CHAPTER I

INTRODUCTION

Glutamine is one of the most important compounds in nitrogen metabolism. It is not only a building block for the synthesis of proteins, but is also a source of nitrogen for the biosynthesis of numerous other compounds including glucosamine-6-phosphate (Glnc-6-P), para-amino benzoic acid (PABA), carbamyl phosphate, and nucleotides. The significance of glutamine in these biosynthesis is shown in fig.1 (1). Two major pathways of ammonium assimilation which response for generating glutamic acid and subsequently to glutamine are: the "glutamate dehydrogenase pathway" (L-Glutamate:NAD^+oxidoreductase, EC.1.4.1.2,GDH) and the "glutamine synthetase-glutamate synthetase (GS-GOGAT) pathway" (L-Glutamate:ammonium ligase, EC.6.3.1.2, GS; L-Glutamate:$NADP^+$oxidoreductase, EC.1.4.1.3,GOGAT) (1)

GDH PATHWAY

$$NH_4^+ + \text{2-oxoglutarate} + NAD(P)H + H^+ \xrightarrow{GDH} \text{L-glutamate} + NAD(P)^+ + H_2O$$

GS-GOGAT PATHWAY

$$NH_4^+ + \text{L-glutamate} + ATP \xrightarrow{GS} \text{L-glutamine} + ADP + Pi$$

$$\text{2-oxoglutarate} + \text{L-glutamine} + NAD(P)H + H^+ \xrightarrow{GOGAT} 2\text{L-glutamate} + NAD(P)^+$$

These two ammonium-assimilating pathways are species and environmental dependent. The enteric bacteria utilize the GDH pathway in ammonium rich medium. In ammonium limited medium, such as in low concentrations of ammonium or when using nitrate or molecular nitrogen as nitrogen sources, these bacteria derepress GS and assimilate NH_4^+ by the GS-GOGAT pathway. Almost all N_2-fixing procaryotes reported to date have been shown to assimilate ammonium by the GS-GOGAT pathway during N_2-fixation(2-4).

The activity and concentration of GS in enteric bacteria are regulated by the availability of nitrogen sources (5-7).The pathways which regulate GS gene expression and enzymatic activity involve the covalent modification of specific proteins as shown in fig.2. The regulation of GS activity in Gram-negative bacteria, have been shown to be involved with the adenylylation and deadenylylation at the hydroxyl group of Tyr_{397} (8) in

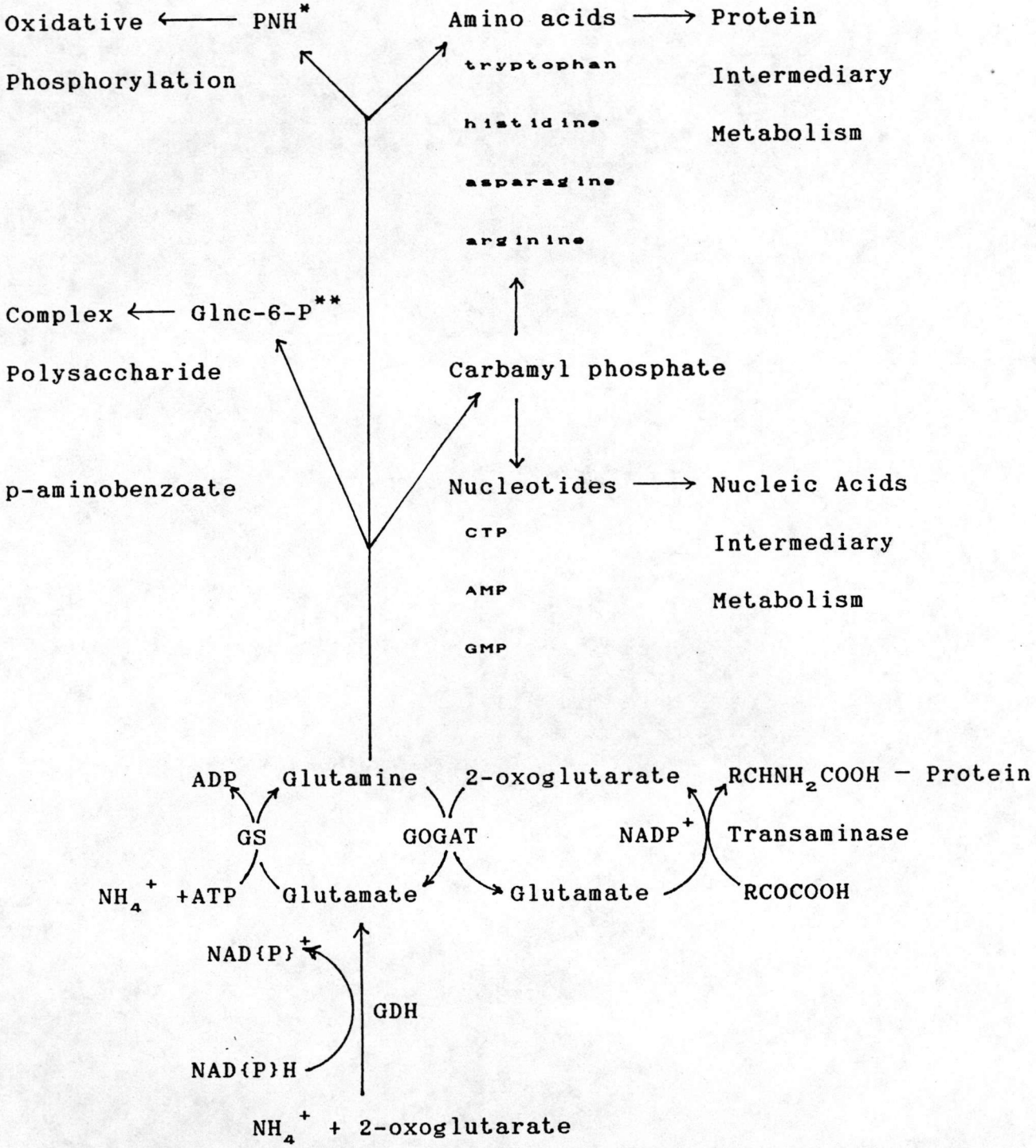

Fig.1 Pathways of ammonium assimilation in the enteric bacteria for the production of glutamate and glutamine, Some of the roles of these compounds in intermediary metabolism are also shown. PNH*=phosphonucleotide, Glnc-6P**=glucosamine-6-phosphate.

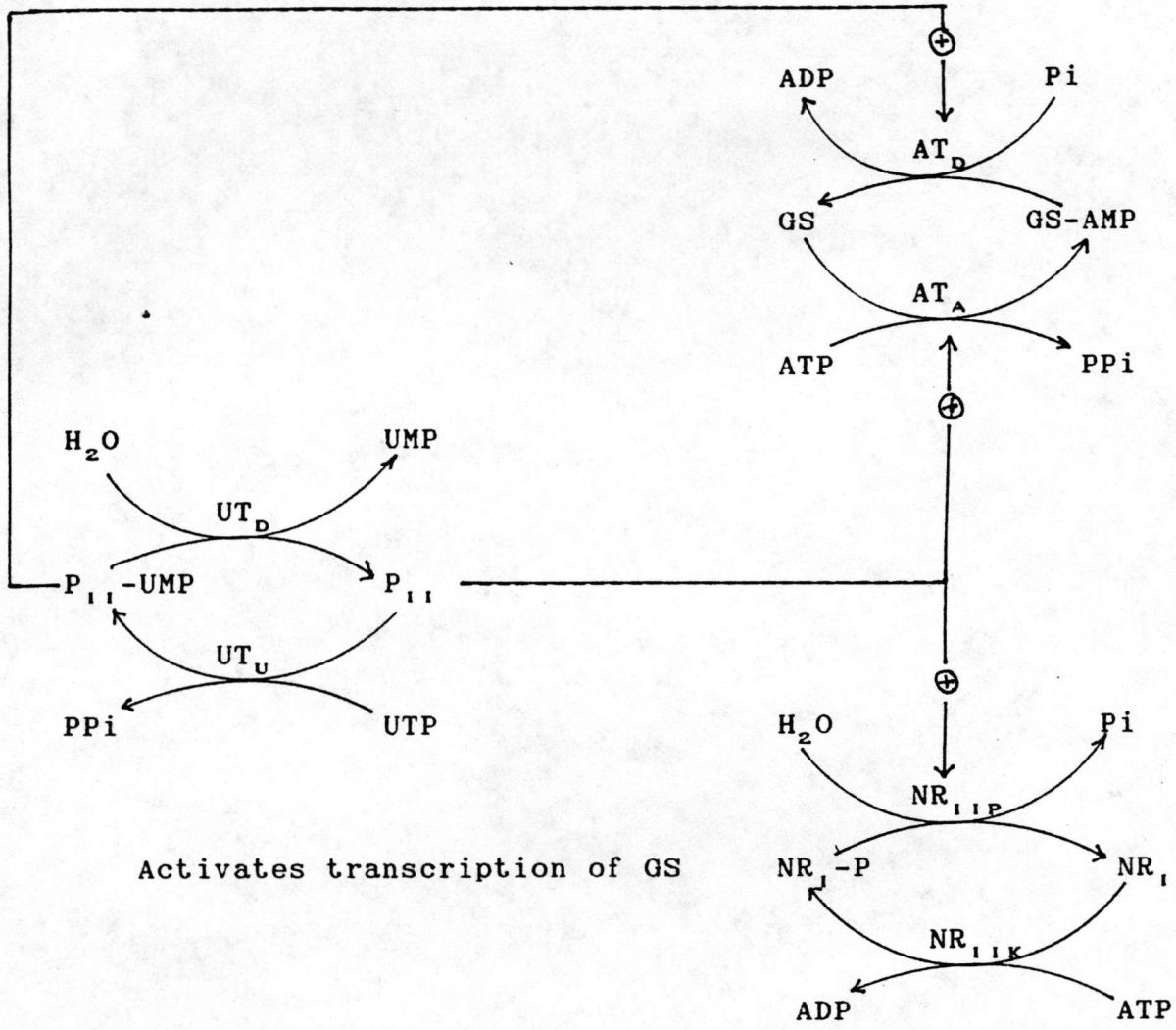

Fig.2 Three protein interconversions involved in the regulation of glutamine synthetase in E.Coli. AT_D/AT_A, adenylyl-transferase catalyzing the adenylylation (AT_A) and deadenylylation (AT_D) of glutamine synthetase; UT_D/UT_U, uridylyl transferase catalyzing the uridylylation (UT_U) and de-uridylylation (UT_D) of P_{II} ; NR_{IIP}/NR_{IIK}, catalyzing the phosphorylation (NR_{IIK}) and dephosphorylation (NR_{IIP}) of NR_I protein.

each subunit of the dodecameric enzyme (9). In physiological condition, the adenylylation and deadenylylation modifications of GS are both catalyzed by the adenylyltransferase [ATase]. The enzyme ATase contains separate catalytic sites which response for the adenylylation (AT_A) and deadenylylation (AT_D) process (10-11). However, the direction of catalysis is dictated by the regulatory protein P_{II} (12-13), of which the activity is also modulated by reversible covalent modification (uridylylation-deuridylylation reaction). The unmodified form (P_{II}) activates the adenylylation reaction (AT_A),whereas the uridylylated form (P_{II}-UMP or $P_{II}D$) is required in the deadenylylation reaction (AT_D). As shown in fig.2, a specific uridylyltransferase (UT_U) catalyzes the transfer of a uridylyl group from UTP to each subunit of P_{II} (12-14). Because the P_{II} protein is composed of four identical subunits, up to four uridylyl groups can be attatched per P_{II} molecule (13). The uridylylation and deuridylylation activities reside in the same molecule of enzyme UTase. The uridylylation (UT_U) and deuridylylation (UT_D) reactions are controlled by the concentrations of the metabolites glutamine and 2-oxoglutarate-which reflect the status of available nitrogen. UT_U is stimulated by 2-oxoglutarate but UT_D is stimulated by glutamine (15). Thus ammonium starvation,

which results in a high intracellular ratio of 2-oxoglutarate to glutamine, causes the conversion of P_{II} to P_{II}-UMP. In contrast, growth with excess ammonium, which results in a high intracellular ratio of glutamine to 2-oxoglutarate, causes the conversion of P_{II}-UMP to P_{II}.

The P_{II} protein also plays an important role in the regulation of GS at the biosynthetic level. It activates the dephosphorylation of NR_I-P (or alternatively designated as NTRC (16-17) which, in turns activates the transcription of GS gene.

Thus, the P_{II} protein transmits the metabolic information sensed by UT_U /UT_D to both the post-translational activity modulation system and the transcriptional regulatory system of GS. Covalent modification of the enzyme is the basis of an elaborate mechanism for the fine control of GS activity in the organism. Under appropriate conditions both forms of the enzyme are catalytically active; however, they differ significantly with respect to catalytic potential, pH optimum, divalent cation specificity and susceptibility to feedback inhibition.

In contrast to most N_2-fixing organism, recent studies of ammonium assimilation pathway in nitrogen-fixing Bacillus polymyxa (19) and Bacillus

maceran(20) indicated that ammonium is assimilated predominantly by the GDH pathway during N_2-fixation process. In these two species the GOGAT level is low in both ammonium grown and N_2-fixing cells. These two species of N_2-fixing Bacillus differ from that of other free living diazotrophs studied, in which the GOGAT activity was found to be significantly higher than (4), or at least comparable (21-22) to that of GDH during nitrogen fixation. Consequently, the mechanism of regulation of ammonium assimilating enzyme in the Gram-positive Bacillus appears to differ from the complex mechanism of regulation observed in the Gram-negative bacteria described earlier.

In nitrogen fixing bacteria, the ammonium is produced from N_2 by nitrogenase, however in both free living and symbiotic diazotroph, the ammonium concentration is vanishingly small because it is assimilated rapidly into cell materials. The GS-GOGAT assimilating pathway has a higher affinity for NH_4^+ but, because it consumes ATP, it is less economic than the GDH pathway. Diazotrophic Klebsiella pneumoniae assimilates the ammonium formed by nitrogenase via the GS-GOGAT pathway (thus consuming another two ATP molecules for each N_2 fixed) and so do most other diazotrophic heterotrophs. In cyanobacteria the assimilatory pathway is not entirely clear (23) and alanine dehydrogenase (ADH) may be

important in the primary step of assimilation.

$$(NH_4^+ + \text{pyruvate} + NADH + H^+ \xrightarrow{ADH} \text{L-alanine} + NAD^+ + H_2O)$$

Free-living diazotrophs are very efficient in ammonium assimilation, so that NH_4^+ formed cannot escape to the external environment. Cyanobacteria can assimilate NH_4^+ nitrogen into phycobiliproteins, a mobilizable endocellular reserve of fixed nitrogen (24). In heterocytous cyanobacteria the heterocysts lack GOGAT but posses GS. Thus the fixed nitrogen is exported to the vegetative cell as glutamine and is then mobilized by GOGAT for biosynthesis. Symbiotic diazotrophs such as rhizobia, or the symbiotic cyanobacterium of Azolla are leaky:a portion of the ammonium is released into the external environment and, presumably, becomes available to the host (25).

Since glutamine is a compound of central importance in the nitrogen metabolism of many microorganisms including nitrogen-fixers, and GS is the key enzyme of the synthetic pathway in those microbes as well. Kinetic and physicochemical properties have been thoroughly investigated in various bacteria (13, 28-31),

fungi (32), rice (33) and bovine brain (34). These properties may highlight the regulation of the enzyme, especially in related to the nitrogen fixation process. The example of GS from nitrogen-fixing bacteria was that from Azotobacter vinellandii. After Kleiner and Kleinschmidt (35,36) have shown in A.vinellandii that the assimilation of ammonium produced in the nitrogenase reaction occurs almost quantitatively via the glutamine-glutamate (GOGAT) pathway with GS as the key enzyme, the regulation of the enzyme has been studied. However, the experiments described by Siedel and Shelton 1979 (28) have not revealed any fundamental difference between this enzyme and the GS of the non-nitrogen-fixing E.coli. The data presented clearly establish the in vivo regulation of A.vinellandii GS by adenylylation / deadenylylation and further support that this control mechanism might be a general phenomenon in Gram-negative bacteria(37). Moreover, electromicroscopy, ultracentrifugal analysis and SDS-disc gel electrophoretic studies provided strong evidence to support that the structure of A.vinellandii GS is identical to that of the E.coli enzyme and other Gram-negative bacteria with a dodecameric arrangement of its identical subunits in two superimposed hexagonal rings. Nevertheless, some differences exist between the catalytic behavior of

A.vinellandii and E.coli GS in the transferase reaction. Most significance is the almost complete inactivation of deadenylylated A.vinellandii GS in the Mn^{2+}dependent transferase assay. So far, no physiological role has been attributed to this reaction

Unlike A.vinellandii, symbiosis N_2-fixer, Frankia spp.strain CpI1 and all members (except Rhizobium sp.strain ORS571, 32) of the Rhozobiaceae, synthesize two forms of GS that have been designated GSI and GSII (38-39). GSI is similar to GS from enteric bacteria in many respects. It is regulated by reversible adenylylation in response to extracellular ammonium concentrations and by feedback inhibition by metabolic downstreams from GS. On the other hand, GSII is apparently regulated primarily by repression/derepression in the presence/absence of ammonium and also by feedback inhibition by certain metabolites (39-40). It has been proposed that GSII might have originated from plant by gene transfer to a progenitor of the Rhizobiaceae (41). It was indicated that GSII is the primary ammonium assimilating enzyme during nitrogen fixation in CpI1.(30).

Klebsiella spp.R15, the associative N_2-fixing bacteria was isolated from the rhizosphere of rice in Thailand (26). This N_2-fixer has been characterized as Gram-negative and rod shape structure. Its association

with rice seeding (CV.RD7) in sterile water resulted in curl, branch, dense and long root hairs. The bacteria adhere firmly on the rhizoplane as individual cells, clusters and eventually as enveloped micronodule structure of 10-15 u in diameter. Invasion of a few bacteria clusters have also been found in the epidermal and outer cortical layer of rice root (27). These observations raised questions concerning the regulation of nitrogen assimilation in this microorganism. It is very likely that GS plays a major role in generating glutamine from ammonium fixed by Klebsiella spp. R15. Base on the finding that GS from other microorganisms is subject to intensive regulatory control as described before, it is important to investigate the existence of this enzyme.

The aims of this thesis are:

1.to demonstrate the existence of GS in Klebsiella spp.R15.

2.to demonstrate the changes in GS activity in this bacteria with respect to NH_4^+

3.to purify the enzyme and to characterize its regulation.

The understanding on the regulation of this enzyme may lead to the explanation about the possible impact of this microorganism on the improvement of rice crop in Thailand.