Progress in Botany 81

Francisco M. Cánovas
Ulrich Lüttge
Christoph Leuschner
María-Carmen Risueño *Editors*

Progress in Botany

Springer

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Volume 81

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ISSN 0340-4773 ISSN 2197-8492 (electronic)
Progress in Botany
ISBN 978-3-030-36326-0 ISBN 978-3-030-36327-7 (eBook)
https://doi.org/10.1007/978-3-030-36327-7

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The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

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Curriculum Vitae

Dr. José M. Vega was born on March 29, 1946, in Villanueva de la Serena, Province of Badajoz (Spain). He is married to Prof. Rosario Pásaro since 1972 and has two sons, José María (born in 1975) and Julio (born in 1979), and one grandson, Álvaro (born in 2016). He graduated in chemistry from the University of Seville in June 1968, and obtained his Ph.D. degree from the same university in July 1972, under the supervision of Prof. Manuel Losada. His Ph.D. thesis was entitled "NADH-nitrato reductasa de *Chlorella*."

His postdoctoral training was completed in the following three places: (a) firstly, at the Faculty of Biology, University of Virginia (1973–1974), in the research group of Prof. Reginald H. Garrett, which was working on NADPH-nitrate and nitrite reductases from the fungi *Neurospora crassa*; (b) then at Duke University Medical Center (1974–1976), under the supervision of Prof. Henry Kamin, who was working on ferredoxin-nitrite reductase from spinach leaves; and (c) finally, at the Department of Botany, University of Erlangen-Nürnberg (Germany), in the research group of Prof. Walter G. Zumft (1978), which was working on the denitrification mechanism of the bacteria *Pseudomonas perfectomarinus.*

In October 2018, he completed 50 years since his first appointment as an Assistant Professor to teach the techniques in Biochemistry to the students of the first course in Biology degree at the University of Sevilla. It was this university where he developed most of his teaching activities in the field of Biochemistry and Molecular Biology at the Faculties of Biology (1969–1980) and Chemistry (1981–2019). In 1981, he was appointed as Professor of Biochemistry and Molecular Biology at the Faculty of Chemistry, University of Sevilla, where he established his own research group that initially comprised Prof. Francisco Galván and Prof. Antonio J. Márquez. Figure 1 depicts the people belonging to the current Biochemistry group at the Faculty of Chemistry.

Fig 1 Biochemistry group at the Faculty of Chemistry. In the first line, from left to right: Prof. Marco Betti, Mrs. M. José Cubas, Mrs. Aurora Gómez, Prof. Carmen Pérez-Delgado, and Prof. Margarita García-Calderón. In the second line, from left to right: Prof. Francisco Galván, Prof. José M. Vega, and Prof. Antonio Márquez. The picture was taken on July 11, 2018

In 1986, Dr. Pedro J. Aparicio (Spanish National Research Council), Dr. Juan L. Serra (University of the Basque Country), Dr. Jacobo Cárdenas (University of Córdoba), and Dr. José M. Vega (University of Sevilla) together founded the Spanish Groups of Nitrogen Metabolism, which involved the groups working in the field of nitrogen metabolism and comprised of about 100 members. In addition, Dr. José M. Vega was a member of the Management Committee of COST 829 action budget by the European Union regarding "Fundamental, Agronomical and Environmental aspects of Sulfur Nutrition and Assimilation in Plants" (1996–2003). He was also a member of the Scientific Committee of "European Nitrate and Ammonia Assimilation Group" (ENAAG) during the period of 2001–2005.

Currently Dr. Vega has the research scopes regarding "Biotechnology of microalgae" collaborating with the research group directed by Dr. Carlos Vílchez, at the University of Huelva (Spain). This research field is gaining more and more presence in different industrial and economic sectors (Fig. 2).

Fig. 2 People of the Biochemistry area at the University of Huelva (Spain). In the first line, from left to right: Prof. Javier Vigara, Prof. Inés Garbayo, Prof. José M. Vega, Prof. Rosa León, and Prof. Carlos Vílchez. The picture was taken on September 25, 2018

To date, Dr. Vega has published 116 articles in indexed scientific journals, 43 chapters in books, and 12 books, and has also presented 240 communications to various scientific meetings. He has supervised 23 Ph.D. scholars, and has a Hirsch index (*h*-index) of 38. In addition, he has received two important awards in recognition of his excellent academic and research work. In the year 2007, the University of Seville distinguished him with the FAMA awards, and in the year 2016, the village of Villanueva de la Serena (Badajoz) declared him a distinguished person born in the village.

Nitrogen and Sulfur Metabolism in Microalgae and Plants: 50 Years of Research

José M. Vega

To my wife Rosario Pásaro

Contents

Communicated by Prof. Francisco M. Cánovas

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Progress in Botany (2020) 81: 1–40, DOI 10.1007/124_2018_26,
Published online: 14 May 2019

Abstract Microalgae and higher plants are primary producers which convert solar energy into chemical energy associated with biomass, which is used by the other organisms as life support. The knowledge and improvement of mineral nutrition of microalgae and plants are very important in order to obtain a good productivity, even in low fertile soils. This review describes, in chronological order, the research topics in which the author has been involved in the past 50 years, which are mostly connected with the nitrogen and sulfur metabolisms in bacteria, fungi, microalgae, and higher plants, with emphasis on the microalgal model *Chlamydomonas reinhardtii* and the plant models *Arabidopsis thaliana* and *Lotus japonicus*. It is described the outstanding contribution of the author to each topic described, and the state of art actualized. The data from our studies on the nitrate and sulfate assimilation pathways aid in clarifying plant nutrition mechanisms, indicating that there are certain key substrates which also trigger the regulatory signals, commonly participating in the photosynthetic organisms. In addition, nitrate and sulfate participate in the metabolic pathways required for plant adaptation mechanisms to abiotic stresses encountered commonly in agriculture, such as salt and metal toxicity, drought, temperature, and herbicides.

There are many species of microalgae in the world but only a few of them have been studied in terms of biomass producers as source of foods, biofuels and/or high added value products for aquaculture, functional foods, pharmaceutical, and cosmetic industries. Actually we are involved in the use of extremophilic microalga, such as *Coccomyxa onubensis*, as a natural source of C40 carotenes, such as lutein, and the halophilic bacteria *Haloferax mediterranei* as source of C50 carotenoids, such as bacterioruberin.

Keywords *Arabidopsis thaliana*, Biotechnology of microalgae, *Chlamydomonas reinhardtii*, *Coccomyxa onubensis*, *Lotus japonicus*, Nitrogen metabolism, Sulfur metabolism

1 Nitrate Assimilation Pathway in Microalgae and Plant Leaves

The term "microalgae" used in this work refers to eukaryotic microalgae and prokaryotic (cyanobacteria) organisms that convert the solar energy aerobically, with high efficiency, into chemical energy as biomass. These organisms possess high metabolic plasticity that has allowed them to adapt to the most extreme environments, even to ones in which sustenance of life is tough. In addition, microalgae are able to grow in places that are not used for crop production, and their nutritional requirements are inexpensive and simple, for example sunlight, CO_2, a source of nitrogen, phosphate, sulfur, and trace elements, which are present normally in the water that is used for the culture of microalgae (Forján et al. 2015).

In association with other research groups, we contributed to establish that the nitrate assimilation pathway in the eukaryotic microalgae and plant leaves involves four basic steps: (a) nitrate transport into the green cell, which requires ATP-dependent permease systems, of the NRT2 transporter type, to cross the cell cytoplasmic membrane; (b) nitrate reduction into nitrite in the cytosol of cells, catalyzed by NAD(P)H-nitrate reductase. With an exception of cyanobacteria, the reduction of nitrate to nitrite proceeds with reduced ferredoxin as the electron donor for a reaction catalyzed by ferredoxin-nitrate reductase; (c) nitrite transport into the chloroplast of cells, mediated by a specific permease of the NAR1 family, and its further reduction into ammonium, in a 6-electron reaction, catalyzed by ferredoxin-nitrite reductase; and (d) the incorporation of ammonium into the carbon skeleton of 2-oxoglutarate leading to the production L-glutamate, in a pathway catalyzed by the ATP-dependent glutamine synthetase (GS)-glutamate synthase (GOGAT) cycle.

The incorporation of 1N-nitrate into 1N-amino glutamate requires a minimum of four ATPs and ten electrons, which are supplied by the photosynthetic machinery. In my first contributed paper (Paneque et al. 1969), we demonstrated that the illuminated spinach grana supplemented with ferredoxin, $NADP^+$, and ferredoxin-NADP reductase (EC 1.18.1.2), are able to couple the photolysis of water with the stoichiometric reduction of nitrate into ammonium.

The reviews including most of the studies conducted by our research group in this field have been published earlier (Guerrero et al. 1981; Vega et al. 1991). An interesting phylogenetic study on the nitrogen-assimilating enzymes has been published previously (Inokuchi et al. 2002).

1.1 Nitrate Transport Systems

In *C. reinhardtii* cells growing autotrophically with nitrate or nitrite, as the N-source, ammonium per se strongly inhibits the consumption of nitrate, but not that of nitrite, indicating the possibility of different uptake systems for these N-sources (Florencio and Vega 1983a).

The nitrate/nitrite transport systems in *C. reinhardtii* have been studied in detail by the research group of Emilio Fernández and Aurora Galván, in the University of Córdoba (Spain). A minimum of four nitrate/nitrite transport systems in this microalga are responsible for the entry of nitrate into the cells, and these systems correspond to NRT2 transporter type. Three of them operate efficiently at high CO_2 concentration and are considerably sensitive to ammonium inhibition. Among these three systems, system I (NRT2;1, NAR2) is a bispecific nitrate/nitrite transporter with a high affinity for both nitrate and nitrite ($Ks = 1$ μM), system II (NRT2;2, NAR2) is nitrate specific ($Ks = 10$ μM), and system III (NRT2;3) is nitrite specific ($Ks = 3$ μM). On the other hand, system IV (NRT2;4) functions optimally at a low CO_2 concentration and is not sensitive to ammonium. It is bispecific for nitrate and nitrite ($Ks = 30$ μM). In addition, NAR 1 represents a member from another family, which is required for the specific transport of nitrite inside the chloroplasts (Galván and Fernández 2001).

In the microalga *Tisochrysis lutea*, four genes (*Nrt2*) coding for the putative high-affinity nitrate/nitrite transporters (TINrt2) have been identified, and the deduced TINRT protein sequences exhibited similarities to the NRT2 proteins from the other phyla, such as the land plants and green microalgae (Charrier et al. 2015). The high-affinity nitrate and nitrite uptake occurs in different cyanobacteria either through an ABC-type transporter or through a permease from the major facilitator superfamily (MFS) (Flores et al. 2005).

1.2 NAD(P)H-Nitrate Reductase (NAD(P)H-NR)

The NAD(P)H-NR enzyme (EC 1.7.1.3) catalyzes the following reaction:

$$\mathrm{NAD(P)H} + \mathrm{NO_3}^- + \mathrm{H}^+ \rightarrow \mathrm{NO_2}^- + \mathrm{H_2O} + \mathrm{NAD(P)}^+.$$

In the process of transfer of electrons from NAD(P)H to nitrate, two enzymatic activities occur sequentially during the catalysis; one is the NAD(P)H-diaphorase activity, which catalyzes the NAD(P)H-dependent cytochrome c reduction, and the other is the reduced flavin-dependent activity (FNH_2-nitrate reductase), which is able to stoichiometrically reduce nitrate into nitrite (Losada et al. 1970). At the beginning of my Ph.D. thesis, the attention was focused on the isolation and molecular properties of NADH-nitrate reductase (NADH-NR; EC 1.7.1.1) from the eukaryotic microalga *Chlorella fusca*, as well as on the regulation of the activity of this enzyme. The most interesting conclusions observed in our results were as follows:

(a) Molybdenum is essentially required when growing *C. fusca* cells in a culture medium containing nitrate; however, the metal is not required when using a culture medium containing ammonium as a unique nitrogen source. The role of the metal is to assist the reduction of nitrate into nitrite, particularly during FNH_2-nitrate reductase activity (Vega et al. 1971).

(b) The enzyme NADH-NR from *C. fusca* is an interconvertible enzyme, which is inactivated in vivo by the presence of ammonium and reactivated by its absence. This regulatory effect was observed during the FNH_2-nitrate reductase activity, while no such effect was observed during the NADH-diaphorase activity. The synthesis of NADH-NR enzyme is repressed completely by the presence of ammonium in the culture medium (Losada et al. 1970).
(c) Purified NADH-NR from *C. fusca* may be inactivated in vitro by treating the NADH-reduced enzyme with cyanide, which binds tightly to the nitrate-active site. The original enzyme activity may be restored by oxidizing the inactivated enzyme using ferricyanide (Vega et al. 1972). The inactivation of the purified NAD(P)H-NR obtained from the microalga *Ankistrodesmus braunii* (also known as *Monoraphidium braunii*) occurs in two steps: the first one is one-electron reduction of the enzyme, which probably involves the molybdenum cofactor, and the second step, which is also the rate-limiting step for this process, is the interaction of the reduced enzyme with a nucleophilic agent which is either a superoxide or a cyanide. The reactivation of the inactivated enzyme by cyanide is a process that requires the removal of one electron from the enzyme (de la Rosa et al. 1981a).

The absorption spectrum obtained for the purified NAD(P)H-NR from *M. braunii* revealed the presence of FAD and *b*-type cytochrome, as the prosthetic groups of the enzyme. A chemical analysis of the purified enzyme preparations indicated the presence of four FAD, four cytochrome *b557*, and two molybdenum for each enzyme molecule. The molecule was an oligomeric protein of size 467 kDa and was composed of eight similar subunits, each of which was 58.75 kDa in size. Electron micrographs depicted that the eight subunits were arranged alternately in two planes, and eightfold rotational symmetry was deduced from the highly magnified images processed by optical superimposition (de la Rosa et al. 1981b). Conversely, nitrate reductase from *C. reinhardtii* was a homodimeric protein with subunits of about 100–120 kDa in size, and including each one of the three prosthetic groups, namely, FAD, cytochrome *b*557, and molybdenum cofactor (MoCo) (Sanz-Luque et al. 2015).

The molybdenum cofactor (MoCo), which was observed to be involved in the nitrate reductase activity, was further characterized, and it was observed that it is a coordination complex formed between a pterin and molybdenum (VI) oxide; this complex is known to function in other molybdoenzymes involved in carbon, nitrogen, or sulfur metabolisms occurring in microorganisms, plants, and animals (Mendel 2013).

1.3 Ferredoxin-Nitrate Reductase (Fd-NR)

This enzyme Fd-NR (EC 1.7.7.2) has been specifically found in cyanobacteria and catalyzes the following reaction:

$$NO_3^- + 2\,Fd_{red} + 2H^+ \rightarrow NO_2^- + H_2O + 2Fd_{ox}.$$

A molecule of this enzyme has a size of 80 kDa, and contains [4Fe-4S] cluster and Mo-*bis*-molybdopterin guanine dinucleotide as prosthetic groups. The enzyme is associated with thylakoid membranes and forms a 1:1 complex with ferredoxin, the physiological electron donor. This complex is electrostatically stabilized through interactions between the lysine and arginine residues of nitrate reductase and the negatively charged residues of ferredoxin (Flores et al. 2005).

1.4 Ferredoxin-Nitrite Reductase (Fd-NiR)

The enzyme Fd-NiR (EC1.7.7.1) catalyzes the following reaction:

$$NO_2^- + 6Fd_{red} + 8H^+ \rightarrow NH_4^+ + 2H_2O + 6Fd_{ox}.$$

Evidence indicates that siroheme is a unique group present in the unusual 6-electron reductions catalyzed by sulfite reductases (Siegel et al. 1982). Siroheme is a complex of iron (II) with sirohydrochlorin, a class of hydroporphyrins consisting of eight carboxylic acid side chains (Murphy et al. 1973). We purified the NADPH-nitrite reductase (EC 1.7.1.4) obtained from *Neurospora crassa*, an enzyme that catalyzes the six-electrons reduction of nitrite into ammonium, and identified the presence of FAD and siroheme as the prosthetic groups of the molecule of this enzyme (Vega et al. 1975).

Prof. Henry Kamin (Masters 1989) at Duke University, North Carolina, was interested in the identification of prosthetic groups of Fd-NiR from spinach, in order to evaluate if siroheme was present in this enzyme as well. We purified the enzyme obtained from the spinach leaves, and identified the presence of one siroheme and one tetranuclear iron-sulfur cluster [4Fe-4S] as prosthetic group per molecule of size 61 kDa, which function in coordination during the 6-electron reduction of nitrite into ammonium (Vega and Kamin 1977; Lancaster et al. 1979; Vega et al. 1980). Later, we confirmed the presence of one siroheme in the Fd-NiR molecule from the model microalga *C. reinhardtii* (Romero et al. 1987), as well as in the enzyme from *M. braunii* (Vigara et al. 2002). Purified Fd-NiR from cyanobacteria also appears to contain siroheme and the iron-sulfur cluster as prosthetic groups, as observed in the cyanobacteria *Anabaena* sp. (Méndez and Vega 1981; Curdt et al. 2000), and *Phormidium laminosum* (Arizmendi and Serra 1990). The nitrate reducing enzymes from cyanobacteria have been substantially reviewed by Flores et al. (2005).

We established the formation of a 1:1 functional electrostatic complex (Fd:NiR) between ferredoxin and the nitrite reductase from *C. reinhardtii*, and identified two negative surface regions in Fd that were involved in its binding with NiR, namely, the Glu91/Glu92 and the Asp25/Glu28/Glu29 regions (García-Sánchez et al. 1997,

2000). An interesting in silico docking model for the1:1 Fd:NiR complex was proposed by Hirasawa et al. (2009) using the technique of site-directed mutagenesis in the putative amino acid residues involved in both the proteins. These Fd residues are also essential for the formation of a functional 1:1 complex (Fd:SiR) required for the intermolecular electron transfer between Fd and sulfite reductase (SiR) from maize leaves (Saitoh et al. 2006).

Furthermore, there has been a significant progress in the area concerning the crystal structure and reaction mechanism involved in spinach NiR. In silico docking model studies have suggested that the spinach Fd residues Phe63, Glu92, and Glu93 (which correspond to Phe65, Glu92, and Glu93 in *Anabaena* Fd) are supposed to be involved in the binding with NiR (Hirasawa et al. 2009). Using NMR spectroscopy, Sakakibara et al. (2012) studied the Fd:NiR interaction using proteins from cyanobacteria and identified three negative regions in Fd, Tyr25 to Leu37, Ser61 to Gln70, and Glu95 to Leu97, which were involved in the interaction with NiR. The spinach Fd:NiR complex is stabilized by the interaction of Ser43 and Glu93 in Fd with Lys80, Lys83, and Lys100 in NiR, by a salt bridge between Asp60 in Fd and Arg504 in NiR, and by a H-bond between Ser43 in Fd and Lys100 in NiR (Swamy et al. 2005; Hase et al. 2006).

Spectroscopic evidence has suggested that the prosthetic groups in NiR are functionally coupled, probably through the sharing of a sulfur ligand between the siroheme iron and the [4Fe-4S] cluster (Wilkerson et al. 1983). In the Fd:NiR 1:1 complex established with reduced Fd, the six-electrons required for the reduction of nitrite into ammonium flow one-to-one through the enzyme, and it is probable that NiR is first reduced by one electron and then the Fe^{2+}-siroheme binds to the nitrite rapidly. The delivery of a second electron from the reduced ferredoxin produces a well-characterized Fe^{2+}-siroheme/NO adduct as a reaction intermediate, which has already been observed in the EPR studies conducted a few years ago (Aparicio et al. 1975; Lancaster et al. 1979; Kurtnetsova et al. 2004). Another chemical species involved as a possible intermediate in the catalytic cycle of NiR is hydroxylamine (Hase et al. 2006; Hirasawa et al. 2010).

2 Ammonium Assimilation and Re-assimilation in Microalgae and Plant Leaves

Ammonium may be present in the microalgae obtained from different sources, such as those obtained directly from the environment, from the nitrate reduction, from nitrogen fixation (in certain cyanobacteria), photorespiration, protein degradation, and from nitrogen-transport compounds, particularly asparagine, which produces ammonium when it is broken down into aspartate plus ammonium under the catalytic action of asparaginase. In addition, ammonium is also formed in plants and microalgae through the first step of the biosynthetic pathway of secondary metabolites, phenolic compounds. This step is catalyzed by the enzyme

phenylalanine-ammonia-lyase (PAL, EC 4.3.1.5.), according to the following reaction:

$$\text{Phe} \rightarrow \textit{trans}\text{-Cinnamic acid} + NH_4{}^+.$$

This enzyme is very active when plant and microalgae are submitted to abiotic or biotic stress (Mrázová et al. 2017).

Photorespiration is a pathway in C3 plants and microalgae for the recovery of the glycolate formed during the oxidase activity of Ribulose-*bis*-phosphate carboxylase (RUBISCO; EC 4.1.1.39):

$$\text{Ribulose-}\textit{bis}\text{-phosphate} + O_2 \rightarrow \text{3-Phosphoglycerate} + \text{Phosphoglycolate}.$$

This pathway is an energy-dependent O_2-consuming process which involves three organelles, chloroplast, peroxisome, and mitochondria, and releases CO_2 and eventually ammonium, which is produced in the mitochondria under the catalytic action of serine-hydroxymethyltransferase (EC: 2.1.2.1) through the following reaction (Maurino and Peterhansel 2010):

$$2\text{Gly} + NAD^+ + H_2O \rightarrow \text{Ser} + CO_2 + NH_4{}^+ + \text{NADH} + H^+.$$

This ammonium is either recycled in the mitochondria, or is transported to the cytosol and chloroplast, where it is incorporated into 2-oxoglutarate under the catalytic action in the GS/GOGAT cycle (Inokuchi et al. 2002; Pérez-Delgado et al. 2014). Photorespiration is essential for the growth of microalgae and plants, and is favored by low CO_2 and light. The identification of plant-like photosynthetic and photorespiratory genes in cyanobacteria indicated that these genes were endosymbiotically conveyed to the eukaryotic oxygenic phototrophs from the ancient cyanobacteria (Kern et al. 2011).

We performed various studies on the primary assimilation of ammonium (from nitrate reduction) through by the sequential action of glutamine synthetase/glutamate synthase (GS-GOGAT) cycle in *C. reinhardtii* as well as through the putative alternative pathway the amination of 2-oxoglutarate catalyzed by NAD(P)H-glutamate dehydrogenase [NAD(P)H-GDH; (Vega et al. 1991)]. When our studies commenced, the existing knowledge regarding these enzymes in microalgae was scarce. Muro-Pastor et al. (2005) reviewed ammonium assimilation in cyanobacteria, and established that subsequent to its transport through specific permeases, ammonium is incorporated into carbon skeletons through the GS/GOGAT cycle.

The molecular and functional properties of these enzymes are as follows.

2.1 Glutamine Synthetase (GS)

GS (EC 6.3.1.2) is a ubiquitous enzyme present in all the organisms in the form of three different types of proteins: dodecameric GS-I, which is present mostly in prokaryotes, octameric or dodecameric GS-II, which is present in eukaryotes, and the hexameric GS-III, which is also present in prokaryotes (Betti et al. 2012). GS catalyzes the incorporation of ammonium into the carbon skeleton of L-glutamate through the following reaction:

$$\text{Glu} + \text{NH}_4^+ + \text{ATP} \rightarrow \text{Gln} + \text{ADP} + \text{Pi}.$$

Two isoforms of glutamine synthetase that have been isolated from *C. reinhardtii* grow autotrophically in a nutrient medium containing nitrate apparently functioning in the cytosol (GS1) and chloroplasts (GS2) (Florencio and Vega 1983b). This is logical because the source of ammonium may have diverse origins, and the microalgae are required to possess the ability to incorporate it into organic molecules in any region of the cell. On the basis of the occurrence of GS isoforms, microalgae have been classified into three groups: (a) the ones containing only cytosolic GS1; (b) the ones containing only chloroplastic GS2; and (c) a third group characterized by containing both the isoenzymes (Casselton et al. 1986). The green alga *M. braunii* contains only one isoform of GS (García-Fernández et al. 1994), while *Haematococcus pluvialis*, a microalga that is used widely in biotechnology and has high economic potential, contains two distinct isoforms of GS (Reinecke et al. 2016). GS is widely distributed in plants and occurs in two major forms, one (GS2) in the chloroplast and other (GS1) in the cytosol. However, the pattern of distribution of GS in the roots, leaves, and specialized tissues and organs has been observed to be highly sophisticated (Miflin and Habash 2002).

On the other hand, two important metabolic pathways are involved in the intracellular release of ammonium in *L. japonicus*; one is the photorespiratory metabolism, and the other is the asparagine breakdown mediated by asparaginase enzyme. Re-assimilation of the intracellular ammonium is crucial for the normal growth and development of the plant. A photorespiratory mutant of *L. japonicus* that was deficient in plastidic glutamine synthetase (GS2) exhibited ammonium accumulation in the plant, and indicated the existence of a coordinated regulation of genes involved in the photorespiratory metabolism (Betti et al. 2014).

2.2 Ferredoxin-Glutamate Synthase (Fd-GOGAT)

Fd-GOGAT (EC 1.4.7.1) catalyzes the formation of two molecules of L-glutamate, one of which would supply the acceptor for a new ammonium molecule in order to maintain the GS/GOGAT cycle, and the other glutamate molecule may assist in

continuing the nitrogen metabolism in the microalga and plant. The reaction catalyzed by Fd-GOGAT is given below:

$$\text{2-oxoglutarate} + \text{Gln} + 2\ \text{Fd}_{\text{red}} + 2\ \text{H}^{+} \rightarrow 2\ \text{Glu} + 2\text{Fd}_{\text{ox}} + \text{H}_2\text{O}.$$

All the photosynthetic organisms, higher plants, and microalgae contain this enzyme in the chloroplasts of eukaryotic organisms or associated with the photosynthetic machinery in the prokaryotic organisms. An important achievement of our research group was the isolation and purification of two enzymes with glutamate synthase activity from the *C. reinhardtii* extracts, one of which was dependent on reduced ferredoxin as its electron donor, and the other was specific for NADH. We were the first ones to report the purification to the electrophoretic homogeneity of Fd-GOGAT, and to demonstrate through chemical analysis and EPR studies the presence of flavin nucleotides, one FAD, one FMN, and one iron sulfur cluster of the [3Fe-4S] type per enzyme molecule of 146 kDa size (Galván et al. 1984; Márquez et al. 1986b). Other authors have indicated that this enzyme is composed of a single polypeptide chain of size165 kDa which contains FMN and [3Fe-4S] iron-sulfur cluster as its prosthetic groups (Suzuki and Knaff 2005; Varoni et al. 2005). Purified Fd-GOGAT may also catalyze the Fd-dependent reduction of 2-oxoglutarate, in the absence of glutamine (Márquez et al. 1986b), which is compatible with the glutaminase activity and that is the other activity associated with this enzyme complex (van den Heuvel et al. 2004). Fd-GOGAT is present in all the cyanobacteria studied and the enzyme from *Synecchocystis* PCC6803 has been well characterized (Ravasio et al. 2002).

In the cross-linking studies we established that Fd and GOGAT proteins from *C. reinhardtii* were able to form two independent covalent complexes with 1:1 and 2:1 Fd:GOGAT stoichiometry, and that the Glu92 in ferredoxin was involved directly in the stabilization of these complexes (García-Sánchez et al. 1997). Similar data were obtained in the X-ray crystallographic studies of the enzyme, in which both the complexes could be formed depending on the relative proportion of these proteins in the mixture. The E94 residue of *Anabaena* 7120 Fd (=E92 of the *Synechocystis* 6803 Fd) was observed to be necessary for the establishment of functional 1:1 enzyme:substrate complex, as well as for an efficient electron transfer from reduced Fd to GOGAT (Schmitz et al. 1996). With the use of X-ray crystallography, it was identified that the GOGAT enzyme contained an amidotransferase domain, where ammonia was formed through the glutaminase activity associated with this enzyme, thereby producing the first glutamate molecule, following which ammonia was channeled through an intramolecular tunnel to the FMN-binding domain, which was able to bind two molecules of reduced ferredoxin in order to catalyze the reductive amination of 2-oxoglutarate to form the second molecule of glutamate (van den Heuvel et al. 2004).

The ferredoxin-dependent enzymes, glutamate synthase and nitrite reductase, obtained from *C. reinhardtii*, contained a similar structural domain for binding ferredoxin and forming the functional enzyme:substrate complex (García-Sánchez

et al. 2000). The other colleagues identified two important sequence regions in NiR that were apparently similar to those regions in the ferredoxin-NADP reductase that contained amino acids involved in ferredoxin binding; this also suggested the presence of a similar specific domain for binding ferredoxin in these enzymes (Zanetti et al. 1988; Curdt et al. 2000). This appears to be a general characteristic of the ferredoxin-dependent enzymes, such as nitrate reductase from cyanobacteria, sulfite reductase, and thioredoxin-reductase (Hase et al. 2006). Ferredoxins are able to distribute electrons probably originated either from photosynthesis, fermentation, and other reductant-generating pathways, to specific redox enzymes in different organisms. In *C. reinhardtii,* six different ferredoxins have been identified, and the third ferredoxin appears to be involved in nitrogen assimilation (Peden et al. 2013).

2.3 NADH-Glutamate Synthase (NADH-GOGAT)

The NADH-GOGAT (1.4.1.14) consists of a single polypeptide chain (200 kDa) in which two different activities may be measured separately. One is the NADH-diaphorase activity, in which ferricyanide is used as an electron acceptor from NADH, and the other is the reduced methyl viologen-glutamate synthase activity, in which glutamate formation is catalyzed from glutamine and 2-oxoglutarate using reduced methyl viologen as the electron donor (Márquez et al. 1984; van den Heuvel et al. 2004).

This enzyme catalyzes the following reaction:

$$\text{Gln} + \text{2-oxoglutarate} + \text{NADH} + \text{H}^+ \rightarrow 2\ \text{Glu} + \text{NAD}^+ + \text{H}_2\text{O}.$$

In further studies, we suggested that the physiological role of this NADH-GOGAT in *C. reinhardtii* could be the assimilation of ammonium under darkness and/or the recycling of the ammonium released from protein degradation during the low availability of nitrogen (Márquez et al. 1986a). This enzyme is associated with the non-photosynthetic tissues in plants (van den Heuvel et al. 2004). NADH-GOGAT is present simultaneously with Fd-GOGAT in the plants (Miflin and Habash 2002) and eukaryotic microalgae (Márquez et al. 1984). However, this enzyme has been reported only in a few cyanobacteria (Muro-Pastor et al. 2005).

2.4 NAD(P)H-Glutamate Dehydrogenase (NAD(P)H-GDH)

In *Chlorella sorokiniana*, the NADH-GDH enzyme (EC 1.4.1.2) is located in mitochondria, and the NADPH-GDH enzyme (EC 1.4.1.4) is located in chloroplasts (Bascomb and Schmidt 1987). *C. reinhardtii* contains three GDH isoenzymes, one mitochondrial NADH-GOGAT that supports the formation of 2-oxoglutarate in

order to maintain the Krebs's cycle, and the other two NADPH-GDH isoenzymes are presumably distributed in cytosol and chloroplast, which are the main parts of the cells that are putatively involved in glutamate biosynthesis (Moyano et al. 1992). There is a possibility that L-glutamate is also synthesized inside the microalgal glyoxysome, particularly when the cells are grown heterotrophically in a medium containing acetate as a unique carbon source (Pérez-García et al. 2011). Although the deamination of glutamate to form ammonia plus 2-oxoglutarate appears to be the role of NADPH-GDH activity in plants (Forde and Lea 2007) and microalgae, this activity may also be involved in L-glutamate synthesis, particularly when the external concentration of ammonium is high. On the other hand, the alternative ammonium-assimilating GS/GOGAT cycle functions when the ammonium concentration is low. The disadvantage of the second pathway for L-glutamate biosynthesis is its additional energy requirement (Hudson and Daniel 1993).

Under abiotic stresses, such as Cd-toxicity, paraquat, high salinity, and/or high presence of ammonium, in the culture medium of *C. reinhardtii*, the amination activity of the NAD(P)H-GDH isoenzymes for producing L-glutamate manifested, indicating that these isoenzymes have a role in the adaptive mechanisms of the microalga in response to the environmental conditions (Domínguez et al. 2003). In the model legume *Lotus japonicus*, we studied the main genes involved in the assimilatory process for ammonium as well as their levels of expression, using qRT-PCR for these genes in different tissues of the plant (Pérez-Delgado et al. 2014).

Plants and microalgae contain genes for GS/GOGAT cycle and GDH enzymes, which have evolved through different evolutionary processes suggesting that the two pathways will play different physiological role in these organisms.

3 Source of 2-Oxoglutarate for Ammonium Assimilation: Isocitrate Metabolism in Microalgae

Glutamate metabolism functions through transaminase reactions that eventually produce 2-oxoglutarate as the reaction product, which may, in turn, feed the GS-GOGAT cycle, thereby sustaining the rate of glutamate synthesis in the photosynthetic organisms. However, if microalgae or plants demand a higher rate of glutamate biosynthesis, isocitrate dehydrogenase (IDH) isoenzymes may supply the new 2-oxoglutarate molecules by catalyzing the oxidative decarboxylation of isocitrate:

$$\text{Isocitrate} + \text{NAD(P)}^{+} + \text{H}_2\text{O} \rightarrow \text{2-oxoglutarate} + \text{CO}_2 + \text{NADPH} + \text{H}^{+}.$$

According to this reaction, IDH contributes to the modification of intracellular C/N ratio in green cells. There are different types of IDH isoenzymes, which are grouped into two classes on the basis of coenzyme specificity; one of these classes is

the mitochondrial NAD-IDH, which is involved in the Krebs's cycle, and the activity of this isoenzyme is modulated by an extensive allosteric regulation. This regulation, in addition to the presence of a 2-oxoglutarate transporter in the inner membrane of mitochondria, allows NAD-IDH to play an essential role in modulating the carbon flux to nitrogen metabolism in the cytosol and the chloroplasts (Lancien et al. 1998). The other class of IDH isoenzyme is NADP-IDH, which is present in the cytosol, chloroplasts, and peroxisomes, the regions where glutamate biosynthesis would eventually be demanded (Abiko et al. 2005; Leterrier et al. 2016).

We studied the isocitrate metabolism in *C. reinhardtii* to better understand the connection between carbon and nitrogen metabolisms. When the microalga was grown autotrophically in a culture medium containing nitrate, three IDH isoenzymes were identified in it. The first one was mitochondrial NAD-IDH, which was probably involved in the function of Krebs's cycle (Martínez-Rivas and Vega 1998), and two were NADP-IDH isoenzymes, one of which was NADP-IDH1, located in the cytosol and the other was the chloroplastic NADP-IDH2. This distribution of IDHs in the microalga is compatible with the two GS-GOGAT systems operating in the cytosol and chloroplast of *C. reinhardtii* (Florencio and Vega 1983b).

3.1 NAD-Isocitrate Dehydrogenase (NAD-IDH)

The NAD-IDH (EC 1.1.1.14) from *C. reinhardtii* is composed of eight similar subunits of size 45 kDa each (Martínez-Rivas and Vega 1998), while plant enzyme was heteromeric and three subunits existed in tobacco (Lancien et al. 1998). The cytosolic NADP-IDH1 isoenzyme has been purified to homogeneity, and it is a dimer of two similar subunits with an apparent molecular weight of 40 kDa each, and the physicochemical and kinetic properties are similar to the corresponding enzymes in higher plants (Martínez-Rivas et al. 1996).

3.2 NADP-Isocitrate Dehydrogenase (NADP-IDH)

The chloroplastic NADP-IDH2 (EC 1.1.1.42) isoenzyme from *C. reinhardtii* was a dimer of 45 kDa subunits. Antibodies generated against a recombinant tobacco-cytosolic NADP-IDH cross-reacted strongly with the NADP-IDH1, and weakly with the NADP-IDH2 (Martínez-Rivas and Vega 2003). Small changes in the cytosolic NADP-IDH1 activity produce significant alterations in the activities of the enzymes involved in primary nitrate assimilation as well as in the synthesis of 2-oxoglutarate-derived amino acids in tomato plant (Sulpice et al. 2010).

In *Arabidopsis*, a peroxysomal NADP-IDH has been identified; although the role of this IDH in plant metabolism appears to be minor because it is dispensable for the plant growth and redox homeostasis, this enzyme could be involved in plant responses to the environmental challenges such as a biotic stress (Mhamdi and

Noctor 2015). The peroxisome is an organelle involved in the photorespiratory pathway in chloroplast and mitochondria of the C3 plants and microalgae, in which ammonia is released within the mitochondria during the glycine decarboxylation step and is re-assimilated by the chloroplastic/cytosolic GS/GOGAT systems (Hagemann et al. 2016).

Only one type of NADP-IDH has been identified in cyanobacteria, and it is strictly dependent on the divalent cations, such as Mg^{2+} or Mn^{2+}. The enzyme from *Anabaena* PCC 7120 has been purified, and it is composed of two identical subunits (MW = 57 kDa). The corresponding gene *icd* has been cloned, and its sequence presented significant similarity with the sequence of the corresponding gene from *E. coli* (Muro-Pastor and Florencio 1994).

3.3 Isocitrate Lyase (ICL)

When *C. reinhardtii* cells were grown heterotrophically in a culture medium containing acetate, as a unique carbon source, an isocitrate lyase (ICL; 4.1.3.1) activity was induced, while the NADH-IDH activity decreased significantly; this observation was consistent with the functioning of the glyoxylate cycle and with the decreasing level of the Krebs's cycle in the mitochondria (Martínez-Rivas and Vega 2003). ICL catalyzes the following reaction:

$$\text{Isocitrate} \rightarrow \text{Succinate} + \text{Glyoxylate}.$$

This reaction allows glucose biosynthesis from acetyl-CoA and eventually from fatty acids. In general, microalgae growing heterotrophically in acetate induces isocitrate lyase and malate synthetase (EC 2.3.3.9), while light and glucose suppress the formation of isocitrate lyase in the microalgae (Pérez-García et al. 2011).

4 Regulation of Nitrate Assimilation

4.1 Eukaryotic Microalgae

We studied the regulation of nitrate assimilation in *C. reinhardtii* that was grown autotrophically with nitrate as unique N-source, and we contribute the following highlights:

(a) The first step in the regulation of this pathway is performed with the entrance of nitrate into the cells. Although ammonium is a powerful antagonist of nitrate uptake, it must be assimilated by the microalga to exhibit its negative regulatory effect (Florencio and Vega 1983b). The entrance of nitrate into the cells promotes the synthesis of nitrate reductase activity (Florencio and Vega 1982). The

activity of the high-affinity nitrate transport system I (NRT2;1, NAR2) is responsible for the efficient signaling of the nitrate assimilation genes in *C. reinhardtii* (Rexach et al. 2002). The availability of a carbon source and a healthy photosynthetic process modulates the nitrate consumption rate in the microalga.

(b) NAD(P)H-nitrate reductase is an adaptive enzyme in *C. reinhardtii*, which exhibits the highest level of its activity in the cells growing with nitrate in the culture medium, while the activity decreases dramatically in the absence of nitrate, and/or in the presence of ammonium (Florencio and Vega 1983b). Our data obtained from microalgae indicate the existence of an in vivo short-term nitrate reductase activity regulation (Vega et al. 1972; Guerrero et al. 1981).

Nitrate serves both as a nutrient and as a positive signal to activate the nitrate assimilation pathway in *C. reinhardtii*, while nitric oxide (NO) serves as a negative signal by inhibiting the expression of the high-affinity nitrate/nitrite transporters and the nitrate reductase activity (Sanz-Luque et al. 2015). NIT2 is the key transcription factor for the nitrate-dependent activation of several genes for the handling of nitrate assimilation in the microalga. It is important to mention that nitrate can be in vivo transformed into nitric oxide, in a process shared with the molybdoenzyme ARC; nitric oxide may then be converted into nitrate, in a process mediated by nitrate reductase and truncated hemoglobin THB1 (Calatrava et al. 2017).

Nitric oxide signaling regulates various physiological processes in both animals and plants. In animals, NO synthesis is catalyzed by the NO-synthase enzyme (NOS). NOS-like sequences have been identified in the genome of 15 among the 265 algal species analyzed in a study (Jeandroz et al. 2016). On the other hand, *C. reinhardtii* is apparently deficient in such sequences.

In *C. reinhardtii,* the level of cytosolic, but not chloroplastic, GS transcript is repressed by ammonium and induced by nitrate. In *Chlorella sorokiniana* seven ammonia-inducible NADP-GDH chloroplastic isoenzymes have been identified (Inokuchi et al. 2002).

4.2 Cyanobacteria

The regulation of inorganic nitrogen assimilation by the cyanobacteria has been reviewed extensively (Flores et al. 2005; Muro-Pastor et al. 2005; Ohashi et al. 2011). Our contribution in this field has been limited. We reported that ferredoxin-nitrite reductase from the cyanobacterium *Anabaena* sp. 7119 was an adaptive enzyme, the synthesis of which was nutritionally repressed by the presence of ammonium in the culture medium (Méndez et al. 1981).

Nitrate assimilation genes are commonly observed to form an operon with the structure *nir* [nitrite reductase-permease gene(s)-*narB* (nitrate reductase)] which is transcribed from a complex promoter that includes binding sites for NtcA, a global nitrogen-control regulator, and sites for NtcB, a pathway-specific regulator (Flores

et al. 2005). The major intracellular signal for the regulation of nitrate assimilation in cyanobacteria is 2-oxoglutarate (2-OG), the intracellular concentration of which changes with the cellular C/N balance. In addition, Pii, a signal transduction protein, inhibits the ABC-type nitrate transporter and nitrate reductase, under low level of 2-OG. Pii was recently shown to negatively regulate the activity of NtcA by binding to PipX, a small coactivator protein of NtcA (Ohashi et al. 2011).

In N-limited cells, the intracellular accumulation of 2-OG activates the transcription factor NtcA for inducing the transcription of the nitrate assimilation genes, and the expression of the genes encoding GS and IDH. In addition, the 2-OG binds to Pii and prevents the protein from inhibiting nitrate assimilation. In all the cyanobacteria that have been studied, the presence of ammonium in the culture medium immediately promoted the inhibition of nitrate uptake, as well as the repression of the transcription of the operon *nirAnrtABCDnarB*. However, these effects require the assimilation of ammonium, thus indicating that it is not an effect of ammonium per se (Flores et al. 2005).

5 Metabolism of L-Asparagine in *Lotus japonicus*

In legumes, L-asparagine or ureides (allantoin and allantoic acid) are crucial nitrogen-storing molecules and transporters between the organs and tissues, due to their high N/C ratio and stability. In the model legume *L. japonicus*, asparagine supports the vast majority of reduced nitrogen stored and translocated between different organs and tissues (Betti et al. 2014; García-Calderón et al. 2017), and the intracellular concentration of asparagine is dependent on a balance between its biosynthesis and degradation. The main enzymes involved in the asparagine metabolism are asparagine synthetase, asparaginase, and serine-glyoxylate aminotransferase.

5.1 Asparagine Synthetase (ASN)

This enzyme (EC 6.3.5.4) converts aspartate plus glutamine into asparagine plus glutamate, in an ATP-dependent reaction, which is given below:

$$\mathrm{Asp} + \mathrm{Gln} + \mathrm{ATP} + \mathrm{H_2O} \rightarrow \mathrm{Asn} + \mathrm{Glu} + \mathrm{AMP} + \mathrm{PP_i}.$$

ASN is widely distributed in all the living organisms, particularly in humans where it has been observed to be overexpressed in the cancer cells, such as the acute lymphoblastic leukemia. Conversely, ASN deficiency is associated with neurological disorders, such as intellectual disability, microcephaly, and progressive brain atrophy (Lomelino et al. 2017).