Chapter 1

Introduction

5-Aminolevulinic acid (ALA) is the universal biosynthetic precursor of the tetrapyrroles, such as porphyrin, heme, chlorophyll, and vitamins B_{12}. It has several physiological activities such as a photodynamic herbicides effect on several plants (Rebeiz *et al*., 1984), a photodynamic insecticidal effect on several insects i.e. *Trichoplusia ni* (Rebeiz *et al*., 1988), plant-growth-regulating properties and may enhance agricultural productivity (Hotta *et al.*, 1997). Moreover, further applications of ALA in medicinal and pharmacological products were also reported (Sasaki *et al*., 2002).

Research and development to reduce the cost of ALA production has been carried out in various aspects. Relatively large amount of extracellular ALA production from *Rhodobacter sphaeroides* was achieved by adding levulinic acid (LA), a competitive inhibitor of ALA dehydratase (ALAD), intermittently (Sasaki *et al*., 1987, 1990). In this condition, supplementing the precursors for synthesis (glycine and succinate) enhanced ALA accumulation (Sasaki *et al*., 1991). In addition, ALA could be produced extracellularly using volatile fatty acids (VFAs) medium containing acetic, propionic and butyric acids as the carbon substrate (Sasaki *et al.,* 1993). Strain improvement by mutagenesis using NTG, a mutant strain CR-17 of *Rhodobacter sphaeroides* was characterized by the low activity of ALA dehydratase and secreted large amount of ALA comparing to the wild-type strain (Tanaka *et al.,* 1991). Following this approach, ALA production using mutants of photosynthetic bacteria has been established (Nishikawa *et al.,* 1999; Sasaki *et al*., 2002).

This investigation aims to increase the ALA yield from the halotolerant photosynthetic bacterial isolates, both wild type strain and mutant strains, through strain improvement and optimization studies.

Literature Review

1. Phototropic Bacteria

Phototropic bacteria can use light as an energy source and comprise a large and heterogeneous group of organisms. They possess one or more pigment called bacteriochlorophylls and can carry out light mediated generation of ATP, a process called photophosphorylation. Two major groups are recognized, the purple and green bacteria as one group, and the cyanobacteria as the other group. The basic distinction between the purple and green bacteria and the cyanobacteria is based on the photopigments and overall photosynthetic process. Cyanobacteria are oxygenic phototrophs, employing chlorophyll *a* and two photosystems in their photosynthetic process. Purple and green bacteria are anoxygenic phototrophs, employing bacteriochlorophyll (of several different types), but only one photosystem in their photosynthetic process.

In photosynthesis by the purple and green bacteria, water is not photolysed, and O_2 is not produced. Because the purple and green bacteria are unable to photolyse water, they must obtain their reducing power for CO_2 fixation from a reduced substance in their environment. This can be either an organic compound, a reduced sulfur compound, or H_2. (Brock and Madigan, 1991)

1.1 The Purple Phototrophic Bacteria

Family 1. Chromatiaceae such as purple sulfur bacteria. Cells are spherical, ovoid, spiral, rod- or vibrioid-shaped and multiplied by binary fission, with or without gas vacuoles; motile or nonmotile. Motile forms have polar flagella and are either monotrichous or multitrichous. The internal photosynthetic membrane system is continuous with the cytoplasmic membrane and of vesicular type. Only one species (*Thiocapsa pfennigii*) contains tubular membranes and bacteriochlorophyll *b*. All other species contain bacteriochlorophyll *a* and carotenoids of group 1, 3 or 4. In general, culture of strains with carotenoids of group 1 appear orange-brown to brownish-red or pink; those of group 3, purple-red; and those of group 4, purple-violet (Pfennig and Truper, 1984).

Family 2. Rhodospirillaceae such as purple non-sulfur bacteria. Cells are spiral or vibrioid- or rod-shaped, motile by means of polar flagella, divide by binary fission, with or without gas vacuoles, and are Gram-negative. They are able to perform anoxygenic photosynthesis with bacteriochlorophylls and carotenoids as photosynthetic pigments. Growth occurs anaerobically in the light with reduced sulfur compounds as electron donors. Sulfide is oxidized to elemental sulfur, which is deposited outside the cells and may be further oxidized to sulfate. In nature, membrane of the *Ectothiorhodospiraceae* are found in marine to extremely saline environments containing sulfide and having neutral to extremely alkaline pH (Pfennig and Truper, 1984).

The purple nonsulfur bacteria are the most diverse and best studied group of all phototrophic bacteria. This diversity is reflected in their morphology, internal membrane structure, carotenoid composition, utilization of carbon sources and electron donors, and various other characteristics. Most species are motile and have, besides various types of carotenoids, bacteriochlorophyll *a* as their photosynthetic pigment. One or more vitamins are generally required as growth factors, most commonly biotin, thiamine, niacin, and *ρ*-aminobenzoic acid. These compounds are rarely needed by species of the Chromatiaceae and Ectothiorhodospiraceae, which may require vitamin B_{12} as the sole growth factor. Growth of many species is also enhanced by small amounts of yeast extract, and some species have a complex nutrient requirement. Photoorganoheterotrophic growth is preferred by most species, but many species are also able to grow photoautotrophically or to respire under micro aerobic conditions (Pfennig and Truper, 1984).

1.2 The Green Sulfur Phototrophic Bacteria

Green bacteria are morphologically quite diverse, including nonmotile rods, spiral, and spheres (green sulfur bacteria), and motile filamentous gliding forms. Green bacteria are also very phylogenetically diverse, as the green sulfur bacteria and Chloroflexus represent two distinct branches of eubacteria. The gliding green bacteria could easy be classified with the rest of the gliding bacteria, but it is generally considered that the ability to grow phototrophically is a more fundamental characteristic. Thus these gliding organisms are classified with the rest of the

phototrophic bacteria. Several other green bacteria have complex appendages called prosthecae, and in this connection resemble the budding and/or appendaged bacteria (Brock and Madigan, 1991).

2. Synthesis Pathway of 5-Aminolevulinic Acid

2.1 The Shemin Pathway (C_4 Pathway)

The Shemin pathway is present in the group of purple sulfur phototrophic bacteria, in yeast and in the mitochondria of mammalian cells. ALA is formed by the enzyme ALA synthase which catalyzes the condensation of succinyl-CoA and glycine (Nishkawa *et al*., 1999) with the liberation of carbon dioxide and CoA (Ano *et al*,. 1999).

The two major substrates for ALA biosynthesis are glycine and succinyl CoA. Succinyl CoA is an intermediate of the TCA cycle and its carbon atoms originate therefore from acetyl CoA. It was then established that first (and committed) step in the biosynthesis of porphyrins is a condensation reaction between glycine and succinyl CoA catalysed by a pyridoxal phosphate-dependent synthetase, the product is 5-aminolevulinate (ALA) (Figure 1).

This condensation takes place in two stages with the intermediate formation of an enzyme-bound (Schiff's base) derivative of α-amino-β-adipate which, on decarboxylation and release from the enzyme, gives ALA. Two molecules of ALA then condense, with loss of two molecules of water, to generate porphobilinogen. The reaction is catalyzed by 5-aminolevulinate dehydratase. This product, with a single pyrrole ring, has one acetate and one propionate side chain. Further condensation of four porphobilinogen molecules then takes place in a head to tail manner to give an enzyme-bound tetrapyrrole. The linear product (aminomethylbilane) is released, after loss of ammonium ions, when each pyrrole ring is linked by a methylene group. Thus, three ammonium ions are lost at this stage, with a fourth eliminated later on ring closure to uroporphyrinogen III. This reaction requires two enzymes, uroporphyrinogen I synthase which catalyzes a head to tail condensation of four porphobilinogen molecules, and uroporphyrinogen III cosynthase, which inverts one of the units and closes the ring (Packter, 1992).

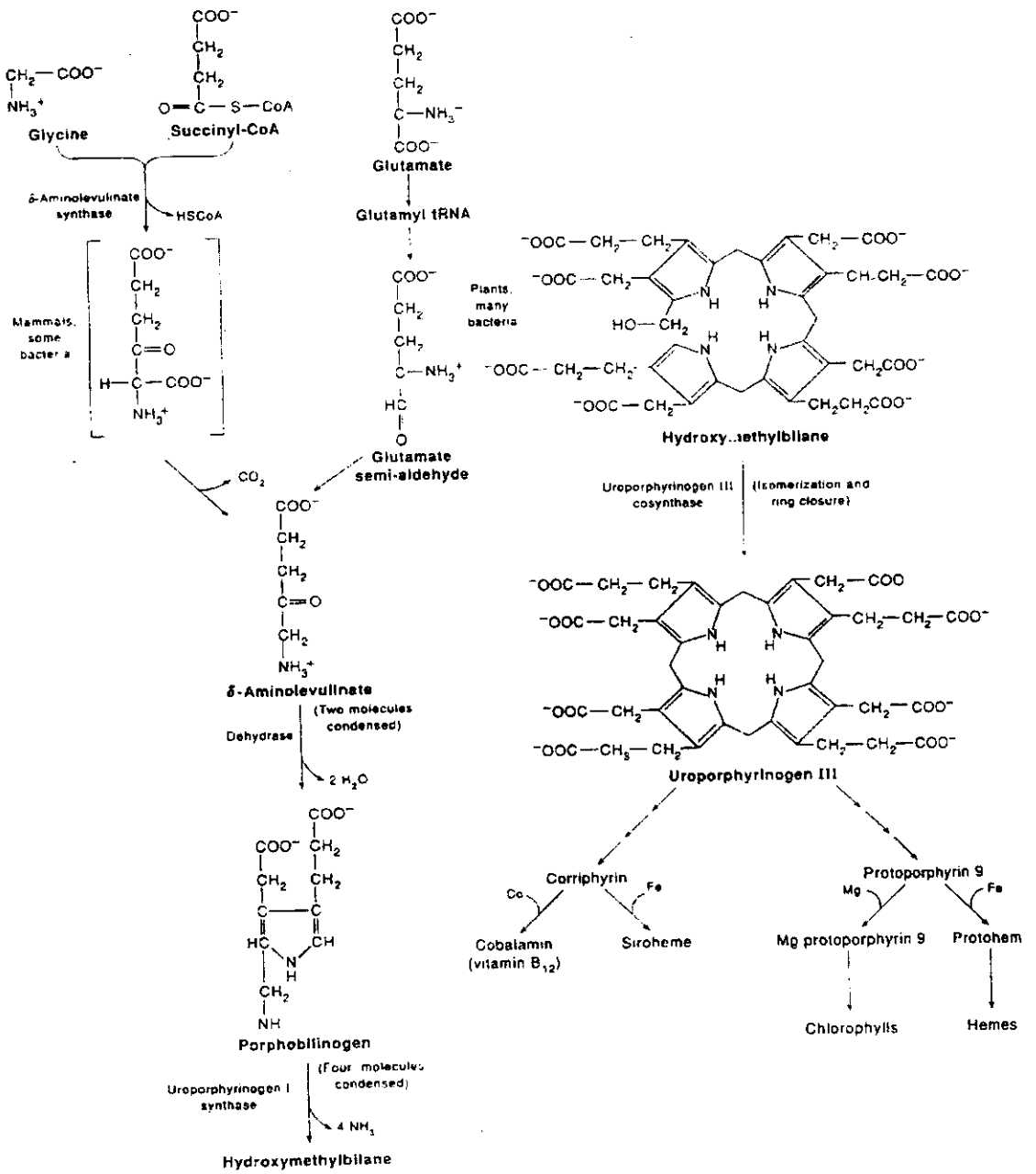


Figure 1 Tetrapyrrole biosynthesis

Source: Umbarger and Zubay, 1993

2.2 The Beale Pathway (C_5 Pathway)

ALA is formed a three step sequence beginning with glutamate and a glutamyl-tRNA intermediate (Tait, 1973) which is present in the chloroplasts of plants, algae such as the unicellular green algae *Scanedesmus obliquus* (Ano *et al.*, 1999), cyanobacteria, most eubacteria, and some photosynthetic bacteria such as *Chlorobium vibrioforme* (Rieble *et al.*, 1989). The first reaction links glutamate, through its carboxyl group, to a specific transfer RNA, just as occurs in protein synthesis (Eisenberg, 2002). The initial metabolite is the unusual Glu-tRNA^{Glu} (glutamyl-tRNA^{Glu}), which is converted by the action of an unusual enzyme, glutamyl-tRNA reductase (GluTR). The carboxyl group thus activated is then reduced by NADPH, giving glutamate 1-semialdehyde with the concomitant release of tRNA^{Glu} . Glutamate-1- semialdehyde may also exist in a cyclic form, hydroxyamino-tetrahydropyranone (HAT)(Figure 2). Both compounds are converted to ALA by a specific transaminase, glutamate-1-semialdehyde 2,1-aminomutase (GSA-AM; E.C.5.4.3.8). Thus, glutamate-1-semialdehyde is the first committed precursor of porphyrin synthesis in organisms and organelles that use this pathway (Jahn *et al.*, 1992).

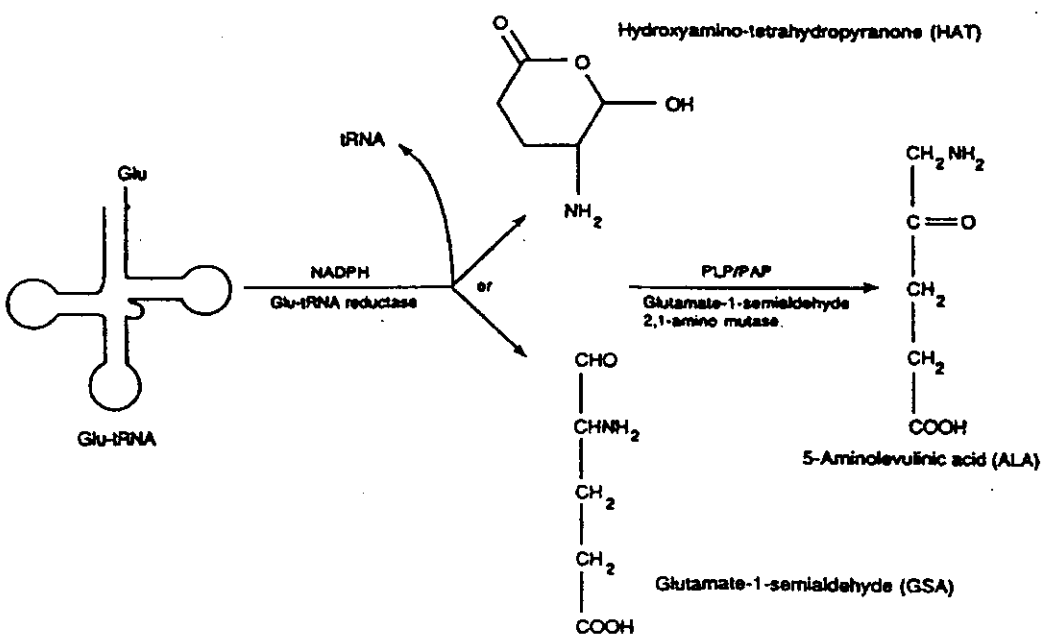


Figure 2 Scheme of the C5-pathway

Source : Jahn *et al.*, 1992

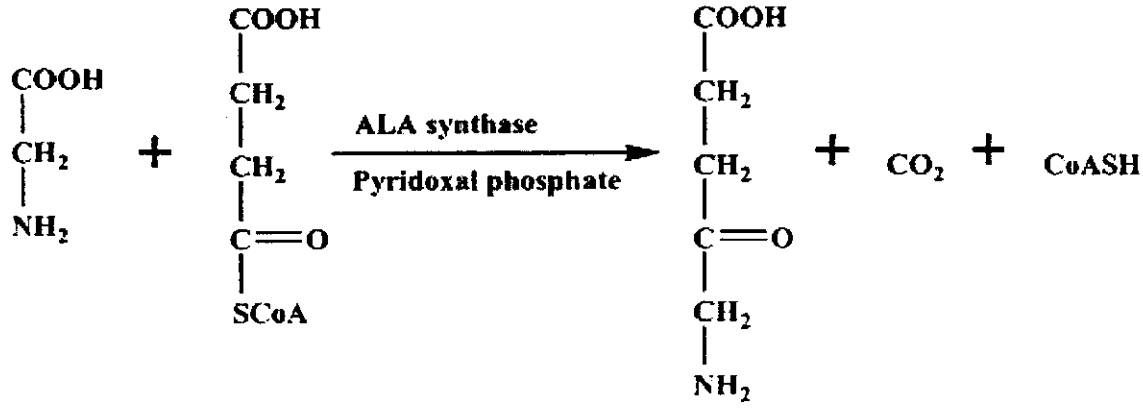
3. Enzymes Relating to 5-Aminolevulinic Acid Production

3.1 5-Aminolevulinic acid synthase

5-Aminolevulinic acid synthase or ALA synthase (ALAS, EC 2.3.1.37) catalyzes the condensation of glycine and succinyl coenzyme A with pyridoxal phosphate (PLP) as a cofactor (Succinyl-coenzyme A : Glycine C-succinyl transferase [decarboxylation]) to yield 5-aminolevulinate, carbon dioxide and Co A (Azeilslls-ryalls and Kaplan, 1995), the first and rate-limiting step in tetrapyrrole biosynthesis (Freist *et al.*, 1997).

The ALA synthase from *Micrococcus denitrificans* was reported to have a short biological half-life (≈ 60 min)(Tait, 1972). Both the synthesis and the activity of the enzyme were subject to regulation by a variety of substances. Inhibition of activity by 50% occurred in the presence of 5 μM of hemin and virtually complete inhibition was noted at a 20 μM concentration. The enzymatic reaction involves the condensation of a glycine residue with a residue of succinyl Co A. The reaction has an absolute requirement for pyridoxal phosphate (Figure 3); the latter interacts with the nitrogen of glycine from a Schiff's base. This generates a carbanion intermediate on the α –carbon of the glycine, allowing a nucleophilic attack and condensation with the succinyl group from succinyl Co A. The bound intermediate, α-amino-β-ketoadipic acid, decarboxylates to the released product, ALA. Pyridoxal deficiency and drugs competing with pyridoxal phosphate lead to a decrease in enzyme activity (Wells and Award, 1992). ALA synthase is synthesized in cytosolic ribosomes as a pre-enzymes, which is then imported into and processed within the mitochondria to yield the mature form of the enzyme (Goodfellow *et al.*, 2001).

Being the first committed step in heme synthesis, the ALA synthase reaction is the major control point. Heme and related compounds exhibited feedback inhibition to the enzyme. Heme also has two other important effects; a low concentrations heme inhibits the synthesis of ALA synthase at the translational level. At higher concentrations, heme somehow blocks the translocation of ALA synthase from the cytosol, where it is synthesized on ribosomes, into the mitochondrion where it acts (Eisenberg, 2002).



Glycine **Succinyl-CoA** **5-Aminolevulinic acid**

Figure 3 Reaction catalysed by ALA synthase. The enzyme catalyses the condensation of glycine and succinyl-CoA in a reaction that requires pyridoxal phosphate PLP as a cofactor. Carbon dioxide, CoA and ALA are generated during the reaction

Source: Bolt *et al.*, 1999

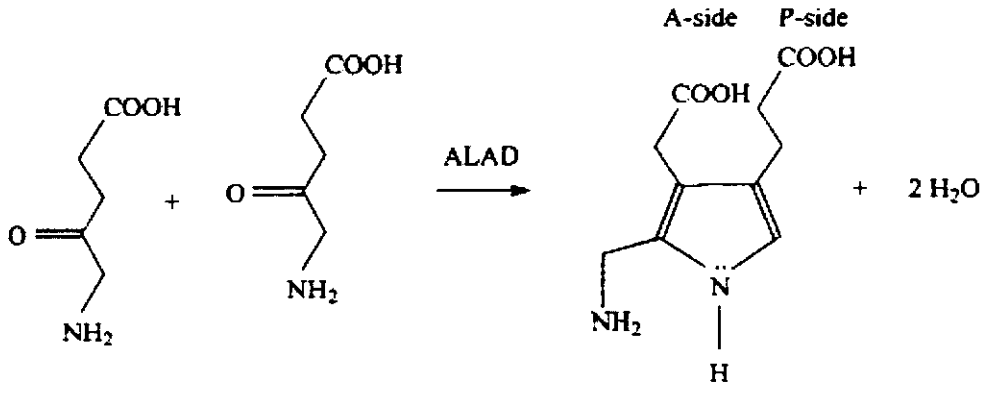
3.2 5-Aminolevulinic acid dehydratase

5-Aminolevulinic acid dehydratase (ALAD, E.C.4.2.1.24) also called porphobilinogen synthase (PBGS), is the second enzyme involved in the biosynthesis of natural tetrapyrroles compounds and condensed two molecules of ALA through a nonsymmetrical pathway to pyrrole-derivative porphobilinogen (PBG) (Jarret *et al.*, 2000) (Figure 4). The two ALA substrate molecules are distinguished as A and P-side-ALA due to the acetic acid and propanoic acid side-chains that they contribute to the product. The corresponding binding sites of ALA dehydratase are termed A-site and P-site, respectively (Frere *et al.*, 2002).

ALA dehydratase is metalloenzymes and divided into two major classes. $Zn^{2\pm}$dependent ALA dehydratase is found in mammals, yeast and some bacteria including *Escherichia coli*, while $Mg^{2\pm}$ dependent ALA dehydratase is present mainly in plants and other bacteria (Frankenberg *et al.*, 1999).

ALA dehydratase is a soluble cytosol component with a molecular weight of 280,000 daltons and consisting of eight subunits, of which only four interact with the substrate. This protein also interacts with the substrate to a Schiff's base, but in this case

the ε – amino group of a lysine residue binds to the ketonic carbon of the substrate molecule. Two molecules of ALA condense asymmetrically to porphobilinogen. The ALA dehydratase is a sulfhydyl enzyme and is very sensitive to inhibition by the absence of an elevation of porphobilinogen (Wells and Award, 1992).



5-Aminolevulinic acid **Porphobilinogen**

Figure 4 Two molecules of 5-ALA condense to porphobilinogen

Source: Erskine *et al.*, 2003

3.3 Glutamyl-tRNA synthetase

Glutamyl-tRNA synthetases (GluRs ; Glutamate-tRNA ligase, E.C. 6.1.1.17) belongs to the class I aminoacyl-tRNA synthetases and is responsible for the formation of Glu-tRNA^{Glu} from glutamate and tRNA^{Glu} in the presence of ATP (Figure 5). Among the twenty aminoacyl-tRNA synthetases, glutamyl-tRNA synthetase occupies a special position. It is one of only two enzymes of this family which is not found in all organisms, being mainly absent from Gram positive eubacteria, archaebacteria. The *Escherichia coli* GluRs is relatively small with 553 amino acids and a molecular mass of 64.4 kDa and functions as a monomer. The mammalian enzyme are somewhat larger and can be parts of mutienzyme complexes (Freist *et al.*, 1997; Neidle and Kaplan, 1993).

GluRS has been considered as the first enzyme of the C_5-pathway. However, a definition of a pathway is that its enzymes are regulated in an interdependent fashion or that their activity may be subject to feedback inhibition by

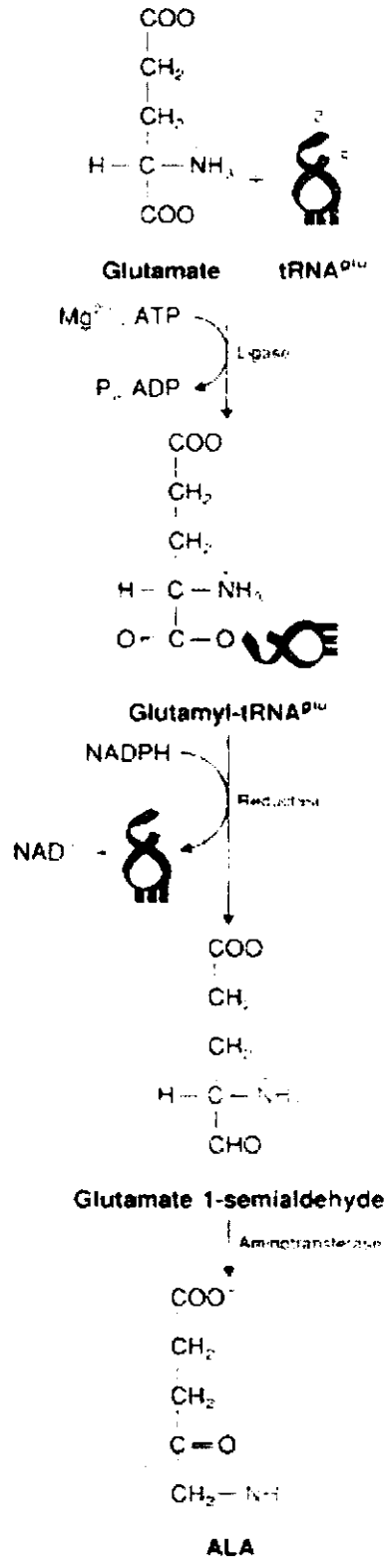


Figure 5 Scheme of the C5-pathway

Source : Drews *et al.*, 1991

downstream metabolites. *In vitro* inhibition of *Chlamydomonas reinhardtii* GluRS by heme (> 90 % inhibition at 5 μM) and of *Scenedesmus* enzyme by protochlorophyllide (83% inhibition at 45 μM) was reported. However, heme in very low concentrations (≤ 1 μM) has been shown to disrupt protein-nucleic acid interactions by non-specific inhibition of a number of enzymes, for example, restriction endonucleases, DNA polymerases, RNA polymerases, DNA ligases, DNases and transcription factor-DNA complexes. Thus, the physiological significance of the observed GluRS inhibition is unclear. In addition, *Synechocystis* GluRS was not inhibited by high amounts (10 μM) of heme (Jahn *et al.*, 1992).

3.4 Glutamyl-tRNA reductase

Glutamyl-tRNA reductase (GluTR) is encoded by the *hemA* gene and catalyzes the reduction of Glyamyl-$tRNA^{Glu}$ to glutamate 1-semialdehyde (GSA) with concomitant release of intact $tRNA^{Glu}$ (Loida *et al.*, 1999). This reaction is the indispensable inhibition step of tetrapyrrole biosynthesis in plants and most prokaryotes (Moser *et al.*, 2001). Investigations of this reaction have been hampered by the scarcity of purified enzyme which is apparently present in low amount in the cell. In *Chlamydomonas*, a monomer of 130 kDa was purified, while two *E. coil* proteins had GluTR activity with molecular masses of 45 kDa (GluTR45) and 85 kDa (GluTR85) (Beale, 1999).

Two different mechanisms for the GluTR-catalysed reduction have been suggested. One mechanism is formally analogous to the back reaction of glyceraldehydes-3-phosphate dehydrogenase (E.C.1.2.1.12), where the phosphorylation activated carboxyl group of 3-phosphoglycerate is reduced to its aldehyde in the presence of NADH via an acyl-enzyme intermediate. In the case of GluTR, the glutamate is activated by ligation to tRNA. The other proposal suggests the presence of a complex of GluRS, GluTR, Glu-$tRNA^{Glu}$ and GTP, which acts as the 'substrate' for another enzyme initiating the reduction. However, the generation of glutamate-1-semialdehyde from Glu-$tRNA^{Glu}$ by all purified GluTR enzymes in the presence of NADPH alone. GluTR is an especially engaging biological target because no exogenous inhibitors are known and because limited data are available regarding its enzymatic properties (Loida *et al.*, 1999).

3.5 Glutamate 1-semialdehyde aminomutase

Glutamate 1-semialdehyde aminomutase (GSA-AM ; E.C. 5.4.3.8), is encoded by the *hemL* gene and catalyses the conversion of GSA to ALA transamination (Loida *et al.*, 1999) in the presence of pyridoxal 5'-phosphate (PLP) or pyridoxamine 5'-phosphate (PAP). It has been purified from bacteria and chloroplasts of higher plants, and the genes encoding this protein have been characterized. Most of the enzymes have a homodimeric structure with subunit molecular masses of 44-46 kDa. A nuclear gene (gsa) encodes this enzyme in higher plants (Jahn *et al.*, 1992). Like other aminotransferases, GSA-AM utilizes pyridoxal phosphate as a cofactor.

4. Regulation of 5-Aminolevulinic Acid Production

4.1 Carbon and nitrogen sources

Glucose has the advantage of being inexpensive source for the industrial production of ALA. In the batch fermentation, the mutant strain of *Rhodobacter sphaeroides* CR606 accumulated ALA to level of 20 mM after 18 h with the production rate of 1.1 mM/h and the yield coefficient for ALA was 40 % (mol/mol of glucose) (Nishikawa *et al.*, 1999).

R. sphaeroides can utilize volatile fatty acid (VFA) such as acetic, propionic and butyric acid as carbon and energy sources (Sasaki *et al.*, 1987). In addition, VFA produced from the anaerobic digestion liquor of sewage sludge was reported to be the carbon source for production of ALA, up to 9.2 mM, by *R. sphaeroides* with repeated addition of the glycine and glutamic acid as the organic nitrogen source (Tanaka *et al.*, 1994).

4.2 Precursors

Glycine and succinate (succinyl Co A) are direct precursors of ALA biosynthesis in the Shemin pathway. Addition of glycine in cultivation of *Rhodobacter sphaeroides* mutants under aerobic-dark condition was extremely effective for increasing ALA accumulation, whereas no significant effect on the addition of succinate on ALA accumulation (Nishikawa *et al.*, 1999). Addition of glycine in the heterotrophic culture of *Chlorella regularis* YA-603 can significantly increase the amount of ALA produced (Ano *et al.*, 1999). Twice addition of glycine resulted in the ALA production 1.5 times higher of ALA production than one time addition to the culture. This indicated

that there is an optimum glycine concentration for ALA production. However, addition of glutamate to the heterotrophic medium culture did not enhance ALA production and Shemin pathway was suggested to contribute to ALA production of this strain.

4.3 Inhibition of ALA dehydratase

Levulinic acid (LA) is a competitive inhibitor of ALA dehydratase (ALAD) in the biosynthesis of tetrapyrrole. LA addition in the middle log phase (active growth phase) effectively blocked ALA metabolism, resulting in the retardation of cellular growth and excretion of the ALA formed.

In photoheterotrophic culture of *R. sphaeroides*, repeated addition of LA resulted in the moderate cell growth suppression while extracellular ALA was not produced during the cultivation without the addition of LA. The amount of LA should be as small as possible since LA is expensive compared with glycine and 30 mM LA were recommend to use for saved cost (Sasaki *et al.*, 1990). However, over 50 mM LA completely suppressed both growth and ALA formation (Sasaki *et al.*, 1987).

Addition of 3-oxoadipic acid, a stronger competitive inhibitor for ALA dehydratase from *Pseudomonas freudenreichii,* to the medium at 15 mM resulted in the increase of extracellular ALA to 2.5 mM. This was about two folds greater than the amount obtained with the use of conventional inhibitor (LA) under the same conditions (Tanaka *et al.*,1995). Furthermore, propionic acid could inhibit ALA dehydratase and acted as a competitive inhibitor (Tanaka *et al.*, 1995). When propionic acid was present with LA, ALA dehydratase activity reduced by 50-60 % as compared with the case of LA only. The high accumulation of ALA in a VFA medium might therefore be caused by the additional inhibitory effect of propionic acid for ALA dehydratase together with the inhibition by LA.

4.4 Metal ions

Metal ions particularly iron and cobalt are other important elements for regulating tetrapyrrole biosynthesis in the microorganism. Co^{2+} is essential for vitamin B_{12} biosynthesis, while Fe^{2+} or Fe^{3+} is necessary for the conversion of coproporphyrinogen III to protoporphyrinogen IX and for the formation of heme compound. Under iron (Fe^{2+} and Fe^{3+}) sufficient conditions, ALA synthase is regulated by heme compound as feedback inhibition or repression (Sasaki *et al.*, 1991). Therefore,

ALA production medium should contain neither cobalt nor iron to enhance ALA accumulation (Sasikala *et al.*, 1994).

Potassium and sodium phosphates are needed for the formation of organic phosphorus compound such as nucleic acids and lipids. Magnesium and sodium are also essential for the synthesis of bacteriochlorophyll and the photosynthetic apparatus (Drews and Imhoff, 1991).

4.5 Light intensity

Light intensity was an important factor for enhancing ALA formation. Illumination intensity is examined as a function of light intensity. Growth was found to be independent of light intensity in the range of 1-5 klux, while the amount of ALA secreted was clearly affected by light intensity (Sasaki *et al.*, 2002) and reached the maximum value at 3 klux. High illumination (over 5 klux) was not effective for ALA production and low illumination (below 1 klux) gave quite a low growth rate and virtually no formation of ALA (Sasaki *et al.*, 1987).

The tungsten lamps are more suitable for growth because they generate emissions in the near infrared (IR) region of 800-900 nm. These wavelengths of light are effectively absorbed by bacteriochlorophyll in the purple non-sulphur phototrophic bacteria. Moreover, tungsten lamps can supply much higher light intensities. With the increase in light intensity, bacterial growth increased until cells in the medium were saturated with light (Kim *et al.*, 1982). No further increase in growth occurred after the saturating light intensity. At very high intensities, depression in the synthesis of photopigments could also occur (Firsow and Drews., 1977). This would reduce the ability of photoheterotrophic growth in the bacteria (Getha *et al.*, 1998).

4.6 pH

ALA production by *Rhodobacter sphaeroides* was investigated at various pH with levulinic acid addition. At neutral pH (6.7 and 7.0) extracellular ALA production was up to 16 mM while low production of ALA (less than 3.5 mM) was observed at acidic pH (lower than 6.5) and less than 3.9 mM of ALA produced of alkaline pH (higher than 7.5) (Sasaki *et al.*, 1993).

Noparatnaraporn *et al.*, (2000) Extracellular formation of ALA by *Rhodovulum sp.* PS88 correlated with the consumption of the undissociated form of levulinic acid (LA) in an intact cell system. The concentration of the undissociated form

of LA, governed the extracellular formation of ALA at various culture pH values. At pH 5.5, ALA formation dropped in spite of a relatively high concentration of undissociated form of LA in culture broth due to the very low pH.

5. Strain Improvement

Strain improvement is an essential and important part of process development for a biotechnology process. The overall is to reduce costs by developing strains with increased productivity, ability to use cheaper or alternate substrates, or other unique and desirable characteristics.

5.1 Random Screening

Random screening has been used reliably for many years for titre improvement first in penicillin production and then in a variety of other fermentations. It still plays a central role in many industrial fermentations todays. Random screening has benefited from advances in many fields which is making it more likely to obtain improved mutants.

5.2 Mutagenesis

Mutagenesis is the source of all genetic variations, but no single mutagenic treatment will give all possible types of mutation. There are two major types of mutagens.

(1) Physical mutagen such as UV, X-ray, gamma radiation

(2) Chemical mutagen such as ethyl methane sulfonate (EMS), nitrosomethyl guanidine (NTG), mustards.

The type of mutations induced in any cell type depends on the follows factors;

1. The type of DNA damage caused by the mutagen. Most mutagens produce more than one type of DNA damage. Some mutagens produce more of one type of DNA damage than others.

2. The cell's DNA repair mechanisms. DNA repair systems may non-mutagenic (error-free) or mutagenic (error-prone). Some mechanisms include photoreactivation, excision repair, recombination repairs and SOS repair (http://web.uconn.edu/mcb235/secure/strainDevelopment.pdf).

For the improvement of ALA production, the NTG mutant strain CR-17 of *Rhodobacter sphaeroides* was characterized by the low activity of ALA dehydratase

compared to the wild-type strain. Without addition of LA, the mutant secreted ALA under anaerobic-light (64.5 μM) or microaerobic-dark (32 μM) conditions and 82.5 μM of ALA was secreted under aerobic-dark condition with addition of 15 mM LA (Tanaka *et al.*, 1991). The mutant strain CR-606 which mutated from *R. sphaeroides* CR-286 by NTG was found to accumulate up to 20 mM ALA from glucose and glycine under dark conditions (Nishikawa *et al.*, 1999).

Previous research work on the strains used in this study

Madmarn (2003) studied on ALA production from *Rhodobacter sphaeroides* ES16 under aerobic-dark condition. It was found that cultivation in glutamate-glucose (GG) medium gave higher extracellular ALA (28.70 mM) than cultivation in glutamate-malate (GM) medium (22.83 mM) at 72 h incubation. The mutant strains of *R. sphaeroides* ES16, namely U7 and N20 which were mutated by UV and NTG, respectively, could give 2.2 and 1.5 folds increase of ALA (59.45 mM and 42.59 mM, respectively) than the wild type strain (ES16) under aerobic/dark condition. Furthermore, cultivation of the mutant strain N20 in analytical grade GG medium and in different commercial grade media revealed that MGSY medium (containing commerial monosodium glutamate, glucose, salt (NaCl) and yeast extract) gave lower extracellular ALA production (40.94 and 44.87 μM, respectively) than GG medium. Nevertheless, addition of 15 mM levulinic acid enhanced the secretion of extracellular ALA from the mutant strain to 82.5 mM under the same condition.

Sattayasmithstid (2001) compared the production of extracellular ALA and intracellular ALA from *Rhodobacter capsulatus* SS3 cultivated in the original medium (GM medium with 3% NaCl) and the optimized medium (10 mM glycine, 10 mM saccinate, 0.5 g/l propionic acid, 15 mM $MgCl_2$, 10 μM pyridoxal phosphate and three addition of 15 mM LA at 24, 48 and 72 h cultivation). Results indicated that the maximum extracellular ALA values were 0.025 and 0.75 mM, respectively while the intracellular ALA were 0.12 and 1.05 mM, respectively. Therefore, cultivation of *R. capsulatus* SS3 in the optimized medium resulted in 30 folds and 8 folds increase of the extracellular and intracellular ALA, respectively.

Objectives

1. To increase ALA production from the strains of halotolerant photosynthetic bacteria, *Rhodobacter capsulatus* SS3 (wild type) and *Rhodobacter sphaeroides* N20, U7 (mutants), by mutagenesis
2. To increase ALA production from the selected mutant strain by optimization studies to optimize the medium and environmental condition for ALA production from the selected mutant strain
3. To compare the effect of analytical grade medium and commercial grade medium on 5-aminolevulinic acid production from the selected strain.