further purification, the culture was grown in NF medium. High activity of GS in both biosynthetic and transferase assays were observed in late log phase of cultural growth

(fig.5).

Results from pH profile (fig.14) show pH optimum at 6.6-6.8 and the isoactivity pH at 7.6. Because the transferase assay was performed at pH 6.8 the activity did not show the total amount of enzyme exactly. However, deadenylylated form was measured in the biosynthetic assay, and the activity is parelled to transferase activity. According to this result, the cells were always harvested in early stationary phase.

4.3 Comparative studies of GS, GDH and GOGAT activities in crude enzyme

The GS in NF culture was found to be more active than that in the NFA100 culture for about 15 fold (fig.6). GDH in NF culture could not be detected even the crude extract was 5 fold concentrated. It was then suggested that Klebsiella R15 use GS-GOGAT pathway for assimilating ammonium during ammonium-limited condition.

The presence of excess ammonium caused the repression in GS total activity. Even though GS activity was repressed in high ammonium salt condition, GDH activity was not increased adequately. Thus ammonium should be assimilated by GS-GOGAT pathway during ammonium excess condition. High activity of GOGAT in NFA100 confirms this conclusion.

These results suggest that GS is the regulatory enzyme in ammonium assimilation and being controlled by ammonium in the environment. Further characterization (later discussed) on covalent modification and feedback inhibition strongly confirm this key role of the GS.

4.4 GS purification

Sharad, and Nicholas(46) have obtained, from Nitrobacter agilis, GS with 60% yield and 42-fold purified by affinity column chromatography. The purification was increased to 431 fold with 58% yield by Sepharose-4B gel filtration. These two steps were then chosen for the purification of Klebsiella spp.R15 GS enzyme.

The results from table 3 indicate that Sepharose CL-6B affinity column chromatography could be used successfully with the Klebsiella enzyme, only a single step could purify the enzyme to about 61-fold with 291% recovery. This success may be due to two factors, i.e. the specific binding of the enzyme molecule to the ligand site and the specific elution of the enzyme with its substrate.

Affinity Blue Sepharose CL-6B is Cibacron Blue F3G-A covalently attatched to the cross-linked agarose gel, Sepharose CL-6B (fig.24). The blue dye in Sepharose CL-6B is capable of binding strongly to many proteins or

enzyme mostly required adenylyl-containing cofactors e.g.NAD^+ (52). This structure serves as the adenylyl group analogue, binding specifically to the adenylylated site on the enzyme molecule. Thus only the deadenylylated or slightly adenylylated enzyme can strongly bind to the column while highly adenylylated form cannot. ADP, with its adenine moiety, competes this binding and thus elutes the enzyme from the column.

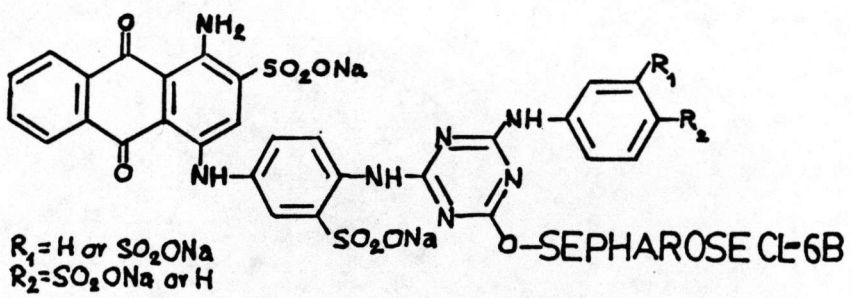

Fig.24 Partial structure of Blue Sepharose CL-6B

Though the Sepharose-4B chromatography was efficient in the removal of contaminated smaller proteins (fig.8 and fig.10), the purification fold did not increase but even decrease to 35-fold and the % recovery was very low (39%). This may be the result of the instability of the enzyme in very diluted solution after the Sepharose-4B chromatography. This problem may be solved by concentrating the enzyme immediately after the active fractions were pooled. Although the recovery was low,

results from polyacrylamide gel electrophoresis show that the GS was pure (fig.9 and fig.10).

Of all various characteristics observed, the change in transferase activity upon SVP treatment is the first evidence to show that covalent modification exists in Klebsiella R15 GS. The supporting evidence was the patterns on isoelectric focusing gel and the retardation effect on affinity column as will be described later.

4.5 Preparation of highly adenylylated GS

After purification, the molecular weight of GS from Klebsiella R15 was determined as 430,000. Unlike the enzyme of E.coli, this Klebsiella GS is an octamer of identical subunit , similar to that reported in eucaryotes (32-34).

The affinity chromatography on Blue Sepharose Cl-6B could resolve the GS into two peaks (fig.15). The enzyme in the wash off peak was considered to be the highly adenylylated form while the second peak was slightly adenylylated and/or deadenylylated form. These conclusions are based on the following reasons:-

1.Total GS activity of heat treated fractions from ammonium-shocked cells (50 units, table 5) was higher than that of heat treated- fractions from non-shocked cells (28 units, table 3). Thus the increase in total enzyme

activity caused by ammonium induction =50-28=22 units.

2.About 25 units of GS activity (calculated from fig.15) was eluted slightly after the wash off fraction (pool GS_A).

3.The activity in reason item 1 and 2 was comparable. It was then fair to conclude that the higher adenylylated GS caused by ammonium induction (22 units) was the GS (25 units), which was slightly retarded in affinity column.

The slight retarding effect could be explained in the sense that GS_A was not completely adenylylated, some sites of on the enzyme was available to bind to the column. Retarding effect was more if less sites of GS were occupied by adenylyl group and vice versa.

4.Because of its capability of binding to the column and being eluted specifically with 2mM ADP buffer, the second peak enzyme should be in the slightly adenylylated form, similar to that obtained in fig.7. However, both forms of the enzyme were further distinguished as described later.

4.6 The stability of purified GS

The purified GS from ammonium-limited cells and ammonium-shocked cells showed no significant difference in their stability upon storage at -20°C (fig.16). These

results imply that the degree of adenylylation of the purified GS from these two sources should not be significantly different due to the purification by affinity column. The effect of adenylylation on the stability of GS remained open, because comparison with SVP-treated GS has not been performed. However, these results clearly show that the purified GS could be kept for 2 months with only 40% loss of the activity.

Heat lability of GS_A and GS_B, the two forms of the enzyme on affinity column was similar to the pattern of stability upon storage. Either no significant difference in adenylylation state of GS between GS_A and nontreated GS_B or adenylylation has no effect on heat stability could be considered. The former explanation was supported by feedback inhibition study, and K_m for glutamine of GS_A and GS_B. However, the results implied that heat stability pattern of Klebsiella R15 GS is the special characteristic of the genus, unlike GS in other gram negative bacteria but similar to that of GSII which has been suggested to be involved in nitrogen fixation in Rhizobium spp.(39) and Frankia spp.(30).

4.7 Kinetic studies

The other specific characteristic of Klebsiella R15 GS is the transferase activity of adenylylated form,

which exceeds that of the deadenylylated form at all pH range 6.0-7.6. This behaviour was also found in some other nitrogen-fixing bacteria, e.g. Azotobacter vinellandii (28), of which the activity was assayed at pH 7.1. Klebsiella R15 GS has pH optima 6.6-6.8 and 8.0. Frankia spp. possesses GSI which has an optimum pH at 6.9, with no shift to other pH upon deadenylylation (30) as observed in Klebsiella R15. In Klebsiella aerogene (44) the pH optimum of GS is lowered by adenylylation over the pH range tested (7.0-7.6), and its isoactivity point was rather closed to the Klebsiella R15 GS (pH 7.6).

The K_m values of Klebsiella R15 GS for glutamine and hydroxylamine of the highly adenylylated and deadenylylated enzyme were compared. Table 6 shows that the K_m for glutamine of the SVP-treated GS_B=2.0mM and the higher adenylylated states possess higher K_m value (15.4 and 18.2mM). This is similar to that reported in Klebsiella aerogene(44) (deadenylylated form 2.7mM, adenylylated form 30mM). This finding clearly explains that the process of adenylylation is responsible for decreasing the affinity of the enzyme to glutamine substrate.

4.8 Feedback inhibition

Among various amino acids and nucleotide tested,

Ala, Gly and AMP showed inhibition on the transferase activity of the enzyme. The inhibition by Ala and Gly was more preference on the deadenylylated form (table 7) while AMP showed strong inhibition on the adenylylated enzyme. This pattern of inhibition was similar to that observed in E.coli enzyme (51).

CONCLUSION 1.GS plays a key role in ammonium assimilation in Klebsiella spp.R15. The bacteria used GS-GOGAT pathway when grown in both NH_4^+-limited and NH_4^+-excess conditions.

2.GS in free living Klebsiella R15 is regulated by ammonium level at both synthetic and post synthetic steps. Post synthetic regulation was through the reversible covalent modification:the adenylylation-deadenylylation process.

3.Klebsiella spp.R15 GS is an octamer with a molecular weight of 430,000. The molecular weight of each subunit was 54,000. pH optimum in transferase assay was in the range of 6.6-6.8 and 8.0 while the isoactivity pH was at 7.6.

4.GS activity was regulated by feedback inhibition in the manner of cumulative effect. AMP demonstrated different degree of inhibition on the two forms of GS.