CHAPTER I

INTRODUCTION

1.1 Ammonium assimilation in nitrogen-fixing bacteria.

In bacteria there are two known pathways for ammonium assimilation via glutamate dehydrogenase (GDH;EC1.4.1.3) (reaction 1), and via glutamine synthetase (GS;EC6.3.1.2) and glutamate synthase (GOGAT; EC1. 4.1.13) reaction (2) respectively. The end product of both pathways is glutamate, from which nitrogen compound of cell are synthesized.

GDH

NH_4^+ + 2-oxoglutarate + NAD(P)H-----> glutamate + NAD(P) (1)

GS

NH_4^++ glutamate + ATP---------> glutamine+ ADP + Pi

GOGAT

glutamine + 2-oxoglutalate + NADPH----> glutamate +NADP (2)

GS/GOGAT is the major enzyme pathway in inorganic nitrogen assimilation in cyanobacteria (Orr and Haselkorn, 1981). This is supported by Russi *et al.*, 1989, with the study in *Rhizobium leguminosarum*, who found that ammonium assimilation in the

organism appeared to take place only through the GS/GOGAT pathways. The control of GS activity as reviewed by Tyler, 1978, showed that GS activity can be regulate by three major different mechanisms, 1) by the interconversion of relaxed (inactive) and taut (active) form in the response to variation in concentration of divalent cation, 2) by cumulative feedback inhibition with various end products of glutamine metabolism and 3) by covalent alteration of the enzyme with reversible adenylylation of a specific thyrosyl residue on each subunit. The ability of GS to catalyze the formation of glutamine can be rapidly decreased by the attachment of adenyl group on the enzyme and ,conversely, rapidly increased by the removal of these groups. Maximum biosynthetic activity is obtained when the enzyme is completely unadenylylated (E_0) and decrease over a wide range as the degree of adenylylated (adenylylation state) increases. However, the fully adenylylated (E_{12}) biosynthetically inactive enzyme still retains glutamyl transferase activity, therefore adenylylated form and unadenylylated form have the same transferase activity measured by the capacity of transferring the glutamine residue of glutamyl to hydroxamate in the present of ADP, Arsenate and Mn^{2+} (reaction 3) (Farnden and Robertson, 1980)

$$\text{glutamine} + NH_2OH \xrightarrow{Mn^{2+}} \text{glutamyl hydroxamate} + NH_3 \quad \text{------ (3)}$$

arsenate, ADP

Adenylylation and deadenylylation are catalyzed by the same enzyme, adenylytransferase (ATase). The reaction with ATase catalysis is regulated by regulatory protein (PII). The unmodified form of PII, piIA stimulates adenylylation of ATase, whereas, a uridylated form ,PIID, is required for deadenylylation. The interconvertion of PIIA and PIID is accomplished by the third enzyme, uridylyltranferase (UTase). UTase is activated by substrate of ammonium assimilation reaction, α-ketoglutarat, and inhibited by product of the same reaction, glutamine. Consequently a high ratio of α-ketoglutarate to glutamine, ammonia deficiency, will stimulate deadenylylation of GS, and conversely, a low ratio of α-ketoglutarate to glutamine, an excess of ammonia, GS will be largely present in the adenylylated form.

1.2 Ammonium assimilation in plant.

Several studies on ammonium assimilation in higher plants indicate that, glutamine synthetase is the key enzyme in ammonium assimilation (Datta *et al.*, 1991: Miao *et al.*, 1991: Kamachi et al., 1992: Sakakibara *et al.*, 1992). GS in plant exits

as a number of isoenzymes which are associated with specific organ and cell compartments. In barley, Mann *et al.*, (1979) found that there are two forms of GS namely, GS_1 and GS_2. GS_1 is cytosolic enzyme and has a molecular weight (MW) of 349,000, pH optimum 7.0, stable at 30°C. GS_2 is chloroplastic enzyme and has a molecular weight of 363,000, pH optimum 7.5, lose activity at 30°C. Stasiewiez and Dunham (1979) studied GS in soybean hypocotyl and found two forms of GS which are characterized by similar molecular weight of 365,000, a little different in optimum pH and temperature of 7.0, 6.5 and 50°C, 45°C respectively. McNally and Hirel,1983, studied GS in several higher plants and reported that higher plants can be classified into four groups according to the pattern of GS. The first group is characterized by the presence only cytosolic GS, whereas the second group is distinguished by the presence only chloroplastic GS, the third group is characterized by cytosolic GS being minor component of total GS activity and the fourth group is distinct from the other groups in having both high cytosolic and chloroplastic GS. Purification, subunit structure, kinetics and physicochemical properties of plant GS have been investigated in various plants. Ericson (1985) studied GS in spinach leaves and found that purified GS has a MW of 360,000 and consists of eight subunits, each has MW of 44,000.

The Km is 6.7 mM for glutamine, pH optimum 7.3. Tingey *et al.*,1987, studied in pea and found that the GS polypeptide predominated in the chloroplast stoma has the MW of 44 kDa. with different from root's (MW of 37 kDa). Datta *et al.*, 1991, studied in *Phaseolus vulgaris* found two form of GS, GS_{n1} and GS_{n2}, both forms of GS are octamer composing of two subunits of polypeptide B and α which differ in charge but not in size.

The GS in plants is regulated by repression and derepression in response to change in environmental levels of combined nitrogen and by several amino acid which are derived from glutamine, by some organic and inorganic substances and by energy change. In addition the genetic of plant is one factor that affect the respond of GS to the mentioned factors. Kanamori and Masumoto, (1972) found that metals, including Cu^{2+}, Hg^{2+}, Cd^{2+} and Zn^{2+} (5 millimolar), strongly inhibit the activity of rice root GS enzyme (90% inhibition), α-ketoglutarate and citric acid, 20 millimolar, increased the enzyme activity about 1.5 fold. Kamachi *et al.*, 1992, studied in cytosolic and chloroplastic GS in rice plant during natural senescence show that through the senescence period (98-140 day old) the cytosolic GS remained constant whereas total GS activity declined to less than a quarter of its initial level. Edward and Coruzzi, 1989, studied on *Pisum sativum* and

Sakakibara *et al.*, 1992, studied in *Zea may* L. found that light could induced expression of chloroplastic GS of both plants, the level of chloroplastic GS protein and mRNA increased after exposed to the light for 24 h. Then it can be concluded that in higher plants GS is the key enzyme in ammonium assimilation, existing as a number of different isoenzyme, GS_1: is isoform in cytosol, GS_2 is isoform in chloroplast. GS in higher plants have MW of 350-400 kDa. consisting of eight subunits of which subunit MW ranging from 43-47 kDa. Regulation of GS is probably a very complex phenomenon considering the diversity of the isoenzyme structure and distribution with in the different organ of the same plant.

1.3 Ammonium assimilation in plant-microbe interaction.

GS is the key enzyme of ammonium assimilation either in plant or in nitrogen-fixing bacteria. Several investigators reported that in natural condition, plant-microbe interaction could alter the GS enzyme in some manner. The plant-microbe interaction can classified in two categories.

1.3.1 Symbiotic condition

1.3.2 Associative condition

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In lower plants symbiotic association between *Lichen*, bryophytes *Azolla* and *Gunnera* and heterocyst-forming cyanobacteria. Nitrogen-fixing cyanobacteria symbiotic with water fern *Azolla* constitute a major group of microorganism in paddy field ecosystem, their potential in rice cultivation are of interest, because dinitrogen reduced by cyanabacteria was assimilated and served as the nitrogen source for growth of the eucaryotic host (Meek 1983). In higher plant, the best known and of the most interesting and important plant-microbe interaction is that between leguminous plant and bacteria of the genera *Rhizobium* and *Bradyrhizobium*. Legumes are a large group of plant which include several economical important plants. *Rhizobium* and *Bradyrhizobium* are Gram negative motile soil bacteria which can infect, and inhibit usually in root nodules, always found in nodule of leguminous plants. Infection of the roots of leguminous plant of the appropriate species of the bacteria lead to the formation of root nodule, which are able to convert gaseous nitrogen into combined nitrogen.

There are a wide variety of leguminous plants-*Rhizobium* symbiosis. The results are vary depending on the species of the plants and bacteria, however it can be concluded that in free-living *Rhizobium*, the ammonium derived from dinitrogen fixation

are assimilated via GS/GOGAT pathways in the organism, but in symbiotic condition most of the ammonia produced by nitrogen-fixation in bacteria is assimilated by GS/GOGAT in plant. Brown and Dilwort, 1975, studied in several *Rhizobium* symbiosis with some leguminous plants found that in associative condition the activity of GS/GOGAT in bacteroid were quite low comparing with the free-living state. As review by Cullimore, 1983, that the labelling studied using ^{15}N and ^{13}N showed that ammonium produced by dinitrogen fixation in the bacteroid is excreted into the plant fraction of the nodules and assimilated there by the combined action of GS and GOGAT. The study in legume (Schuller *et al.*,1986) showed that in cytoplasm of legume nodule, ammonium produced by nitrogen fixing bacteroid is incorporated to glutamine and glutamate via the GS/GOGAT pathway of plant. Datta, 1991, observed GS enzyme in kidney bean (*Phaseolus vulgaris*) by immunocytochemistry, and immunogold labelling in the cell cytoplasm of each plant organs fonud that in nodules, the labeling was more intense in the infected cells than in the uninfected cells. Moreno *et al.*, 1991, an d Matin and Chelm, 1991, studied in *Bradyrhizobium japonicum* found the difference between wild type and *glnA* mutant strain. The glnA mutant strain could produce a few number of nodules on soybean root and unable to grow on

nitrate or ammonium as the sole nitrogen source. The results indicate that *glnA* gene of bacteria was also involved in the role of symbiosis.

1.3.2 Associative condition.

The associative bacterial-plants interaction was found in *Gramineae* plants such as tropical grass rice, wheat, maize, sorghum with various genera of bacteria namely *Enterobacter*, *Azospillium*.

Enterobactor species are widespread in rhizophere of grasses grown in both tropical and temperate climates (Kleeberger *et al.*, 1983). The association of nitrogen-fixing bacteria and cereal such as wheat, maize and barley is of interest, because the most important plant for the nutrition of the world population are the cereal, like wheat,corn and rice (Quispel,1991). The biochemistry of ammonium assimilation and transferring of them have been unclear. Michiel *et al.*, (1990) reported that inoculation of crop plants with *Azospirillium* has resulted in significant yield increases only under certain conditions. Bashan, 1991. reported that, inoculation of wheat seedling with *Azospillium brasilense* significantly increased the proton efflux of the roots. The previous study on ammonium assimilation in

Klebsiella R15-rice association, Saengduan, 1992, indicated that in associative condition the nitrogenase activity in *K. oxytoca* R15 increased 400-500 fold and the GS activity increased 5-7 fold comparing with free-living bacteria, suggesting that part of the fixed-nitrogen were assimilated in bacteria. The GS activity of the inoculated rice roots decreased slightly (10%) comparing with free-living rice plant, therefore it remained unclear, how the fixed- nitrogen be assimilated in the rice plant.

1.4 problem.

Rice is the most important plant for economic and nutritional status of Thai people. In general rice plants utilize about 20 kilograms nitrogen per ton of seeds (Swaminathan, 1984). Either from added chemical fertilizer or organic and inorganic nitrogen in the soil (combined nitrogen) chemical nitrogen fertilizer are increased and the soil fertility are decreasing by intensive cropping in the past. The alternative way to approach this problem is the use of atmospheric nitrogen for the nitrogen source nutrition of economic crops. The intensive studying about associative nitrogen fixation and ammonium assimilation are there fore essential. Recent study (Saengduan, 1991) on rice-nitrogen *Klebsiella oxytoca* R15 indicated that, nitrogenase activity was

increased 400-500 fold in suitable associative system, which the *K. oxytoca* R15 showed increase in GS protein to 3-5 fold of the free-living condition and the GS specific activity to 7-9 fold. Saengduan (1991), had proposed that the associative *K. oxytoca* R15 can fix dinitrogen more effectively and assimilated most of the fixed-N_2 by their GS enzyme to glutamine and other amino acids. Since these results showed that the specific activity of GS in rice roots were constant or slightly decreased (about 10 %). The non corresponding figures of the nitrogenase activity and the root GS activity do not support the notion that root GS plays and important role in assimilating the fixed-N_2. It is possible that the GS enzyme in the rice leaves were responsible for the assimilation of the fixed-N_2 indirectly via amino acids produced by bacteria. If this hypothesis is true, the transferase activity of GS enzyme in the rice leaves should be increased in associative condition to assimilate fixed-N_2 transferred from bacteria.