PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF NITROGEN METABOLISM IN *ANTHOCEROS — NOSTOC* SYMBIOSIS

By

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ABBREVIATIONS

ADP	= Adenosine 5'-diphosphate
ATP	= Adenosine 5'-triphosphate
BOD	= Biological oxygen demand
BSA	= Bovine serum albumin
C	= Carbon
°C	= Degree centrigrade
C_2H_2	= Acetylene
C_2H_4	= Ethylene
Chl	= Chlorophyll
cm	= Centimeter
cm^{-2}	= / Square centimeter
EDTA	= Ethylene diamine tetra acetic acid
E.M.	= Electron microscopy
Fd	= Ferredoxin
Fv	= Flavodoxin
g	= Gram
GOGAT	= Glutamate synthase
GS	= Glutamine synthetase
HEPES	= 4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid
HRP	= Horse radish peroxidase
IgG	= Immuno globulin G
l	= Litre
m	= Mole
M	= Molar

mA = milli ampere

MES = 2-(N-morpholino) sulphonic acid

mg = Milligram

Min = Minute (s)

ml = Milliliter

mM = Milli molar

µmol = Micro mole

µM = Micro molar

μm^2 = micro meter square

MSX = L-Methyonine-DL-sulphoximide

N = Nitrogen

NAD = Nicotinamide adenine dinucleotide

NADH = Nicotinamide adenine dinucleotide reduced

NH_4^+ = Ammonium

Nif = Nitrogen fixing

NO_3^- = Nitrate

nm = nano meter

NR = Nitrate reductase

OD = Optical density

PAGE = Poly acrylamide gel electrophoresis

PBS = Phosphate buffered saline

PE = Phycoerythrin

pH = Hydrogen ion concentration

PS = Photosystem

psi = Pounds per square inch

RuBisCo = Ribulose 1,5-bisphosphate carboxylase/oxygenase

s = Second (s)

SDS = Sodium dodecyl sulphate

SE = Standard error

TBS = Tris buffered saline

TEM = Transmission electron microscopy

W = Watt

Wt. = Weight

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CHAPTER 1

General Introduction

Nitrogen is one of the most abundant elemental constituents of all living matter. It is an integral component of enzymes, proteins, nucleic acids, chlorophylls and other related compounds which are essential for maintaining structural and functional integrity of living systems.

Molecular nitrogen, which constitutes about 78% of the earth's total atmosphere, can not be directly used as nitrogen source by vast majority of organisms. Instead, they require combined nitrogen. Therefore, in modern day agriculture, chemical fertilizers are being used increasingly as the source of combined nitrogen. However, certain prokaryotic organisms possess the ability to reduce atmospheric nitrogen to ammonia under normal physiological conditions and therefore, are self sufficient. There is a global attempt to understand the physiological, biochemical and the genetic aspects of these primitive organisms so as to help find an alternative to chemical fertilizers. Among such organisms, cyanobacteria which are diazotrophic in nature and constitute one of the largest sub-group of gram negative prokaryctes, are of special interest as they have simple growth requirements combining oxygenic photosynthesis and nitrogen fixation (Singh, 1961; Sprent, 1979; Stewart, 1980; Carr & Whitton, 1982; Gallon & Chaplin, 1987; Fay & Van Baalen, 1987; Rai, 1990).

1.1 Cyanobacteria:

Cyanobacteria are prokaryotes possessing chlorophyll *a* and phycobiliproteins. They are photoautotrophs performing oxygenic photosynthesis, using water as eletron donor, in light. Some are also capable of performing photosynthesis using H_2S as electron donor, many fix atmospheric N_2, and some are facultative photo- or chemoautotrphs. Among cyanobacteria, a wide range of morphological diversity exists. They include unicellular, multicellular filamentous, branched filamentous and non-filamentous colonial strains. The filamentous forms consist of a maximum of three types of cells: vegetative cells, heterocysts and akinets (*see* Carr & Whitton, 1982; Fay & Van Baalen, 1987).

Vegetative cells are the site of photosynthesis and CO_2-fixation. CO_2 fixation occurs through calvin cycle (Stanier, 1977; Stewart, 1977; Allen, 1984). Oxidative pentose phosphate pathway is the main catabolic route. Oxidative phosphorylation and cyclic and non-cyclic phosphorylation generate ATP. Vegetative cells contain a number of storage bodies such as cyanophycean starch (glycogen) (Simon, 1971), carboxysomes (polyhedral bodies) which contain ribulose 1,5-bisphosphate carboxylase/oxygenase (Stewart & Codd, 1975; Codd & Marsden, 1984), and polyphosphate bodies (Grillo & Gibson, 1979).

Morphologically, heterocysts are distinctive cells with thick cell envelopes. They are enclosed by a unique glycolipid layer surrounded by polysaccharides which together impede the entrance of oxygen (Wolk, 1982; Murry & Wolk, 1989). They

represent about 3-7% of the total cells present in N_2-grown cultures. This proportion increases to almost double in nitrogen starved cultures (Kulasooria *et al.*, 1972). In presence of combined nitrogen sources, the frequency of heterocysts in the filaments of filamentous cyanobacteria decreases (Fogg, 1949). Heterocysts are larger than the vegetative cells and show a number of structural, biochemical and genetic changes during their development from vegetative cells (Wolk, 1982; Golden *et al.*, 1985; Haselkorn *et al.*, 1987). Heterocysts are the site of dinitrogen fixation (Fay *et al.*, 1968; Stewart, 1980; Janaki & Wolk, 1982; Bergman *et al.*, 1986). The key enzyme of nitrogen fixation, nitrogenase, is present and functional in the vegetative cells of non-heterocystous cyanobacteria (Nagatani & Haselkorn, 1978; Gallon & Chaplin, 1987). Heterocysts lack photosystem II (PS II) activity, thereby avoiding evolution of O_2 from water (Reinmann & Thornber, 1979; Alberte *et al.*, 1980) and have a high respiratory O_2 consumption rate (Haury & Wolk, 1978; Walsby, 1982; Jenson & Cox, 1983; Sprent *et al.*, 1987). These two processes together contribute to the protection of nitrogenase from O_2 damage. Heterocysts lack glutamate synthase (GOGAT) (Thomas *et al.*, 1977; Rai *et al.*, 1982) and nitrate reductase (Kumar *et al.*, 1985; Rai & Bergman, 1986; Rai *et al.*, 1992) and hence avoid competition for molybdenum and reductant (reduced Fd) with nitrogenase. They also lack ribulose 1,5-bisphosphate carboxylase enzyme and therefore do not fix CO_2 (Codd & Stewart, 1977; Codd *et al.*, 1980; Cossar *et al.*, 1985). The demand for fixed carbon is met from the neighbouring vegetative cells (Wolk,

Stewart, 1980; Bothe *et al.*, 1984; Stewart *et al.*, 1985; Herman *et al.*, 1986). They possess very little or no phycobiliproteins (Stewart & Rodgers, 1977; Stewart *et al* 1983; Lindblad, 1987). Ammonia produced by the action of nitrogenase is assimilated by the action of glutamine synthetase (GS) which is present in the heterocysts at levels two-fold higher than that in vegetative cells (Stewart *et al.*, 1975; Dharmawardene *et al.*, 1973; Thomas *et al.*, 1977). Such high level of GS are probably required in heterocysts for the assimilation of N_2-derived ammonia (Renstrom-Kellner *et al.*, 1990). Glutamine thus produced in the heterocysts is transported to adjacent vegetative cells where it is converted to glutamate by glutamate synthase (GOGAT) (Stewart *et al.*, 1975; Stewart, 1977). Part of the glutamate produced in the vegetative cells through the activity of GOGAT is transported back to the heterocysts to provide substrate for glutamine synthesis by GS (Thomas *et al.*, 1977).

Akinets are the single cells serving as perenating bodies. They are usually larger, their cell wall thicker and protoplasm more granular than the vegetative cells. They are associated with reproduction and survival under adverse conditions (Fogg *et al.*, 1973; Nichols & Adams, 1982).

1.2 Cyanobacterial symbiosis:

In general, symbiosis means the permanent living together of two or more dissimilar organisms involving exchange of metabolites between the symbionts. In most circumtances, this

interaction is mutually beneficial for both (all) partners. N_2-fixing cyanobacteria form symbiotic associations with a variety of plants as well as animals. Among plants representative hosts are found among algae (diatoms), fungi (about 8% of all lichens), bryophytes (liverworts, hornworts and mosses), pteridophytes (*Azolla*), gymnosperms (Cycads), and angiosperms (*Gunnera*). Among animals marine sponges and echiruoid worms as well as non-photosynthetic protists belonging to group glaucophyta and bacteria form symbiosis with cyanobacteria (Rai, 1990a; Bergman *et al.*, 1992).

Table: 1.1 N_2-fixing symbioses involving cyanobacteria:

Host plant	Cyanobacteria	Comments
Angiosperms:		
Gunnera	*Nostoc* sp.	Cyanobacterium located inside host cell in stem nodules.
Gymnosperms:		
Cycads (e.g. *Cycas angulata*, *Macrozamia communis*)	*Nostoc* sp.	Nodules found on coralloid roots. Occurs naturally only in southern hemisphere.
Pteridophytes:		
Azolla	*Anabaena azollae*	Occupies mucilage-filled cavities on the ventral surface of the dorsal lobes of the leaves. Widely used in as green manure/ biofertilizer in rice cultivation.
Bryophytes:		
Anthoceros Blasia Blasia Dendroceros	*Nostoc* sp.	Cyanobacteria occupy mucilage-filled cavities on undersurface of liverwort/hornwort gametophyte thallus.
Sphagnum	*Nostoc* sp.	Cyanobacteria occupy the hyaline cells of the moss.

Host	Cyanobacteria	Comment
Fungi:		
Cyanolichens:	*Nostoc* sp. *Scytonema* sp. *Fischerella* sp. *Calothrix* sp.	Nitrogen-fixing lichens consist of two-membered associations between fungi and cyanobacteria or three-membered associations which contain a green alga as well. About 8% of the 18,000 species of lichens contain cyanobacteria.
Algae:		
Rhizosolenia *Hemiaulus* *membranaceus* *Rhopalodia*	*Calothrix* Coccoid cyanobacteria	Marine diatoms. Thin-walled unicellular cyanobacteria.
Host animals:		
Helminth worms		
Marine sponges:	*Aphanocapsa* *phormidium*	Occurs inter or intracellularly throughout the sponge tissue or in superficial tissue.
Hair of polar bear	*Aphanocapsa* *Gloeocapsa*	
Prokaryotes:		
Bacteria:	*Pleurocapsa minor.*	
Nonphotosynthetic protists:		
Rhizophytes Radiolaria	*Cyanelles*	

1.2.1 Association with Angiosperms:

Gunnera is the only angiosperm genus capable of entering into a symbiotic association with *Nostoc*. The symbiosis between the two partners is facultative; the two partners can be

isolated, cultivated and reconstituted easily (Bergman *et al.*, 1992; Johansson *et al.*, 1992; Bonnett, 1990). The cyanobiont provides fixed nitrogen to the host plant which fully meets the latter's requirements for combined nitrogen (Osborne, 1989; Bonnett, 1990). The cyanobacteria infect specialized mucilage-filled glands at the base of the leaves on the stem of the host. This symbioses is unique in the sense that here, in contrast to other symbiosis, the cyanobiont enters the *Gunnera* cells and resides intracellularly (Johansson *et al.*, 1992; Stewart *et al.*, 1983; Rai, 1990a). The cyanobiont undergoes pronounced morphological, physiological and biochemical alterations. A gradient in the heterocyst frequency occurs, being about 20% in the younger colonies and higher in the older parts (Silvester, 1976). However, the nitrogenase activity is highest in the younger colonies near the plant apex, where the heterocyst frequency has not yet reached the maximum (Soderback *et al.*, 1990). Photosystem II (PS II) and the accessory pigments of PS II are absent and *in vivo* CO_2-fixation is negligible in the cyanobiont and the fixed carbon requirements of the latter is met by the host (Silvester, 1976). The primary ammonia assimilating enzyme, glutamine synthetase (GS), is found to be evenly distributed in the vegetative cells and heterocysts of the cyanobiont. The GS activity is more in the apex where the nitrogenase activity is also high (Soderback, 1992).

1.2.2 Association with Gymnosperms:

Within gymnosperms, all members of the order cycadales,

develop symbiotic associations with cyanobacteria. The cyanobiont (*Nostoc* sp.) occupies the middle cortical zone of the coralloid roots (Lindblad & Bergman, 1990). The vegetative cells possess chlorophyll *a* and phycobiliproteins and have a fully developed photosynthetic apparatus (Lindblad *et al.*, 1985). The vegetative cells also contain carboxysomes with the CO_2 fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo) (Lindblad *et al.*, 1985). Thus, the cyanobiont is similar to the free-living *Nostoc*. However, the heterocyst frequency increases gradually from being low in the younger parts to being higher in the older parts of the coralloid roots (Lindblad & Bergman, 1990). Nitrogenase activity has been found to be about seven times higher in the cyanobiont than in the free-living isolate (Kumar *et al.*, 1986). The primary ammonia assimilating enzyme (GS) activity and cellular and subcellular level of GS is found to be comparable to those of free-living N_2-fixing cyanobacteria (Lindblad & Bergman, 1986). However, the activity of GS declines towards the basal parts of the roots. The CO_2-fixation is negligible in the cyanobiont and fixed carbon may be provided by the host plant (Lindblad & Bergman, 1990). Using $^{15}N_2$, it has been clearly demonstrated that the fixed nitrogen is rapidly transferred from the cyanobiont to the host *Cycas* and *Macrozamia* (Renaut *et al.*, 1975; Bergersen *et al.*, 1965). Analysis of freshly extracted xylem sap from coralloid roots in a number of cycads showed that glutamine and a smaller amount of glutamic acid are the principal translocated N-solutes from coralloid roots to the rest of the cycad (Pate *et al.*, 1988).

1.2.3 Association with Pteridophytes:

Among pteridophytes, water fern *Azolla* develops symbiosis with the nitrogen-fixing cyanobacterium, *Anabaena*. The cyanobiont resides in the cavities on the ventral surface of the dorsal lobe of each leaf (frond). Within the cavities simple and branched hair-cells developed, having the structural characteristics of transfer cells (Peters *et al.*, 1982; Calvert *et al.*, 1985). The branched hair-cells have been suggested to be involved in transfer of fixed-nitrogen to *Azolla* from *Anabaena*, while single hair-cells may participate in the transfer of fixed carbon in the opposite direction (Calvert *et al.*, 1985). Sucrose is the major product of photosynthesis in *Azolla* and seem to be transported to the *Anabaena*. Several ultrastructural changes occur in the process of heterocyst differentiation (Neumueller & Bergman, 1981). Heterocyst frequency increases with leaf age, reaching upto 30% in the mature cavities (Hill, 1975; 1977; Braun-Howland & Nierzwicki-Bauer, 1990). Nitrogen-fixation takes place in heterocysts and supplies the total nitrogen requirements of both *Azolla* and *Anabaena* (Peters & Mayne, 1974). Symbiotic *Anabaena* *azollae* has been found to fix nitrogen at a rate 4 to 18 times greater than that of free-living *Anabaena cylindrica* (Tang *et al.*, 1981). Both the activity and protein levels of the primary ammonia assimilating enzyme, GS, is drastically decreased in the cyanobiont (Orr & Haselkorn, 1982). Such low levels of GS are unable to assimilate all the ammonia produced during N_2-fixation by the cyanobiont resulting in its liberation and subsequent

uptake by the host. Levels of primary carboxylating enzyme RuBisCo are also significantly lower in the cyanobiont as compared to the free-living ***Anabaena***, thus making it dependent on fixed carbon availability from the host ***Azolla*** (Braun-Howland & Nierzwicki-Bauer, 1990).

1.2.4 Association with Bryophytes:

The division bryophyta includes three classes of terrestrial plants: musci (mosses), hepaticeae (liverworts) and anthocerotae (hornworts). The nitrogen-fixing cyanobacteria symbiotically associated with bryophytes, are species of ***Nostoc***. In mosses, except for two species of ***Sphagnum***, the cyanobacteria form epiphytic associations. Stewart in 1966 first reported endophytic association of ***Sphagnum***. The cyanobacteria in ***Sphagnum*** are localized in hyaline cells. The cyanobacteria have been identified as ***Nostoc*** sp. Endophytic association of ***Nostoc*** with ***Sphagnum*** is neither frequent in occurrence nor as active in nitrogen fixation as epiphytic association (Basilier, 1980). Morphologically, both epiphytic and endophytic ***Nostoc*** are similar. Nitrogen fixed by the epiphytic ***Nostoc*** is transferred to the host showing a metabolic interaction between the two partners (Basilier, 1980). Association of liverworts with the nitrogen-fixing cyanobacteria is rare, only two instances each of both epiphytic and endophytic associations (***Blasia*** sp. and ***Cavicularia*** sp.) are reported till date (Meeks, 1990). The hornworts are represented by six genera which undergo symbiotic association with endophytic nitrogen-fixing cyanobacteria. These are

Anthoceros laevis, *A. husnotti*, *A. punctatus*, *Phaeoceros laevis*, *Notothylas* sp. and *Dendroceros* sp. In all cases the cyanobiont is *Nostoc*. The *Nostoc* communities are visible as dark, round spots (0.4 to 0.7 mm in diameter), with a distinct organized structure in the gametophytic tissue and are referred as the *Nostoc* colonies (Rodgers & Stewart, 1977; Enderlin & Meeks, 1983). The cellular morphology of the cyanobiont is markedly different from its free-living isolates (Rodgers & stewart, 1977; Duckett *et al.*, 1977; Enderlin & Meeks, 1983). The vegetative cells during symbiotic association enlarge and become spherical. Connection between adjacent vegetative cells and heterocysts and vegetative cells appears to be fragile. The heterocyst frequency increases relative to free-living cultures (30-45% as against 5-10%) and the cyanobiont heterocysts have less distinct polar nodules (Rodgers & Stewart, 1977; Enderlin & Meeks, 1983). The cyanobiont vegetative cells contain cyanophycin granules, phycobilisomes and carboxysomes and numerous glycogen granules. The heterocysts lack these inclusions, but may possess phycobiliproteins in soluble state (Meeks, 1990). The bryophyte responds to the association with increase in cavity surface area and formation of multicellular filaments that mediate the interchange of metabolites (Duckett *et al.*, 1977). The photosynthetic CO_2-fixation (Steinberg & Meeks, 1989) and exogenous NH_4^+ assimilation (Meeks *et al.*, 1985) rates in symbiotic *Nostoc* are five to eight times lower than that in the free-living isolate. Cyanobiont assimilates N_2-derived NH_4^+ via glutamine synthetase - glutamate synthase (GS -GOGAT) pathway.

The activity of GS is three times lower than free-living isolate but the GS protein concentration is found to be comparable, suggesting a post-translational modification of the enzyme in the cyanobiont (Meeks, 1990).

1.2.5 Association with Fungi:

Lichens are symbiotic associations between one or two photobionts and a fungus (mycobiont). The lichen thalli are quite distinct from either of the symbionts occurring in the free-living state. They represent an integration of a heterotrophic fungal partner (mycobiont) and an autotrophic photosynthetic partner (photobiont) in bipartite lichens. The photobiont in such lichens is either a cyanobiont or a phycobiont. In tripartite lichens, apart from the mycobiont there are two photobionts: a green alga (phycobiont) and a cyanobacterium (cyanobiont). About 8% of lichen species have cyanobacteria as photobiont. Such lichens are called cyanolichens. Within the lichen thallus, cyanobiont develops extracellularly except in the case of ***Geosiphon pyriforme***, where the ***Nostoc*** invaginates the plasmalemma of the fungus. The cyanobiont either occurs throughout the thallus or is restricted to a distinct symbiont layer in the upper cortex in bipartite lichens. In tripartite lichens, the cyanobiont occurs in special structures called cephalodia. Cephalodia may be external, occuring on the surface of the thallus (e.g. in ***Peltigera aphthosa, Placopsis, Pilophorus, Stereocaulon*** etc.) or internal, occuring inside the thallus. (e.g. in ***Lobaria*** and ***Sticia***).

Cyanobacteria occuring as lichen cyanobionts may be unicellular (*Chroococcidiopsis, Gloeocapsa, Gloeothece, Synechocystis, Hyella*), filamentous heterocystous (*Calothrix, Nostoc, Scytonema*) or heterocystous branched filamentous (*Fischerella*). All are diazotrophic. Reproduction in lichens is mainly by vegetative means.

A comparative study between *Nostoc* isolate from *Peltigera canina* and the cyanobiont in the symbiotic thallus indicates that ultrastructure of both is similar except increase in cell size (Bergman & Hallbom, 1982). Heterocyst frequency in case of bipartite lichens is similar to that in the free-living isolate (maximum of 8%). However, in case of tripartite lichens heterocyst frequency is higher reaching upto 20% with corresponding high rates of nitrogen fixation (Bergman & Hallbom, 1982; Koriem & Ahmadjian, 1986). Cyanobiont is autotrophic in all lichens. It fixes CO_2 and transfers fixed carbon to the mycobiont. The mechanism of carbon fixation in cyanobiont is similar to that in free-living cyanobacteria. Primary carboxylation reaction mediated by RuBisCo fixes CO_2 via calvin cycle (C_3 cycle) (Stewart *et al.*, 1981; Drew, 1966). In several lichens dark CO_2 fixation also occurs via C_4 cycle. In *P. aphthosa* dark CO_2 fixation rates are about 17 to 20% of that in the light (Rai *et al.*, 1981b). Cyanobiont provides both fixed carbon and fixed nitrogen to mycobiont in bipartite lichens. The fixed carbon moves in the form of glucose. The transfer of fixed nitrogen is in the form of ammonia. In tripartite lichens, cyanobiont mainly provides fixed nitrogen. The fixed carbon comes

from phycobiont in the from of mannitol (Smith, 1974; 1980) which is converted to ribitol in mycobiont. Reasons for carbon transfer is not known, but nitrogen transfer is due to low levels of GS synthesis and activity in the cyanobiont (Rai *et al.*, 1980; Rai 1981a, 1983).

1.2.6 Association with Algae:

There are a few reports of occurrence of cyanobacteria in symbiosis with marine diatoms. The filamentous heterocystous cyanobacterium *Richelia intracellularis* occurs in the marine pennate diatom *Rhizosolenia*. The cyanobiont is reported to be responsible for most of the CO_2 fixation in the association (Mague *et al.*, 1974). However, the major function of the cyanobiont is to fix nitrogen and transfer the fixed nitrogen to the diatom (Rai, 1990a). Inclusion bodies resembling thin walled unicellular cyanobacteria characterised as coccoid cyanobacteria are found in the diatom *Rhopalodia* (Drum & Pankratz, 1965). The cyanobiont fixes nitrogen in the association (Floener & Bothe, 1980). Both partners fix CO_2.

1.3 Nitrogen Fixation and Nitrogenase:

The enzyme responsible for biological N_2-fixation (reduction of dinitrogen to ammonia) is nitrogenase (Gallon, 1980; Stewart, 1980; Hallenbeck, 1987; Smith *et al.*, 1987). It is a cold-labile and oxygen-sensitive enzyme present in some prokaryotes only. The enzyme complex consists of two component

proteins: Mo-Fe protein (dinitrogenase) and Fe-protein (dinitrogenase reductase). The dinitrogenase is a tetramer (mol. wt. 245 KD) of two pairs of different subunits. It also contains two molecules of Mo-Fe cofactor. The dinitrogenase reductase (mol. wt. 64 KD) is a dimer of two identical subunits. N_2 reduction occurs on dinitrogenase and the electrons for reduction are supplied by dinitrogenase reductase which in turn is reduced by ferredoxin (Fd) or flavodoxin (Fv). The latter receives electrons from reductants generated in intermediary metabolism. The reaction is highly endergonic requiring 4 to 5 ATP per pair of electrons transferred. In addition to N_2, nitrogenase can reduce a number of other substrates such as acetylene, azides, cyanides, nitrous oxides etc. During nitrogen reduction there is a concomitant reduction of protons resulting in H_2 formation. This represents a waste of energy and reductants. However, H_2 recycling by uptake hydrogenase in some diazotrophs reduces such losses.

Two alternative nitrogenases, in addition to the molybdenum nitrogenase, have been reported in some diazotrophs (Bau, 1991). There are preliminary indications that these may be present in some cyanobacteria also (Kentemisch *et al.*, 1988; Dillon & Chaplin, 1988; Bothe *et al.*, 1991).

Nitrogenase protein from various diazotrophs show remarkable similarity in molecular mass (Burris *et al.*, 1980; Braun-Hawland *et al.*, 1988; Bergman & Rai, 1989; Zehr *et al.*, 1990). Futhermore, antibodies raised against nitrogenase from one

diazotroph specifically cross react with nitrogenase from other diazotrophs (Smoker *et al.*, 1989; Stal & Bergman, 1990; Rai *et al.*, 1989, 1992). The structural genes for nitrogenase (*nif* H,D,K) are also highly conserved (Haselkorn, 1986). This enables use of immunological and genetic probes to study nitrogenase protein and the concerned genes in a variety of diazotrophs.

As N_2-fixation demands high input of energy, the process is subjected to rigorous control both at the nitrogenase activity and the synthesis levels. Oxygen rapidly and irreversibly inactivates nitrogenase and also represses its synthesis (St. John *et al.*, 1974; Eady *et al.*, 1978; Smith *et al.*, 1987a). Combined nitrogen sources such as nitrate, nitrite, ammonia and amino acids are preferred sources of nitrogen and nitrogenase is synthesized only when such sources are absent in the medium. Detailed studies on effect of ammonia suggest that it inhibits nitrogenase activity as well as synthesis. Ammonia inhibits nitrogenase activity by lowering membrane potential (Haaker *et al.*, 1980) or causes irreversible inactivation by modification of the Fe-protein (Gotto & Yoch, 1982; Reich & Böger, 1989). Primarily though, ammonia through its assimilation via GS, causes repression of nitrogenase synthesis in all diazotrophs (Nagasanik, 1977; Shanmugam *et al.*, 1978; Stewart, 1980).

Diazotrophic cyanobacteria are broadly of two types: heterocystous and nonheterocystous. Among heterocystous forms nitrogenase is synthesized only in heterocysts (Bergman *et al.*, 1986). These are specialized cells having undergone a number of

biochemical and structural changes, including loss of O_2-evolving photosystem II, conducive to nitrogenase functioning (Wolk, 1982). Among nonheterocystous cyanobacteria nitrogenase is synthesized in all the cells although this poses a problem of protecting it from O_2 evolved during photosynthesis (Stal & Bergman, 1990; Rai *et al.*, 1992). Such organisms however employ a number of strategies including temporal separation of N_2-fixation and photosynthesis, to protect nitrogenase (see Fay, 1992).

1.4 Hydrogenase:

Hydrogenases catalyze H_2 oxidation and/or proton reduction. Considerable work has been done on hydrogen metabolism and hydrogenases of bacteria, cyanobacteria and diazotrophic symbioses (Adams *et al.*, 1981; Gogotov, 1986; Lambert & Smith, 1981; Houchins, 1984; Ewart & Smith, 1989a, b; Evans *et al.*, 1987; Huss-Danell, 1990). The aim has been to investigate hydrogenase-nitrogenase relationships with regard to the efficiency of N_2-fixation. At least two types of hydrogenases have been reported in diazotrophs. A reversible hydrogenase and an uptake hydrogenase. The latter plays an important role in recycling of hydrogen evolve during N_2-fixation by nitrogenase. It has been suggested that such recycling improves the N_2-fixation efficiency because it generates ATP and reductant, consumes O_2, thereby contributing to oxygen protection of nitrogenase, and prevents build up of hydrogen which is inhibitory to nitrogenase and photosynthesis (*see* Adams *et al.*, 1981; Lambert & Smith, 1981; Houchins, 1984; Antarikanonda *et*

1980). In heterocystous cyanobacteria, reversible hydrogenase was suggested to be located in cytoplasm of both heterocysts and vegetative cells while uptake hydrogenase bound to the membranes in heterocysts (Houchins, 1984). However, later studies showed presence of both uptake and reversible hydrogenase activities in membrane bound as well as in soluble fractions (Rao & Hall, 1988; Ewart & Smith, 1989a, b; Kentemisch *et al.*, 1989; Papen *et al.*, 1986). In addition, H_2-evolution also occurs via nitrogenase in heterocysts (Chen *et al.*, 1986; Almon & Boger, 1988).

1.5 Ribulose 1,5-Bisphosphate Carboxylase / Oxygenase (RuBisCo):

In cyanobacteria, RuBisCo is present both in the cytoplasm (soluble form) and in specialized structures called carboxysomes (polyhedral bodies). The latter contain a very high concentration of RuBisCo. Each vegetative cell on an average contains 1-2 carboxysomes (Lang & Whitton, 1973; Wolk, 1973; Stewart & Codd, 1975; Stanier & Cohen-Bazire, 1977). In contrast to the vegetative cells, the heterocysts do not contain RuBisCo and they also lack carboxysomes (Stewart & Codd, 1975; Codd & Stewart, 1977; Cossar *et al.*, 1985; Rai *et al.*, 1989). Cyanobacteria fix CO_2 via calvin cycle. The key reaction of calvin cycle where CO_2 is introduced to react with ribulose 1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglyceric acid (3-PGA), is catalysed by RuBisCo (Shively *et al.*, 1973; Shively, 1974; Codd, 1988). RuBisCo is amongst the most abundant proteins in the autotrophic cells. It has a molecular weight of 550,000 and has

eight large and eight small subunits. This enzyme needs Mg^{+2} ions for its catalytic activity (Lorimer, 1981; Miziorko & Lorimer, 1983). In all autotrophs RuBisCo is a bifunctional enzyme capable of catalysing carboxylation or oxygenolytic cleavage of RuBP.

1.6 Glutamine synthetase:

In all N_2-fixing cyanobacteria, glutamine synthetase (GS), plays a central role in the assimilation of the ammonia produced by the action of nitrogenase (Haselkorn, 1978; Bothe, 1982). GS catalyses the synthesis of L-glutamine in the reaction:

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

(at pH 7 and 37° C with a free energy change -5.2 Kcal $mole^{-1}$).

GS is the key enzyme in the flow of ammonia nitrogen to organic compounds. The amide nitrogen of glutamine thus produced through the action of GS is utilised in the biosynthesis of AMP, CTP, tryptophan, histidine, glucosamine 6-phosphate and carbamoyl phosphate (Meister, 1974). In addition, GS may be coupled to GOGAT (Tempest *et al.*, 1970) and various transaminases to provide a pathway for ATP-dependent synthesis of most amino acids. GS-GOGAT pathway serves as the sole route of primary ammonia assimilation in diazotrophic cyanobacteria (Wolk *et al.*, 1976; Singh *et al.*, 1991). In heterocystous cyanobacteria, the ammonia produced by the action of nitrogenase in the heterocysts, is assimilated in the heterocysts by the action of GS. The GS level in nitrogen fixing heterocysts is generally two-fold higher than that of the vegetative cells. The increased level of GS in

heterocysts shows a correlation with expression of nitrogenase and seems essential for assimilation of N_2-derived ammonia (Bergström-Kellner *et al.*, 1990). GS is also involved in mediating the effect of ammonia on nitrogenase repression in all diazotrophs (Magasanik, 1977; Shanmugam *et al.*, 1978; Stewart, 1980). Heterocysts lack GOGAT hence the glutamine is transported to the adjacent vegetative cells where it is further metabolised by the action of GOGAT (Wolk *et al.*, 1976). The native GS enzyme has twelve identical subunits of approximately 50,000 dalton molecular weight each arranged in two superimposed hexagonal rings. The biosynthetic activity of GS is Mg^{+2}-dependent and the transferase activity requires Mn^{+2} ions (Sampaio *et al.*, 1979). GS activity has been shown to be regulated as a function of nitrogen source available in the growth medium. In the unicellular cyanobacterium ***Synechocystis***, intracellular carbon-nitrogen balance plays an important role in the regulation of GS (Merida, *et al.*, 1991). In ***Anabaena*** sp. associated with ***Azolla*** and ***Nostoc*** cyanobiont of lichens, GS activity is regulated by repression of synthesis (Nierzwicki-Bauer & Haselkorn,1986; Orr & Haselkorn, 1982; Sampaio *et al.*, 1979; Stewart *et al.*, 1983). But GS is regulated by posttranslational modification in ***Nostoc*** sp. associated with ***Anthoceros punctatus*** (Joseph & Meeks, 1987). Genes encoding GS has been found to be induced directly by available ammonia in ***Rhizobium*** (Hirel *et al.*, 1987).

1.7 Phycobiliproteins:

Phycobiliproteins are accessory photosynthetic pigments in cyanobacteria. They constitute well over half of the total soluble proteins of the cyanobacterial cells (Bennet & Bogorad, 1973). Cyanobacteria possess phycobiliproteins in their vegetative cells along the thylakoid membranes (Stewart *et al.*, 1983; Lindblad, 1987) and heterocysts are found to be deficient in them (Stewart, 1980; Rai *et al.*, 1989). Phycobiliproteins are assembled into particles called phycobilisomes which are attached to the external surface of the thylakoid membranes. They occur as chromoproteins and are involved in the light phase of the photosynthesis along with chlorophylls and carotenoids. They are divided into two major groups on the basis of their colour. The red phycoerythrin and blue phycocyanin. Phycoerythrin and phycocyanin are composed of two different protein subunits designated as α (mol. wt. 19 KD) and β (mol. wt. 21 KD), which occur in 1:1 stoichiometric ratio. Each subunit carries covalently bound phycobilins. Depending upon its source, a subunit may carry 1-4 molecules of phycobilins. The phycobiliproteins also serve as a nitrogen reserve and undergo degradation during nitrogen limitation (Cohen-Bazire & Bryant, 1982; Ho & Krogman, 1982). Cyanobacteria under symbiotic condition possess phycobiliproteins in their vegetative cells along the thylakoid membranes (Stewart *et al.*, 1983; Lindblad, 1987) and heterocysts are found to be deficient in them (Stewart, 1980; Rai *et al.*, 1989).

1.8 *Anthoceros*:

Anthoceros belongs to the division bryophyta. It is cosmopolitan in distribution, mainly occurring in temperate and tropical regions and has about 200 species. About 25 species of *Anthoceros* have been reported in India.

The plant body is small, dorsiventral, prostate and dark green thallose gametophyte. The thallus is sub-orbiculous or variously lobed. The lobes may be divided, several layers thick in the middle, midrib either broad indistinct or absent. The dorsal surface of the thallus is smooth or velvety. On the undersurface of the *Anthoceros* thallus mucilage-filled cavities are present in which *Nostoc* colonies develop (Enderlin & Meeks, 1983). From the ventral surface rhizoids of smooth walled type develop. Scales and mucilage hair are absent. Vegetative reproduction takes place by the growth of apical region and progressive death of the older parts. *Anthoceros* may be homothallic or heterothallic. Sex organs are deeply embedded in the thallus. Antheridia develop in clusters from the hypodermal cells of the thallus and archegonia develop simply from the superficial cells of the thallus. Sporophytic generation begins as soon as fertilization takes place. A mature sporophyte consists of expanded or bulbous foot and smooth, slender, erect cylindrical capsule. A capsule presents all the stages of spores production at a time.

1.9 The cyanobiont:

The cyanobiont, ***Nostoc***, is a filamentous heterocystous cyanobacterium capable of photoautotrophic as well as heterotrophic growth (Rippka *et al.*, 1979). There is an apparent correlation between symbiotically competent cyanobacteria and the presence of hormogonia (Stewart *et al.*, 1980; Enderlin & Meeks, 1983). The hormogonia of symbiotic ***Nostoc*** strains are highly motile, small-celled filaments that lack gas vacuoles. The stimulation of hormogonia formation depends upon an extracellular low molecular weight, heat labile product of ***A. punctatus*** metabolism which is inhibited by excess NH_4^+ (*see* Meeks, 1990). The cyanobiont forms discrete colonies within preformed cavities in the ***Anthoceros*** gametophyte tissue (Enderlin & Meeks, 1983).

1.10 *Anthoceros-Nostoc* symbiosis:

Anthoceros-Nostoc symbiosis is an endophytic association where ***Nostoc*** colonies develop in the mucilage-filled cavities on the undersurface of the ***Anthoceros*** thallus. The ***Nostoc*** colonies are macroscopically visible as distinctly organised, dark, round spots (0.4 to 0.7 mm in diameter) in the gametophytic tissues of the ***Anthoceros*** (Rodgers & Stewart, 1977; Enderlin & Meeks, 1983). Oxygen microelectrode profiles of symbiotic ***Anthoceros-Nostoc*** tissue reveals an anaerobic environment in the symbiotic cavities containing ***Nostoc***. It appears that the symbiotic cavities of ***Anthoceros punctatus*** can physiologically replace the function of

heterocyst outer wall (Campbell & Meeks, 1992). ***Anthoceros*** responds to the endophytic presence of ***Nostoc*** by an increase in the cavity surface and prolification of multicellular papillae which acquire the characteristics of transfer cells and facilitate the exchange of metabolites between the two partners (Rodgers & Stewart, 1977; Duckett *et al.*, 1977). The symbiotic *Nostoc* has a filament and cellular morphology markedly different from free-living cultures (Rodgers & Stewart, 1977; Enderlin & Meeks, 1983; Meeks, 1990). The symbiotic ***Nostoc*** vegetative cells enlarge and become spherical. The connection between adjacent vegetative cells and heterocysts and vegetative cells become fragile (Meeks, 1990). Symbiotic heterocysts tend to have less distinct polar nodules and the difference in the pigmentation is muted. The heterocyst frequency goes upto 43 - 45 % (Rodgers & Stewart, 1977; Enderlin & Meeks, 1983). The symbiotic vegetative cells contain nitrogen reserves in the form of cyanophycin granules, phycobilisomes and carboxysomes as well as numerous glycogen granules. The relative amount of these cellular inclusions are equal to or greater than those observed in the free-living vegetative cells of N_2-grown cultures. The heterocysts lack these inclusions both in free-living and symbiotic cyanobionts. The eukaryotic partner ***Anthoceros***, exerts some regulatory constraints on the growth, metabolism and cellular differentiation of the symbiotic ***Nostoc*** to bring about a balanced growth (Campbell & Meeks, 1992). The main function of symbiotic ***Nostoc*** is to provide fixed-nitrogen to its eukaryotic partner. *In situ* ^{13}N tracer experiments established that

symbiotic *Nostoc* releases 80 to 90% of its fixed nitrogen as ammonia to support growth of *Anthoceros* tissue (Meeks *et al.*, 1985). The photosynthetic capability of the symbiotic *Nostoc*, as measured by CO_2-fixation, O_2-evolution and the level of accessory pigments for photosystem II, is diminished by five to eight times as compared to free-living cultures (Meeks *et al.*, 1985). It is now established that *Nostoc* is capable of complete photosynthesis when in association with *Anthoceros*, though at a lower rate (Steinberg & Meeks, 1989) and can use its photosynthate to support nitrogenase activity (Steinberg & Meeks, 1991). Free-living *Nostoc* under diazotrophic conditions doubles every 45 h but in symbiosis, *Anthoceros-Nostoc* association doubles its biomass in 10 days as against the doubling time of 5 days for symbiont-free *Anthoceros* (Meeks, 1990). The size of the *Nostoc* colony in *Anthoceros* tissue, its frequency of heterocysts and rate of nitrogen fixation are influenced by the growth conditions of the *Anthoceros-Nostoc* association (Enderlin & Meeks, 1983; Steinberg & Meeks, 1991). Establishment of symbiosis also initiates several biochemical changes in the endophyte. Rate of nitrogen-fixation by symbiotic *Nostoc* is strongly dependent on association with the intact *Anthoceros punctatus* tissue. Rodgers & Stewart in 1977 demonstrated that the rate of acetylene reduction is 3.2 times higher than the free-living cultured isolates. Cyanobacteria assimilate NH_4^+ exclusively by the sequential activity of glutamine synthetase (GS) and glutamate synthase (GOGAT) via GS-GOGAT pathway (Meeks, 1981; 1990). The GS activity in the cyanobiont of *Anthoceros-Nostoc* is reduced by

three-to four-fold without similar decrease in the GS content (Joseph & Meeks, 1987). Thus, the capability to assimilate the fixed-N is reduced greatly and as much as 90% of the fixed-N is transported to the host as ammonia (Stewart & Rodgers, 1977; Meeks *et al.*, 1985).

I.III Present study:

Diazotrophic cyanobacteria efficiently combine oxygenic photosynthesis and nitrogen fixation. Those diazotrophic cyanobacteria which form symbiotic associations with plants undergo major structural, physiological and biochemical changes relating to their carbon and nitrogen metabolism (see Rai, 1990). Some of these changes such as high heterocyst frequency, high nitrogenase activity and ammonia liberation have biotechnological implications such as photobiological production of ammonia and production of biofertilizers. By understanding the underlying mechanisms involved in the physiological/biochemical changes during the symbiotic state of the cyanobiont, it is hoped that free-living cyanobacterial strains could be modified on similar lines. Thus, there is a need to fully understand the symbiotic associations of cyanobacteria, particularly the physiological and biochemical changes in the cyanobiont's nitrogen metabolism and related processes.

Nostoc-Anthoceros symbiosis was chosen for this particular study since the symbionts can be axenically cultured and the symbiosis reconstituted in the laboratory. This thesis

incorporates details of the work done on general characterization of the cyanobiont, reconstitution of symbiosis, electronmicroscopic localization of key enzymes involved in nitrogen, carbon and hydrogen metabolism. For comparative purposes, some of these aspects have also been studied using the non-heterocystous cyanobacterium *Plectonema boryanum*.