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PERSPECTIVE

Location of the photosynthetic carbon metabolism in microcompartments and separated phases in microalgal cells

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Carbon acquisition, assimilation and storage in eukaryotic microalgae and cyanobacteria occur in multiple compartments that have been characterised by the location of the enzymes involved in these functions. These compartments can be delimited by bilayer membranes, such as the chloroplast, the lumen, the peroxisome, the mitochondria or monolayer membranes, such as lipid droplets or plasmoglobules. They can also originate from liquid–liquid phase separation such as the pyrenoid. Multiple exchanges exist between the intracellular microcompartments, and these are reviewed for the CO₂ concentration mechanism, the Calvin–Benson–Bassham cycle, the lipid metabolism and the cellular energetic balance. Progress in microscopy and spectroscopic methods opens new perspectives to characterise the molecular consequences of the location of the proteins involved, including intrinsically disordered proteins.

Keywords: bioenergetics; Calvin–Benson–Bassham cycle; *Chlamydomonas reinhardtii*; cyanobacterium; diatom; intrinsically disordered protein; liquid–liquid phase separation; nuclear magnetic resonance; photosynthesis; structural biology

Photosynthesis is an essential metabolic pathway that allows autotrophic organisms to mobilise inorganic carbon and produce carbohydrates and fatty acids that

are further used by other metabolic pathways. These pathways are differentially localised in compartments that have distinct physicochemical properties, which

Abbreviations

ALA, α -linolenic acid; ARA, arachidonic acid; ATP S.ase, ATP synthase; ATP, adenosine triphosphate; BPGA, bisphosphoglyceric acid; CA, carbonic anhydrase; Carb., carbamylated; CBB, Calvin–Benson–Bassham; CCM, CO₂ concentration mechanism; CEF, cyclic electron flux; DAG, diacylglycerol; DES, deep eutectic solvents; DGAT, diacylglycerol acyltransferase; DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceryltrimethylhomo-Ser; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EPYC1, essential pyrenoid component 1; ER, endoplasmic reticulum; FA, fatty acid; FAS, fatty acid synthase; FBPase, fructose-1,6 bisphosphatase; Fdx, ferredoxin; FNR, ferredoxin NADP⁺ reductase; FRAP, fluorescence recovery after photobleaching; GAP, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IDP, intrinsically disordered protein; LD, lipid droplet; LEF, linear electron flux; LLPS, separated liquid–liquid phases; MGDG, monogalactosyldiacylglycerol; MLDP, major LD protein; NaDES, natural DES; NADPH, nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; NTR, N-terminal repeat regions; OPP, oxidative pentose phosphates; PC, phosphatidylcholine; PDB, protein data bank; PG, phosphatidylglycerol; PGA, phosphoglyceric acid; PGD1, plastid galactoglycerolipid degradation 1; PGK, phosphoglycerate kinase; PRI, ribose-5-phosphate isomerase; PRK, phosphoribulokinase; PSI, photosystem I; PSII, photosystem II; PTM, post-translational modifications; PUFA, polyunsaturated fatty acids; Ri5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; RuBisCO, Ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5 bisphosphate; SAXS, small-angle X-ray scattering; SBPase, sedoheptulose-1,7 bisphosphatase; SQDG, sulfoquinovosyldiacylglycerol; SSUL, RuBisCO small subunit like domain; TAG, triacylglycerol; TPI, triose phosphate isomerase; Trx, thioredoxin.

can affect the physiology. The complete photosynthetic carbon metabolism is complex, and only three main stages will be discussed here: The first stage is the

acquisition of inorganic carbon and its accumulation at the location of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO; Fig. 1). In the

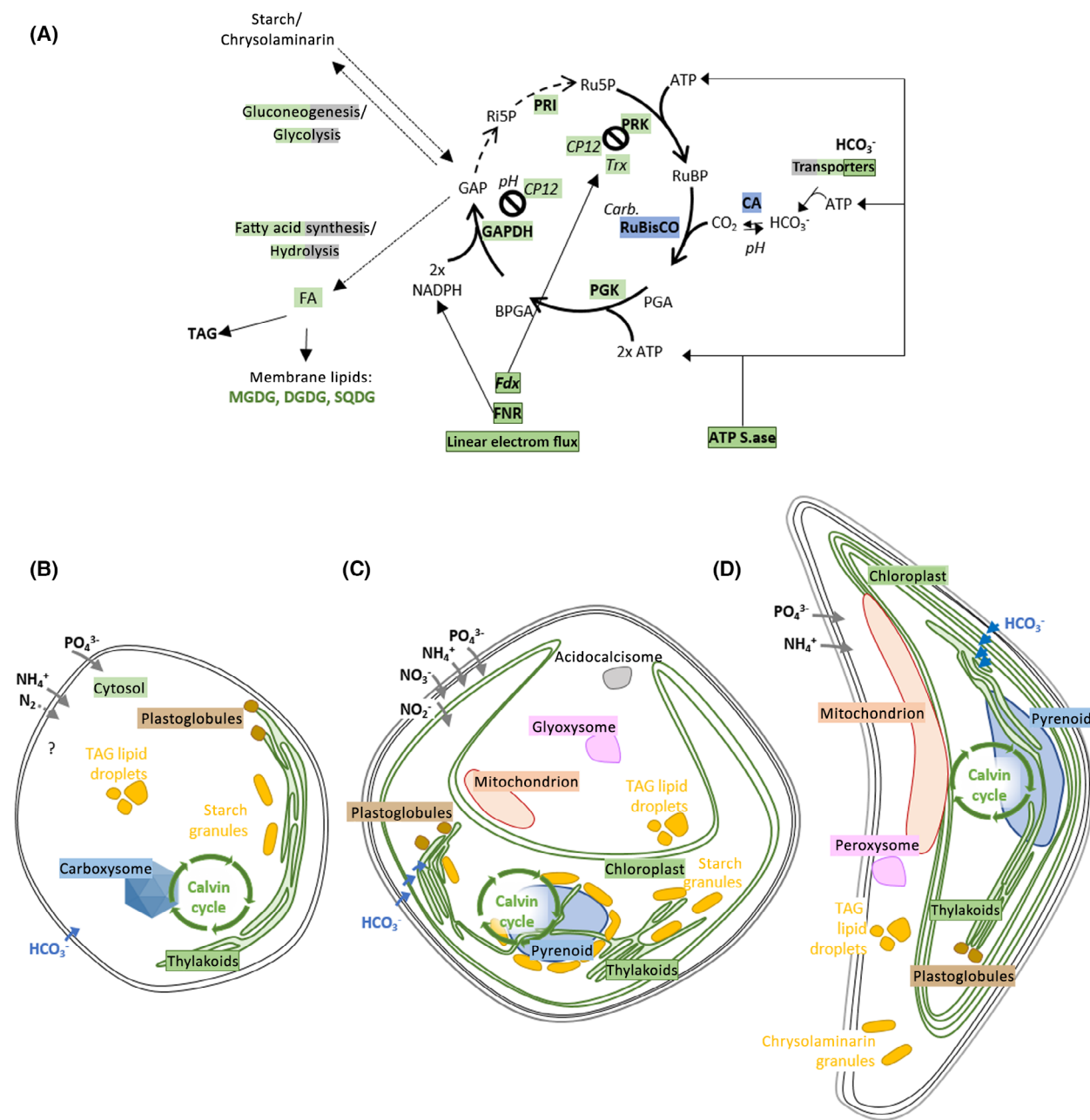


Fig. 1. Schematic of the carbon metabolism pathways that are discussed in this Review (A) together with schemes of the ultrastructures of a cyanobacterial cell (B), of *C. reinhardtii* (C) and of *P. tricornutum* (D). The location of some enzymes has been determined experimentally (refer to the main text) and the colour of the rectangle surrounding their name indicates their localisation: blue: pyrenoid or carboxysome; light green: cytosol of cyanobacteria or chloroplast of eukaryotic algae; dark green: thylakoids; grey: cytosol of eukaryotic algae. ATP S.ase, ATP synthase; ATP, adenosine triphosphate; BPGA, bisphosphoglyceric acid; CA, carbonic anhydrase; Carb., carbamylated RuBisCO; DGDG, digalactosyldiacylglycerol; FA, fatty acid; Fdx, ferredoxin; FNR, ferredoxin NADP⁺ reductase; GAP, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MGDG, monogalactosyldiacylglycerol; NADPH, nicotinamide adenine dinucleotide phosphate; PGA, phosphoglyceric acid; PGK, phosphoglycerate kinase; PRK, phosphoribulokinase; Ri5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; RuBP, ribulose-1,5 bisphosphate; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol; Trx, thioredoxin.

second stage, RuBisCO fixes CO₂ with a carboxylation step and produces two molecules of phosphoglyceric acid (PGA), a three-carbon molecule that is further converted into reduced carbohydrates in the Calvin–Benson–Bassham (CBB) pathway. The CBB pathway relies on the availability of a reducing power and adenosine triphosphate (ATP) generated by the photochemical phase of photosynthesis. Finally, the third stage is the synthesis of the carbon storage compounds in the form of either carbohydrates or fatty acids further esterified into membrane and storage lipids (triacylglycerol lipids). Each of these three stages is finely tuned according to light intensity, availability of the inorganic carbon source or environmental stresses (nutrient shortage, temperature, salinity, drought, pressure, etc.).

The molecular mechanisms underlying the regulation of carbon acquisition, assimilation and storage pathways are well-described in the literature. These involve physicochemical transitions such as changes in pH, redox potential, metabolites and metal ion concentrations within the cellular compartments where photosynthesis occurs [1–4], post-translational modifications [5–7], structural transitions of key enzymes [8–10] and reorganisations within supramolecular complexes [11–14]. Recent high-resolution cryo-electron microscopy images and tomograms from the cyanobacterium *Synechocystis 6803* [15], the green alga *Chlamydomonas reinhardtii* [16–18] and the diatom *Phaeodactylum tricorutum* [19] enabled the cellular localisation of some metabolic pathways. The location of enzymes within the cells (Fig. 1B) can be correlated with the molecular description of the biochemical pathways (Fig. 1A) thanks to progress in biophysical methods such as microscopy, mass spectrometry, several spectroscopic methods such as fluorescence spectroscopy and nuclear magnetic resonance (NMR) and machine-learning-assisted-data interpretation [16,20,21].

Here, we will focus on unicellular photosynthetic organisms, where all stages of the carbon cycle occur in a single cell, unlike most higher plants. In multicellular organisms, cell specialisation can allow to separate physiological functions on the macroscopic level. In eukaryotic unicellular photosynthetic organisms, different organelles participate in the compartmentalisation of some metabolic pathways. This partitioning helps prevent futile cycles of antagonist metabolic pathways that share the same metabolites. For example, the oxidative pentose phosphate (OPP) cycle in diatoms is localised in the cytoplasm, and its antagonist pathway, the CBB, also named reductive pentose phosphate cycle, is localised in the chloroplast [22]. In eukaryotic microalgae, inorganic carbon is imported

and assimilated in the chloroplasts (Fig. 1B). Carbon storage is then ensured by the synthesis of polysaccharides that accumulate in the form of starch granules in the chloroplast of green algae, of starch-like polymers in cyanobacteria [23], or of chrysolaminarin granules in the vacuoles of diatoms [24,25]. Alternatively, carbon can be stored through the synthesis of triacylglycerols (TAG) that assemble in lipid droplets (LD) [26]. Even though these carbon metabolic pathways are separated in different spaces within the cell, they are interconnected by exchange of metabolites between the different partitions and across their boundaries.

The physical–chemical nature of these cell compartments varies. For example, LDs encapsulate an organic liquid phase [27], whereas polysaccharide granules are composed of amorphous and semicrystalline layers of polysaccharides [27–29]. Traditionally, the term ‘organelle’ refers to large aqueous cellular compartments surrounded by a bilayer membrane such as the chloroplast, the vacuole or the mitochondrion. The term organelle has been also proposed to name LDs that are smaller organic partitions surrounded by a monolayer membrane [27,30]. Organelles can be themselves divided in different regions of space that we chose to name microcompartments. For example, polysaccharide granules that localise within the chloroplast of green microalgae or within the vacuole of diatoms can be referred to as a microcompartment. One can also refer to the self-assembled granules of polysaccharide as ‘biomolecular condensates’. Indeed, the term ‘condensation’ has been used to denominate the spontaneous clustering of biomolecules that result in a high local concentration surrounded by a dilute phase and the partitioning of the cell [31]. Different types of biomolecules can spontaneously cluster in biomolecular condensates, including ribonucleic acid, polysaccharides, proteins or lipids [32–34]. The pyrenoid matrix is a well-described example of protein biomolecular condensate that is described in details in [Pyrenoid and carboxysome](#) section. Because biomolecular condensates do not possess membranes, they are also referred to as membrane-less organelles [32–34]. The physicochemical properties of these biomolecular condensates are the focus of intense scrutiny.

Microcompartments can be observed in microscopy images of prokaryotic unicellular organisms, such as carboxysomes, in nonheterocystous cyanobacteria, where inorganic carbon and RuBisCO are condensed [35]. The thylakoid membranes can also be considered as a microcompartment because they define a new region of space in the chloroplast where the photochemical phase of photosynthesis produces ATP and NADPH. Their associated plastoglobules are another

example of partition, the function of which is multiple [36]. Plastoglobules encapsulate a hydrophobic core with varying compounds that change according to culture conditions, surrounded by a monolayer membrane [37]. They have been proposed to provide storage for thylakoid components, and to be involved in insoluble isoprenoid synthesis or degradation, and in stress response, as well as in the storage of compounds that can be toxic for the cell such as terpenes [36,38].

This perspective review aims to discuss the organisation of carbon metabolism in different microcompartments and at different scales within the cell. This includes the molecular description of the regulatory processes with the physical separation of their key actors, as well as the formation of supramolecular assemblies/biocondensates leading to the segregation of metabolites and enzymes.

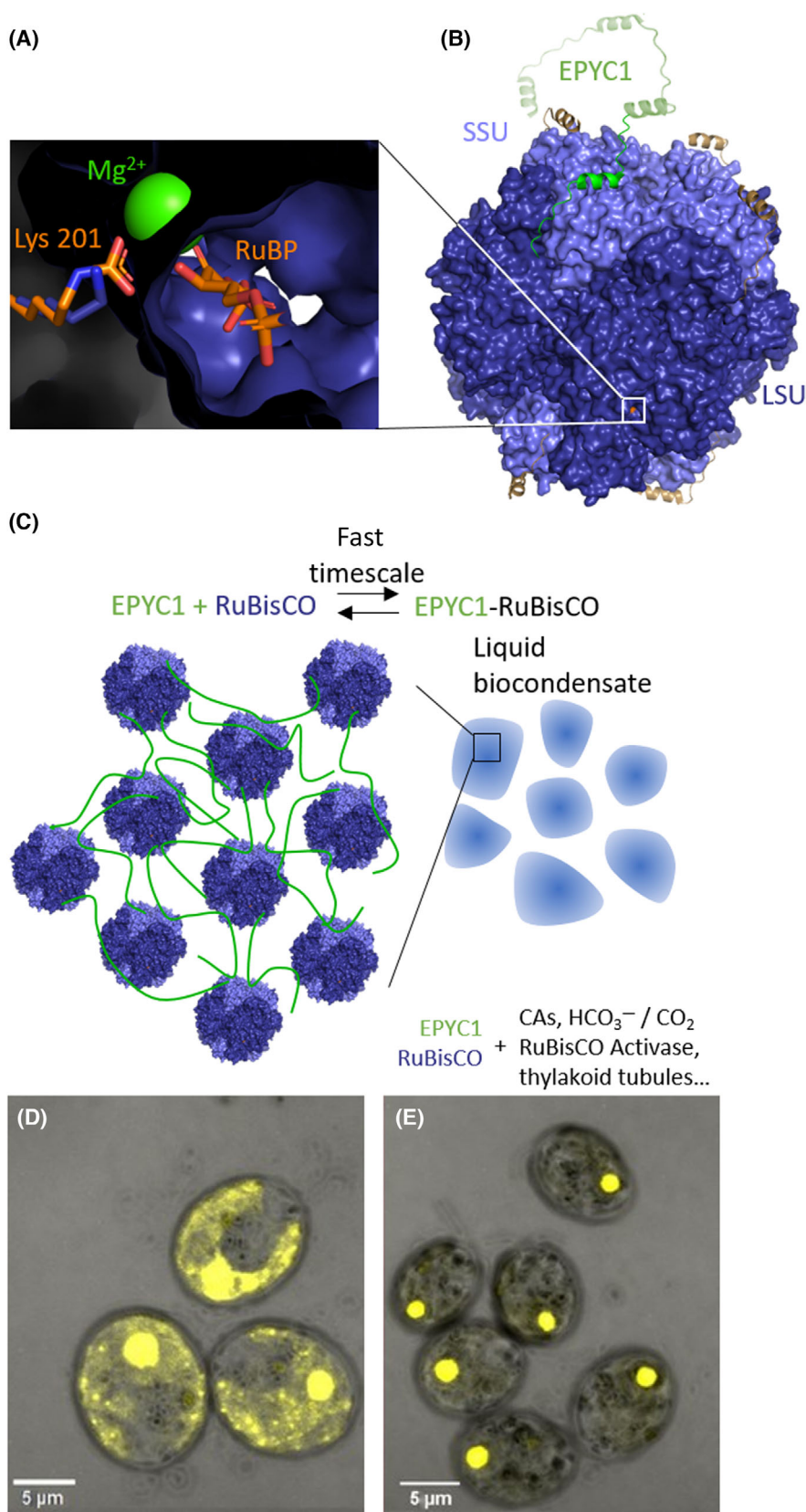
Illustration from the emblematic case of RuBisCO

The study of RuBisCO, which is a complex made up of large (L) and small (S) subunits (Fig. 2A), is emblematic of the structure–function relationships in a complex [39]. Since the first X-ray structure of the active RuBisCO in 1989 [40], more than 240 structures from all the photosynthetic lineage have been deposited in the Protein Data Bank (PDB). The mainly described oligomeric state of the enzyme is the L₈S₈ hexadecameric form, but other oligomeric states exist [41]. The regulation of the enzyme, notably by pH, a Mg²⁺ cation and an ‘activating’ nonsubstrate CO₂ molecule via the carbamylation of Lysine 201 in the active site of the large subunit is well-understood [7] (numbering from *C. reinhardtii*, Fig. 2A). Binding of RuBP to noncarbamylated RuBisCO results in an inhibited state. This state is resolved by interaction with a RuBisCO activase or possibly by CBB X protein (CbbX) in diatoms [42,43]. The ‘structure–function’ correlation of this model enzyme is thus well-established. Since more than 30 years, its localisation in an ultrastructure has been observed on microscopy images: in the pyrenoid in eukaryotic microalgae

or in the carboxysomes in cyanobacteria [35,44–46]. More precisely, in *C. reinhardtii* and under atmospheric CO₂ (~400 ppm) and O₂ concentrations, RuBisCO is present both in the chloroplast stroma and in the pyrenoid microcompartment (Fig. 2D). Under CO₂ concentrations lower than atmospheric (<200 ppm) or high O₂ conditions, RuBisCO relocates from the stroma to the pyrenoid (Fig. 2E) [18,47,48]. Only very recently, the physicochemical nature of the pyrenoid has been described. This biomolecular condensate is detailed in the following section [32,49–52]. Also, recent metabolic flux models provide insights into the consequence of this ‘segregation’ or ‘condensation’ of RuBisCO within these demixing condensates as regard to the CBB metabolic flux [18].

From the example of RuBisCO, one can now extend the ‘structure–function’ relationships from quaternary structure (oligomeric enzyme complex) to an additional level of supramolecular assembly that could be named ‘ultrastructure–function’ relationships. Such an organisation raises new questions. What is the significance of the heterogeneity within a cell, in other words ‘roughness’, observed in microscopy images [53,54]? What are the physicochemical properties of the various microcompartments? Some are mesoscopic macromolecular complexes such as carboxysomes or starch granules. Some are separated liquid phases that demix because of the presence of different water-insoluble compounds such as lipids. Some are separated liquid phases that demix because of condensation of protein constituents through multivalent transient intermolecular interactions such as the pyrenoid [33,34,55]. While the phase separation of lipids appears obvious, why do carbohydrates (starch) and proteins (pyrenoid) also phase separate? These microcompartments cannot be considered as strictly confined zones, as metabolites diffuse in and out. How is their formation/disassembly regulated? How do proteins fold in these different microcompartments? All these questions can be raised for each stage of the carbon cycle of microalgae, but this is also true for all metabolic pathways in all types of cells.

Fig. 2. RuBisCO regulation at the molecular and supramolecular levels. (A) Structure of the active site of RuBisCO (PDB ID: 1IR2). (B) Hexadecameric L₈S₈ RuBisCO bound to eight Essential Pyrenoid Component 1 (EPYC1) proteins, obtained by cocrystallisation with saturating concentrations of synthetic peptides shown in brown (PDB IDs: 7JSX and 7JFO). In addition, one full-length EPYC1 protein is artistically depicted in green. (C) Schematic of the molecular contacts between EPYC1 and RuBisCO underlying the phase separation. (D, E) Confocal microscopy images of *C. reinhardtii* showing Venus-labelled RuBisCO that indicating the location of the hexadecameric complex. (D) Location of RuBisCO in both the chloroplast stroma and the pyrenoid at atmospheric CO₂ concentration with 5 mM bicarbonate. (E) Hyperoxia phenotype and the relocation of RuBisCO induced by 100 μM H₂O₂ to the culture. Panels D and E are adapted from [48]. The same relocation is observed under low CO₂ conditions without hyperoxia.



Ideally, to grasp the above-defined ‘ultrastructure-function’ relationships, one would like to be able to describe all the ultrastructural elements observed on electron microscopy images at the molecular level and provide a rationale on their role for the regulation of the metabolic pathways they are hosting. Most of the recent technological developments that allowed deciphering some of these aspects on the carbon metabolism of microalgae will be mentioned.

Pyrenoid and carboxysome

As mentioned above, the most emblematic enzyme for CO₂ fixation is RuBisCO, which is responsible for the conversion of 10¹⁴ kilogrammes of carbon each year [56]. Surprisingly, the maximum catalytic activity for the carboxylation of RuBP by RuBisCO is achieved at significantly high CO₂ concentrations (ranging from 25 to 100 μM). These concentrations are higher than the atmospheric CO₂ concentration (c.a. 15 μM in the oceans) [57,58]. The pH of the compartment where RuBisCO is localised, such as the chloroplast stroma or the cyanobacteria cytosol (pH 7.9), suggests that the major form of inorganic carbon is bicarbonate. Considering these biochemical data, it can be concluded that RuBisCO is not working at its maximum rate and that the carboxylation reaction is in competition with the oxygenation of RuBP. The latter produces an inhibitor of the CBB cycle, 2-phosphoglycolate, that must be metabolised in the C2 respiratory pathway. This apparent paradox may be resolved when the ultrastructure of the chloroplast is taken into consideration. The chloroplast membranes, including the thylakoids membranes, are permeable to CO₂, and bicarbonate transporters catalyse the import of HCO₃⁻ into the stroma (Fig. 1B). In each compartment, carbonic anhydrases catalyse the HCO₃⁻/CO₂ conversion so that their pH-dependent ratio is instantly achieved. Besides, the equilibrium across multiple microcompartments that have different pH—the thylakoids lumen (pH 6) versus the chloroplast stroma and the pyrenoid (pH 7.9)—drives the import of inorganic carbon to the pyrenoid [57]. The ‘passive’ transport of inorganic carbon through the different microcompartments can support RuBisCO carboxylation activity in the pyrenoid under atmospheric CO₂ conditions, while additional HCO₃⁻ transporters enhance inorganic carbon import under low CO₂ [59]. Finally, the condensation of RuBisCO together with CAs in the carboxysome (cyanobacteria) or the pyrenoid (eukaryotic microalgae) increases the availability of CO₂ in the vicinity of the enzyme, which enables its activity. The pyrenoid is often surrounded by a discontinuous layer of starch granules that could contribute to the confinement of

inorganic carbon that results in a higher CO₂/O₂ ratio compared with the stroma and that supports carboxylation at the expense of oxygenation reaction [47]. Under specific conditions (hyperoxia), the pyrenoid has no starch sheath and presents a large interface with the chloroplast stroma [48]. The existence of such interface will be discussed later.

For decades, it was enigmatic how the colocalisation of RuBisCO and CAs was mediated, even though the functions of the carboxysome and pyrenoid were known [35,44,45]. RuBisCO was copurified in cyanobacterial β-carboxysome as a supramolecular complex with small putative shell proteins (CcmM) that are composed of three RuBisCO small subunit-like domains (SSUL) and one isoform that possesses a CA domain [60]. Reconstitution of the RuBisCO–CcmM complexes *in vitro* induced a liquid–liquid phase separation: One phase is a biomolecular condensate of RuBisCO–CcmM and the other phase is free of proteins [61,62]. In a nutshell, multivalent interactions between the hexadecameric RuBisCO (L₈S₈) and the SSUL domains result in the condensation of the two protein complexes when they are mixed in the appropriate stoichiometry (0.25 : 1 molar ratio). The reversible demixing of these two separated liquid–liquid phases (LLPS) can be observed using fluorescent-tagged proteins and fluorescence microscopy. Demixing does not occur under high salt concentrations that interfere with electrostatic intermolecular interactions. Also excess of CcmM prevented the condensation of the separated liquid phase, indicating that stoichiometry is essential [61]. The fluidic (liquid) nature of the protein condensate was confirmed by dual-colour fluorescence cross-correlation spectroscopy and fluorescence recovery after photobleaching (FRAP) that allowed to quantify the short live-time of the RuBisCO–CcmM contacts.

Similarly, the α-carboxysome biogenesis involves multivalent interactions of the hexadecameric RuBisCO with four N-terminal repeat regions (NTR) of a large (~ 900 residues) intrinsically disordered protein (IDP) named CsoS2. Demixing into two liquid–liquid phases was observed when RuBisCO and CsoS2–NTR are present in the appropriate stoichiometry (1 : 1 molar ratio) [63]. The dynamics that are characteristic for the LLPS biocondensates only have been studied by *in vitro* studies of carboxysomes biogenesis. This biphasic state is not conserved in the final mesoscopic RuBisCO–CA supramolecular complex, that is surrounded by shell proteins. This suggests that the transient interactions are fixed upon the recruitment of other partners of the final aggregates that acquire microcrystalline properties [64].

Unlike carboxysomes, the pyrenoid has no protein-shell. It has been a challenge to isolate this membrane-less microcompartment in order to identify its molecular constituents. It is not a microcrystalline structure and therefore it loses its integrity upon purification steps. The constituents of the pyrenoid were identified by a combination of genetic screening with the creation of a library of GFP-mutant chloroplast proteins, microscopy and proteomic screening [65]. An essential protein for the formation of the pyrenoid is the Essential Pyrenoid Component 1 (EPYC1) that has no enzymatic activity [66]. Like CsoS2, EPYC1 (in *C. reinhardtii*) is an IDP composed of five repeat domains having a small RuBisCO subunit binding site (Fig. 2B). If EPYC1 and hexadecameric RuBisCO are present in a defined stoichiometry (2 : 1 molar ratio), demixing into two phases occurs in a process similar to that of the RuBisCO : CcmM or the RuBisCO : CsoS2 biomolecular condensates [49,51]. As for the RuBisCO–CcmM biocondensates, the demixing is prevented by high salt concentration or by excess of EPYC1 or RuBisCO, indicating that the multivalent electrostatic interactions are essential for the condensation. Similarly, FRAP revealed the very transient EPYC1 : RuBisCO contacts, which is also a hallmark for LLPS and the formation of biocondensates [51] (Fig. 2C).

EPYC1, CsoS2 or CcmM act as scaffolds, ‘smooth mortar’ or ‘stickers’ to confine RuBisCO in a biomolecular condensate due to their multivalent binding sites with this enzyme. This condensate can maintain a high degree of freedom—liquid properties—because the intermolecular interactions are transient. Other factors can contribute to the rigidification as it is the case for the mature carboxysomes. On the contrary, in the cells, the pyrenoid retains a high degree of freedom that characterises liquid droplets. The absence of a stable three-dimensional structure in CsoS2 and EPYC provides an extra level of flexibility to the scaffold. Intrinsically disordered proteins are described as essential in the responsiveness to stimuli, and their roles in the regulatory network also have been demonstrated for the regulation of the carbon metabolism [11]. Their structural flexibility enables fast re-arrangement upon exposition to different conditions, such that they can act as sensors. For example, the intrinsically disordered region of the MAPK phosphatase AP2C3 of *Arabidopsis thaliana* was proposed to be the CO₂ sensor that regulates the phosphatase activity in response to increasing CO₂ concentration [67]. The molecular description of AP2C3 revealed that the IDP fosters the condensation of LLPS droplets at 10 000 ppm CO₂,

whilst the proteins were scattered or dispersed at atmospheric CO₂ concentrations (400 ppm). To our knowledge, the catalytic properties of RuBisCO in the pyrenoid compared with that free in solution is not known but new properties might emerge in this more complex environment. How the condensation of protein-dense phases might contribute to the catalytic efficiency will be discussed at the end of this perspective paper. Intrinsically disordered proteins are also prone to have post-translational modifications (PTMs) as response to signalling pathways, and these PTMs can trigger dissociation of LLPS as it is the case for histone containing LLPS [68]. Altogether, IDPs are considered as key regulators for the formation/dissassembly of LLPS [69].

Intrinsically disordered proteins are also often described as ‘hub’ proteins, a property observed for several scaffold proteins in liquid biomolecular condensates. The full molecular properties of EPYC or CsoS2 in the condensates are not yet described. Only transient contacts between the scaffold protein and RuBisCO have been described by using fusion proteins with small polypeptides (Fig. 2B) [49,63]. It is expected that the entire proteins will remain highly flexible like other IDPs scaffolds for LLPS biocondensates. Several of them have been investigated at the residue-scale using NMR such as FUS [70], hnRNPA2 [71], CAPRIN1 [72,73], HP1 α [74], tau [75,76], Ntail [77] and TCP42 [78] (for global review, refer to [70]). Nuclear magnetic resonance can indeed complement the fluorescence-based methods that have mostly been employed to characterise the RuBisCO-containing LLPS. The NMR chemical shift reflects the structural properties of the protein at the residue-specific scale (are they in folded regions or disordered?). Putative conformational dynamic timescales can be probed using a range of NMR experiments (ns– μ s using spin relaxation experiments, 100 μ s–100 ms using relaxation dispersion or off-field saturation experiments) and the short-range and long-range transient contacts can be probed using nuclear Overhauser effect spectroscopy or paramagnetic relaxation enhancement experiments. A review of the recent NMR method developments and their use in structural biology can be found in Thielliet and Luchinat, Prog. in NMR Spec. (2022) [79]. In addition, recent developments of pulsed-field gradient NMR spectroscopy (or diffusion-ordered spectroscopy) can be used to discriminate the localisation of the proteins within the different phases [73,76]. Nuclear magnetic resonance is thus a unique method that can combine structural and physicochemical information in complex environments such as LLPS.

Regulation of the RuBisCO substrate regeneration

The positive effect of pyrenoid or carboxysome formation for the availability of CO₂ in the vicinity of the RuBisCO active site has been modelled [18,57], but their effect for the availability of the sugar substrate, RuBP, within these microcompartments is puzzling. In the higher plant *Nicotiana tabacum*, the RuBP carboxylation by RuBisCO is not the rate limiting step for CO₂ assimilation, but the RuBP regeneration by the CBB enzymes is limiting [80], and one could assume that it is the same in green microalgae. The last step of RuBP regeneration is performed by the phosphoribulokinase (PRK) that catalyses the phosphorylation of ribulose-5-phosphate with ATP. Decrease of up to 80% of the amount of PRK did not affect CO₂ assimilation in *Nicotiana tabacum* and *C. reinhardtii*, indicating that the rate limiting step is not the last step of the RuBP regeneration [81,82]. Genetic engineering of a fluorescent-tagged protein library in *C. reinhardtii* allowed to localise PRK in the chloroplast stroma around the pyrenoid (Fig. 3A) [16,18], which raises the question of how the PRK product, RuBP, crosses the pyrenoid boundaries. Similarly, phosphoglycerate kinase (PGK) that catalyses the phosphorylation of RuBisCO product PGA into bisphosphoglycerate is also localised in the chloroplast stroma, such that the PGA has to migrate out of the pyrenoid. The bisphosphoglycerate is in turn reduced by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that shares the same stromal localisation (Fig. 3A). It is known that substrate and cofactor availability is a key parameter in the regulation of the enzymes in general and of the CBB pathway in particular [1,83]: PRK and PGK phosphorylate their substrate by using ATP, and the reducing power of GAPDH is provided by NADPH. The availability of ATP and NADPH, both products of the photochemical stage that occurs on thylakoid membranes, is discussed below. In the cyanobacterium *Synechocystis pcc6803*, several CBB enzymes were localised by immunogold labelling and electron microscopy in proximity with the thylakoid membranes: 72% of the PRK particles, together with 66% of PGK and GAPDH and 47% of the observed RuBisCO particles [15].

Regulation of PRK is another emblematic case for the structure–function relationships. Since 2019, the crystal structure of the active PRK dimer is available as well as that of inactive PRK in supramolecular complexes with GAPDH and the small regulatory protein CP12 [9,84–86] (Fig. 4B). CP12 colocalises with PRK and GAPDH in the chloroplast stroma at the

periphery of the pyrenoid [16]. Activity assays of purified PRK revealed that it is regulated by its redox state: The reduction in inactive oxidised PRK is catalysed by thioredoxin f or m [87–89]. Phosphoribulokinase is thus a target of the ferredoxin–thioredoxin network as several other CBB enzymes [5]. The nature of the spatial organisation of the thylakoid membranes discussed below could be considered in this regulation. The molecular description of thioredoxin-mediated regulation of PRK was deciphered by a combination of mutagenesis, activity assays and crystallographic structures and can be summarised as follows. In its reduced state, the active site is localised in a groove that harbours Arg64 (numbering from the model *C. reinhardtii*) that can coordinate Ru5P and Cys16 that binds ATP (Fig. 4A) [9,90]. Upon oxidation, the disulphide-bond formation between Cys16 and Cys55 reorganises the ATP binding site (Fig. 4B). When oxidative conditions prevail, PRK has a high affinity for the GAPDH-CP12 subcomplex [91,92]. The N-terminal hairpin of CP12 that harbours the disulphide-bond Cys23–Cys31 occupies the active site groove of PRK [84,85]. The formation of the ternary complex is mediated by the redox-dependent structural transition of CP12 that we have described using NMR and small-angle X-ray scattering (SAXS) experiments: It is intrinsically disordered in reducing conditions and becomes partially ordered in oxidising conditions [12,93]. This protein is thus conditionally disordered and is a hub for the ternary complex association. The weak electron density observed for CP12 in the cryo-electron microscopy of the GAPDH-CP12-PRK complex suggests that it retains a high degree of flexibility within the ternary complex [84]. Interestingly, reduction and re-activation of PRK occurs faster when it is sequestered within the ternary complex compared with free oxidised PRK, and this is an example of how supramolecular organisation modifies the regulation [14,88,92,94]. Also, in the complex, PRK and GAPDH are protected from oxidative damage [95]. This large assembly also recruits another CBB enzyme: the aldolase, conferring new properties to this enzyme when embedded in this supramolecular edifice [96].

The recent molecular description of the GAPDH-CP12-PRK complex can be confronted to 30-year-old reports of copurification of active proteins (Table 1) [12]. For example in spinach, GAPDH and PRK were shown to associate with phosphoribose isomerase (PRI), RuBisCO, sedoheptulose-1,7-bisphosphatase and ferredoxin-NADP⁺ reductase close to the thylakoid membranes [97]. Other studies revealed the association of at least PRI, PRK and RuBisCO in spinach [13], rice [98], tobacco [99], all

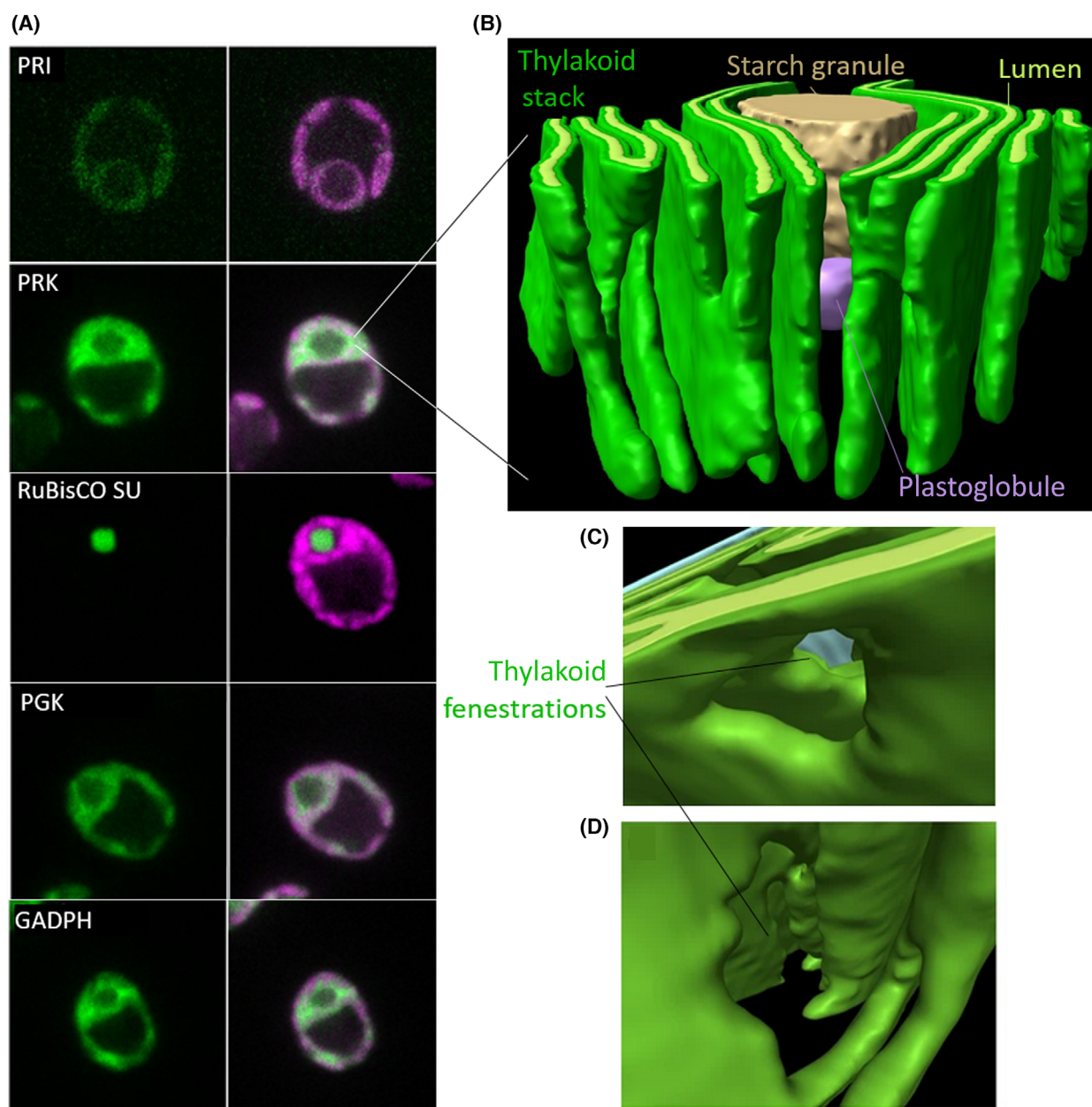


Fig. 3. (A) Location of different enzymes of the CBB cycle revealed by fluorescence microscopy using Venus-fusion constructs shown ingreen. The autofluorescence of chlorophyll is shown in purple. Figure adapted from [18]. (B) 3D organisation of the thylakoids stacks that shows the three continuous phases: the lumen (light green), the bilayer membrane (dark green) and the stroma. (C, D) Show fenestrations through the thylakoids that enable communications between different stroma compartments delimited by the thylakoids. Figure adapted from [17].

higher plants that are devoid of pyrenoids. The colocalisation of successive enzymes for the RuBP regeneration is a strategy to increase metabolite turnover [100]. In *C. reinhardtii* and other microalgae, however, PRI and PRK were not observed in the pyrenoid or carboxysomes. Nonetheless, RuBisCO is not sequestered in the pyrenoid or carboxysome under atmospheric or

high CO₂ conditions and therefore could be available for such supramolecular complexes (Fig. 2D) [15,48]. Despite the tremendous efforts that enable to solve the structure of homomeric complexes for most enzymes of the CBB cycle [1], no other supramolecular complexes except GAPDH-CP12-PRK or the transient complex of RuBisCO-EPYC1 have been reconstituted

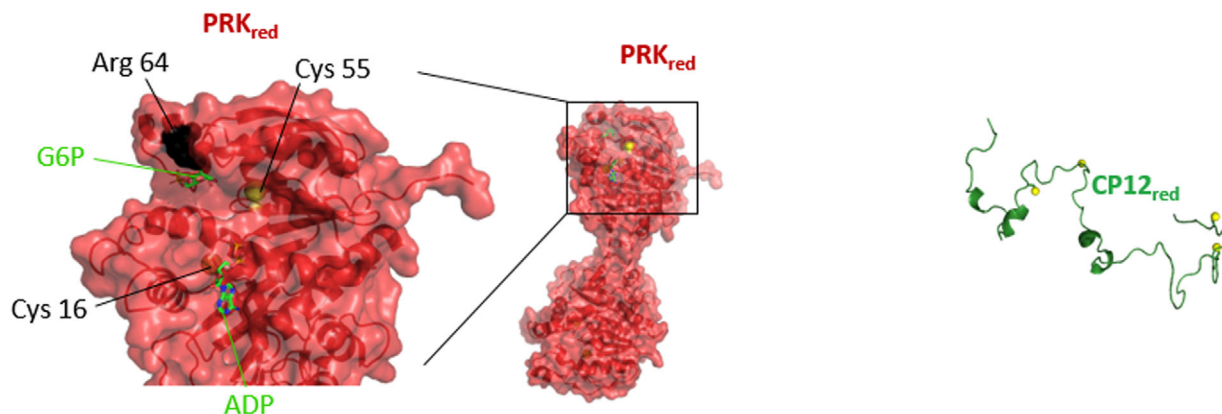
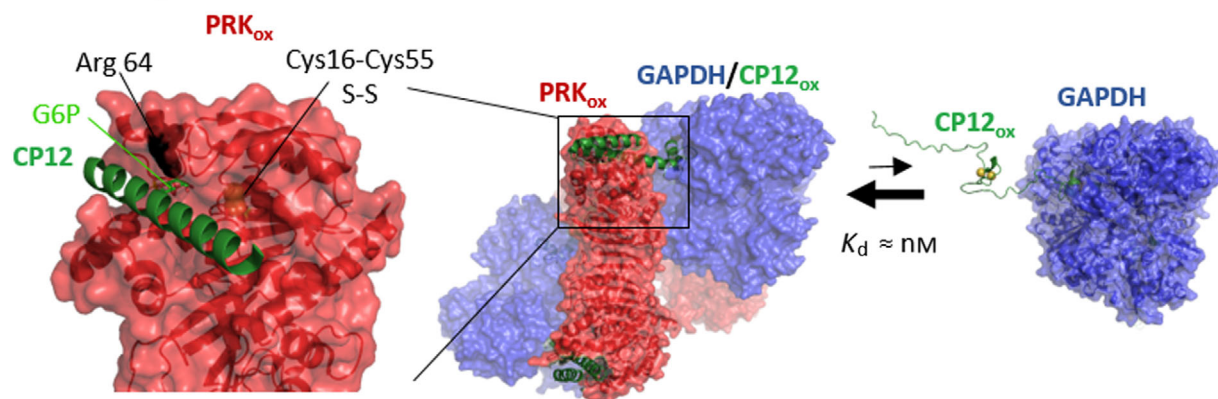
(A) Light, $E'_0 < -350$ mV(B) Dark, $E'_0 > -350$ mV

Fig. 4. Regulation of PRK from the molecular to the supramolecular level: from left to right are shown the structure of the active site of PRK from *C. reinhardtii* in reducing conditions (PDB ID: 6H7G) and from *A. thaliana* in oxidising conditions (PDB ID: 6KEZ). The positions of the ATP/ADP and substrate are modelled thanks to alignment with the structure of *S. elongatus* PRK (PDB ID: 6KEV). In this structure PRK is crystallised with glucose-6-phosphate that can occupy the active site at the position of Ru5P. The Arg64 position is indicated. (A) In the light, reducing conditions prevails in the chloroplast stroma as it is indicated by the low Nernst potential (E'_0). CP12 is intrinsically disordered [204] and the regulatory PRK disulphide Cys16-Cys55 bond is disrupted. (B) In the dark, oxidative conditions prevails in the chloroplast stroma as it is indicated by the higher Nernst potential. CP12 is partially folded, has a high affinity for GAPDH and the GAPDH-CP12 sub-complex in turn has a high affinity for PRK [205].

and studied *in vitro* (let aside the carboxysomes). Both of these complexes involve a hub or scaffold IDP, that has no enzymatic function: EPYC1 or CP12. The identification of CP12 was enabled decades ago thanks to its homology with the C-terminal extension of the B-isoform of GAPDH of higher plants [101]. The RuBisCO-EPYC1 interactions are transient and would not support copurification by classic chromatographic strategies, and it has thus escaped knowledge until recent proteomic studies [66]. The recent advances of mass spectrometry coupled with cross-linking agents could be in future at the origin of the identification of transient protein-protein interactions and of other

protein hubs that may mediate the association of other CBB enzymes in supramolecular complexes [102].

ATP and reducing power generation

The acquisition of one CO₂ molecule (i.e. its transport to the vicinity of RuBisCO in the pyrenoid) consumes 1.3 ATP molecules [57], and its assimilation by the CBB cycle consumes two NADPH and three ATP molecules [1]. Their primary generation occurs in the thylakoid membranes via the proton gradient and the linear electron flux (LEF) that are generated on the photochemical phase of photosynthesis. This

Table 1. Supramolecular complexes containing CBB enzymes, the organism in which they have been observed and the related reference that had reported these complexes. CA, carbonic anhydrase; FBpase, fructose-1,6 bisphosphatase; FNR, ferredoxin NADP⁺ reductase; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; PGK, phosphoglycerate kinase; PRI, ribose-5-phosphate isomerase; PRK, phosphoribulokinase; RuBisCO, ribulose-1,5 bisphosphate carboxylase/oxygenase; SBpase, sedoheptulose-1,7 bisphosphatase; TPI, triose phosphate isomerase.

| Protein content | Organism | References |
|---|------------------------------|-----------------|
| CA/PRK/RuBisCO/PGK | <i>Pisum sativum</i> | [188] |
| PRI/PRK/RuBisCO | <i>P. sativum</i> | [189] |
| PRI/PRK/RuBisCO/PGK | <i>P. sativum</i> | [190] |
| PRI/PRK/RuBisCO/PGK/GAPDH | <i>Spinacia oleracea</i> | [13] |
| | <i>Anacystis nidulans</i> | [191] |
| PRI/PRK/RuBisCO/PGH/GAPDH/ATP synthase | <i>Synechocystis 6803</i> | [15] |
| RuBisCO/PGK/GAPDH/TPI/aldolase/FBpase | <i>S. oleracea</i> | [192] |
| PRI/PRK/RuBisCO/PGH/GAPDH/SBpase/FNR/ATP synthase | <i>S. oleracea</i> | [193] |
| RuBisCO/PGK/GAPDH/TPI/aldolase/FBpase | <i>S. oleracea</i> | [192] |
| GAPDH/TPI/aldolase/SBpase | <i>P. sativum</i> | [190] |
| GAPDH/CP12/PRK | <i>S. oleracea</i> | [101, 194, 195] |
| | <i>P. sativum</i> | [194, 195] |
| | <i>A. thaliana</i> | [196] |
| | <i>Galderia sulfuraria</i> | [197] |
| | <i>Synechocystis 6803</i> | [198, 199] |
| | <i>Synechococcus PCC7942</i> | [200] |
| | <i>C. reinhardtii</i> | [199, 201, 202] |
| GAPDH/CP12/FNR complex | <i>Asterionella formosa</i> | [203] |

photochemical process produces two NADPH and 2.6 ATP molecules, and the ATP : NADPH ratio is modulated by the Mehler reaction, the flavodiiron proteins and the cyclic electron flux (CEF) though their quantitative contribution is difficult to assess [103,104]. Malate valves that are discussed in the next section are also involved [105]. Before expending on the energetic balance across organelles in the case of eukaryotic algae, the supramolecular organisation of the thylakoid membranes (that is shared by prokaryote and eukaryote algae) is discussed. The thylakoids are bilayer membranes of amphiphilic lipids that adopt a well-organised structure that encapsulates a single internal aqueous phase that is the lumen, where protons accumulate from water splitting at the interface of photosystem II (PSII). This proton gradient force provides the energy for the ATP synthase to regenerate ATP. The electrons released from water splitting are transported by the quinone pool to the cytochrome *b₆/f* and the photosystem I (PSI), and finally to ferredoxin and ferredoxin NADP⁺ reductase (FNR). This LEF occurs between spatially separated photosystems: PSII accumulates in thylakoids stacks or grana that are multilayer packing of thylakoids, Cyt *b₆/f* is more evenly distributed, whereas PSI accumulates in unstacked thylakoids lamellae together with ATP synthase [106]. It should be noted that in *C. reinhardtii*, the proportion of stacked over unstacked thylakoid membranes is much lower than that in higher plant, and the above-mentioned distribution can be balanced

[17,107]. Continuous progress of tomographic and cryo-electron microscopy will improve to decipher the localisation of the photosystem constituents [17]. The organic interface of the bilayer membranes and the lumen are continuous phases all through the chloroplast, such that there is a unique boundary between the lumen and the stroma and a unique thermodynamic balance between the two aqueous phases. The thylakoid lamellae where NADPH and ATP are regenerated separate the chloroplast stroma in a series of microcompartments; in other words, they create boundaries within the chloroplast stroma [53]. How do NADPH and ATP distribute through the chloroplast stroma under these circumstances? The recent electron microscopy tomograms of *C. reinhardtii* cells revealed that the thylakoids are pierced by fenestrations (Fig. 3B) that enable the different microcompartments of the stroma to communicate. As a consequence, the stroma is also a continuous tangled phase in which proteins and metabolites can diffuse. The heterogeneity and complex 3D boundaries render molecular motions highly complex in the stroma, and these effects on protein and metabolites are discussed in the last paragraph of the manuscript.

Biosynthesis of thylakoid lipids and their supramolecular assembly

The thylakoid membranes should not be seen as rigid boundary, their assembly in bilayer membranes is

dictated by phase separation and this is highly dependent on temperature stress [108,109]. Furthermore, several molecular mechanisms involved in membrane-remodelling have been discovered, and the role of the lipid polar head and fatty acyl chain composition will be discussed below [110–113]. Membrane-associated protein can also play a role in membrane remodelling. For example, the molecular mechanism by which the protomers of IM30 protein (for Inner Membrane protein of 30 kDa) self-assemble to thylakoid membranes has been described using a range of biophysical techniques such as atomic-force spectroscopy, SAXS and modelling [114]. Their association with the thylakoids results in the formation of a protective carpet and their intrinsically disordered C-terminal tail unfolds and promotes pore forming. The role of IDP in membrane protection and remodelling is another contribution to the ‘ultrastructure’-function relationships.

The polar lipids present in the thylakoid membranes and the inner-chloroplast membrane of eukaryotic microalgae as well as those of cyanobacteria are mainly monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG) and sulfoquinovosyldiacylglycerols (SQDG) and a small proportion of phosphatidylglycerol (PG). These represent 70–80% of the total lipids of eukaryotic microalgae that also possess other phospholipids in the extrachloroplastic membranes [115]. In the most studied microalga *C. reinhardtii*, betaine lipids such as diacylglyceryltrimethylhomo-Ser (DGTS) can act as substitute of phosphatidylcholine (PC). The acyl chains of galactolipids from eukaryotic microalgae and cyanobacteria usually differ from those of higher plants by their enrichment in polyunsaturated fatty acids (PUFA) such as α -linolenic acid (ALA, 18 : 3), the main fatty acid in *C. reinhardtii*, arachidonic acid (ARA; 20 : 4 n-6) and eicosapentaenoic acid (EPA; 20 : 5 n-3) found in *Nannochloropsis gaditana* [116] and *P. tricornutum* [117], docosahexaenoic acid (DHA; 22 : 6 n-3) found in *Schizochytrium limacinum* [118] or hexadecatetraenoic acid (16 : 4 n-3) found in *C. reinhardtii* [115]. Interestingly, lipid biosynthesis in eukaryotic photosynthetic microalgae results from molecular exchanges occurring between different cellular compartments [115,119]. A major part of fatty acid biosynthesis occurs in the stroma of the chloroplast through the action of the fatty acid synthase (FAS) complex on acetyl-CoA as the starting unit further converted in elongated acyl chains by sequential condensation of two-carbon units. Then, acyl chains bound to acyl carrier protein can either be incorporated into acylglycerolipids inside the chloroplast via the so-called ‘prokaryotic’ pathway or be exported outside the chloroplast to reach

the ‘eukaryotic’ pathway in the endoplasmic reticulum (ER). Finally, their incorporation in acyl lipids is performed by various acyl transferases. Approximately 40% of fatty acids synthesised in chloroplasts enter the prokaryotic pathway, whereas 60% are exported to the eukaryotic pathway. About half of the exported fatty acids returns to the plastid after their desaturation in the ER and is used for the synthesis of the thylakoid membrane galactolipids by MGDG and DGDG synthases [120]. Desaturation of fatty acids is tightly linked with the location of the diacylglycerolipids in which they are incorporated and their transfer between the chloroplast envelope and the ER. These fatty acids result from a combination of elongation and desaturation reactions taking place either in the ER where the $\Delta 6$ - or $\Delta 5$ -the desaturase are located [121,122], or in the chloroplast where the $\Delta 4$ desaturase Cr $\Delta 4$ FAD [123] acts on MGDG to generate the 16 : 4 $\Delta 4,7,10,13$ PUFA, a predominant component of MGDG molecular species.

The polar head and fatty acid compositions of the acylglycerol lipids determine their supramolecular organisation [110–112]. While DGDGs are known to form lamellar phases ($L\alpha$) and induce bilayer formation, MGDGs tend to form inverted hexagonal phase (H_{II}) in aqueous solution [112]. Because of MGDG physicochemical properties, the formation of photosynthetic membrane bilayer *in vivo* raises questions about the role of this lipid in the organisation and functional properties of the chloroplast membranes [124]. It has been suggested that MGDG, which has a conical molecular shape due to its small head group, may help pack large protein complexes into biological membranes through lipid–protein interactions [113]. The molar ratio of MGDG over DGDG is known to adapt to stress conditions and to modulate the photosynthesis efficiency [125]. Conversely, fatty acid desaturation depends on the photosynthetic activity and the molecular composition of galactolipids varies according to the growth conditions. The high content of PUFAs in galactolipids contributes to the fluidity of membranes [109]. Lipidomics has provided a rich database of the lipid composition of photosynthetic membranes [126]. Critical physical and thermodynamic properties such as bilayer thickness, 2D motions of lipids, packing of the acyl chains, surface charge distribution and thylakoid membrane packing have been characterised using reconstituted galactolipid membranes and a range of methods such as microscopy, differential scanning calorimetry, solid-state NMR, fluorescence anisotropy, small-angle neutron and X-ray scattering, atomic force spectroscopy and molecular dynamics (reviewed here [109]).

The phase separations possibly occurring in these membranes have also been investigated by tensiometry,

ellipsometry and Langmuir–Blodgett transfer coupled to atomic force microscopy using biomimetic Langmuir monolayers [127]. Enzymes (lipases) interacting with monolayers were found to be preferentially adsorbed at the expanded/fluid lipid phases but compounds such as phytosterols and phospholipids inducing phase heterogeneity also favoured the adsorption of enzymes at the phase boundaries and towards the defects in condensed phases. Thus, the lipid composition of photosynthetic membranes affects both the structure of integral membrane proteins from photosystems and the binding of peripheral proteins.

Other chloroplast microcompartments

Another feature of the chloroplast lipids is that they constitute the boundaries of plastoglobules [54]. The function of these organic phase droplets encompassed by the external thylakoid membrane protuberance is still a matter of debate. It has been proposed that they serve as a reservoir for the maintenance of the protein/galactolipid ratio of the thylakoids, or that their organic phase enables the storage of organic metabolites produced in the chloroplast [36]. In *C. reinhardtii*, recent cryomicroscopy tomograms suggest that their proximity with the thylakoid stack is not obvious [17]. They share some similarities with the eyespots, which are also LDs that locate at the periphery of the chloroplast towards the light. The molecular composition of the plastoglobules and the eyespot is not clear, except that they are phase separated from the chloroplast stroma. They contain mainly prenylquinones and carotenoids and lower amounts of TAGs [128,129]. A protocol for their isolation has been proposed in 2022 that may lift a hurdle for their molecular characterisation [130].

One interesting perspective would be to consider the fact that many metabolites produced in the chloroplast in large quantities form natural deep eutectic solvents (NaDES). These mixtures characterised by a fusion point lower than those of their isolated components are liquid at physiological temperature and they present peculiar solvation properties [131–133]. This is the case for instance for Glucose : Fructose, Fructose : Sucrose, Glucose : Sucrose, Sucrose : Glucose : Fructose at molar ratio [133]. *In vitro* reconstitution of these mixtures studied by NMR showed that in the fluid phase, intermolecular transient contacts act as in liquid crystal to partially order the molecule orientation whilst offering a high fluidity. Chemists have described the good solvation properties of synthetic DES and NaDES for poorly soluble molecules [134,135].

Carbon and energy storage: starch granules and lipid droplets

In the chloroplast, the main carbon storage components are large polysaccharide condensates in the form of a highly organised semicrystalline fraction: the starch or chrysolaminarin granules. These are ordered polymers of polysaccharide where glucose subunits are predominantly linked by α -(1 → 4)-D glycosidic bonds with α -(1 → 6) branches for starch and by β (1 → 3) and β (1 → 6) branches in chrysolaminarin. The starch granules result from a complex organisation of semicrystalline and amorphous concentric layers surrounding an amorphous hilum, and different models have been proposed for their assemblies [27–29]. Contrary to glycogen that is water-soluble and occurs as nanoparticles of limited diameter, the starch/chrysolaminarin molecules can have in principle unlimited size [136]. In the green lineage, starch biogenesis is located in the chloroplast, whereas in the red lineage such as in diatoms, chrysolaminarin synthesis occurs in the vacuole, another membrane-bound microcompartment of the cytosol [137]. The understanding of gluconeogenesis or glycolysis is mainly achieved via genetic studies [138], and recent biophysical tools are being used to explore the molecular structure of this large ordered semicrystalline condensate [139].

The gluconeogenesis vs glycolysis equilibrium is dependent on the availability of the energy source, primarily ATP and NAD(P)H, and the several regulation pathways involve exchange through the several microcompartments within the stroma discussed above, but also exchange in and out of the chloroplast in eukaryotic algae. This will be discussed in the next section.

Another form of carbon storage compartment much studied in microalgae is the LD. This is the most effective form of energy storage with 9 kcal·g⁻¹ for fatty acid and derived acylglycerolipids against 4 kcal·g⁻¹ for carbohydrates. The most common type of LD presents a hydrophobic core made of TAG and surrounded by a lipoproteic monolayer. *Chlamydomonas reinhardtii* LDs contain 95% TAGs, 1–5% polar lipids and 1–5% proteins [140]. The polar lipid monolayer surrounding the LDs contains a specific set of polar lipids including DGTS, SQDG, DGDG and MGDG with relative amounts depending on culture conditions (light level, nitrogen depletion) [141]. Also at the monolayer, LD proteomics has revealed the presence of enzymes involved in several subcellular mechanisms, including lipid synthesis, degradation, trafficking, signalling and lipid homeostasis. For example, in *C. reinhardtii*, LD proteomics has shown that around 30 proteins involved in lipid metabolism were present,

including the betaine lipid synthase (BTA1), a lysophosphatidic acid acyltransferase (LPAT), a putative long chain acyl-CoA synthetase (LACS), a diacylglycerol acyltransferase (DGAT), a major LD protein (MLDP) of unknown function, the phosphatidylethanolamine-binding DTH1 (DELAYED IN TAG HYDROLYSIS1), two lipases and two enzymes involved in fatty acid β -oxidation [141–143]. This has led to the classification of LDs as new cellular organelles [30].

Triacylglycerol biogenesis regulation is complex, and often triggered by stress conditions such as heat or nutrients depletion (e.g. nitrogen) [26,126]. In *C. reinhardtii*, TAG biosynthesis is thought to mainly occur in the cytosol through the same enzymatic steps as in plants [144], and it requires the transfer to the cytosol of the acyl chains initially synthesised in the chloroplast. Galactolipids also appear as the main backbone in which *de novo*-synthesised fatty acids are incorporated before they enter into TAG synthesis. In *C. reinhardtii*, a galactolipase, named plastid galactoglycerolipid degradation 1 (PGD1), was identified as a key player in lipid remodelling following nitrogen deprivation. The galactolipase activity of PGD1 allows the flux of fatty acids from plastid lipids to TAGs in which they are re-esterified to form LDs [145]. It remains however unclear whether the final step of TAG synthesis from diacylglycerol (DAG) also occurs inside the chloroplast. Indeed, some TAG-synthesising enzymes such as DGAT1 and phosphoglycerol acyltransferase (PDAT) were found in the chloroplast [146]. Also, data from microscopy studies have showed that some LDs can be present inside the chloroplast, as well as in the cytosol in close association with the chloroplast envelope [146,147]. In the chloroplast, LD are referred to as plastoglobules, and we have seen above in [ATP and reducing power generation](#) section, that these contain TAG. Lipid droplet synthesis can yet be another function of these enigmatic microcompartments. We have also seen previously in [ATP and reducing power generation](#) section, that polar lipids from membranes can serve as a source of recycled acyl chains for TAG synthesis, besides *de novo* fatty acid synthesis.

The study of LD biogenesis and composition is typically investigated using timely resolved culture sampling and lipid extraction combined with genetic modification [126]. Nevertheless, these techniques require cellular disruption before isolation of LDs and lipid extraction. These steps can generate side reactions such as lipid hydrolysis/degradation due to their mixing with lipolytic enzymes initially present in other cell compartments, and binding to LDs of amphiphilic proteins that are normally not interacting with LDs.

There is therefore a need for other methods allowing the identification and structural characterisation of lipids and proteins within the cell. One common approach is the expression of green fluorescent protein (GFP)-fused protein to localise the protein using fluorescence confocal microscopy and confirm whether it is effectively bound to LDs *in cellula*. Regarding lipids, an emerging methodology using NMR allowed to investigate the accumulation of TAG in the microalga *N. gaditana* [148]. This nondestructive approach allows real-time analysis of lipids in the living algae, without the need of lipid extraction and separation. As discussed above for protein-NMR, NMR can provide information on the chemistry of lipids such as TAG, but also on the physicochemical properties of their supramolecular organisation. For example, the dynamics of LD formation (size changes) in *P. tricornutum*, as well as the lipid-motion within the cells, were monitored by pulse field gradient nuclear magnetic resonance [149].

The two main forms of carbon storage are antagonistic and correlated. In *C. reinhardtii*, as in other species, fatty acid degradation takes place in the peroxisome, that are small organelles in the cytosol, via β -oxidation [150]. The generated acetyl-CoA can be further converted to carbohydrates serving as building blocks or cellular energy through respiration in the mitochondria. This pathway requires a close connexion between LDs and peroxisome to enable the transfer of the fatty acid released from LDs' TAG by lipases. Also, this pathway is linked to the global energetic balance within the cell that implies exchange between organelles.

Exchange of metabolites across the boundaries of organelles

We discussed above the apparent energy unbalance between the production by the LEF (ATP/NADPH ratio of 1.3) and the consumption by CO₂ uptake and the CBB cycle (ATP/NADPH ratio of 2.6). Many other metabolic pathways have been described that modulate the ATP/NADPH ratio, which will not be described here (for review refer to [105]). In eukaryotic algae, the energetic balance occurs between the chloroplast, the mitochondria and the cytosol. Organelle membranes are impermeable to NAD(P)H, and various transporters have been identified together with malate and oxaloacetate transporters [151]. Malate conversion to oxaloacetate by malate dehydrogenase generates reducing power; thus, it is established that the malate/oxaloacetate shuttle through the different cellular compartments is a key factor for their

energetic balance. Malate dehydrogenases are located in the chloroplast, in peroxisome (also named glyoxysomes in *C. reinhardtii* [152]), in the cytoplasm and in the mitochondria. Various other transporters also contribute to the maintenance of the metabolic flux, such as inorganic phosphate, ammonium and nitrate/nitrite transporters [153]. Bicarbonate transporters have been discussed above in **Pyrenoid and carboxysome** section. Also concerning the carbon flux, several sugar transporters are present with specialised functions. For example, the triose phosphate transporter 3 has been described as being essential for the metabolic balance and the export of photosynthetic fixed carbon in green algae [154].

The case of diatoms is more complex because of their evolutionary origin, their chloroplast is surrounded by four membranes. Metabolic exchange between the mitochondria and the chloroplast involves direct exchange between the two organelles through their membranes [19,155]. Membranes of intra- and extracellular vesicles have also been identified as shuttles for transferring information, function and metabolites from cellular compartments and the endosomal trafficking system to other cells and tissues. Still concerning lipids, LDs have been shown to interact with other organelles such as the peroxisomes, the nucleus, the mitochondria and involved in metabolite trafficking [140,150].

Metabolic exchange across organelles is also an example of how the existence of microcompartment modulates the metabolism. To fully understand the energetic balance within the cell, one would like to be able to follow the fate of a metabolite from its synthesis to its conversion. Whilst this challenge remains unrealistic at the single molecule level, progress in fluxomic methods provides precious data to improve the current models [156,157]. Fluxomic or 'flux-metabolomics' enable to characterise the metabolic pathway downstream of an isotopically labelled metabolite using mass spectrometry or NMR. Mass spectrometry enables the characterisation of a high number of metabolites on a wide concentration range and thus provides a huge metabolic flux database [158]. Nuclear magnetic resonance has suffered for some time from a lower sensitivity, but it provides the advantage of being nondestructive, as opposed to mass spectrometry, and is not dependent on extraction using different solvents [159,160]. As discussed above for the on-flow monitoring of TAG accumulation, *in-situ* NMR can also be used to monitor the fate of specific metabolites [161–163].

The boundaries of the different microcompartments are where metabolite exchange occurs at their

interfaces. These boundaries are usually biological membranes where receptors and transporters are present in the form of integral membrane proteins, as well as peripheral proteins that bind to the membrane upon fulfilling their task as molecular shuttle or enzymes. In LDs, the monolayer surrounding the hydrophobic core also contains functional enzymes and structural proteins, such as MLDP in *C. reinhardtii*, that control the biogenesis and fate of these organelles. Such interfaces have been extensively studied in the case of LDs/emulsions dispersed in an aqueous phase [164–167]. The concepts of phase separation, interfacial tension, adsorption and partitioning occurring at the lipid-water interface are well-defined. Interfacial tension, or surface pressure, for instance, has an impact on protein and metabolite adsorption and penetration at the interface, enzyme activity, as well as on protein folding and stability [168,169]. The surface of polysaccharide granules also contains starch granule-associated proteins that are often starch synthetic enzymes but can have other functions such as modulating the overall properties of the semicrystalline phase [170,171]. The membrane-less organelles formed by LLPS create a novel type of interface between distinct phases. Intracellular phase separation differs however from demixing of oil and water, because the compounds involved in LLPS (proteins, nucleic acids, amino acids, sugars and other small metabolites) are water soluble and the condensates they form are hydrated. The interface delimiting two aqueous phases formed by LLPS should also be considered. Some proteins and other compounds are specifically localised at this interface, where they might impact physicochemical properties (surface tension and surface potential). For example, Starch Granules Abnormal 1 and 2 proteins localise in the pyrenoid in small puncta and are responsible for the association of starch granules at the periphery of the LLPS [172]. Deletion of this protein results in the modification of the volume/surface ratio of the LLPS and induces the formation of several pyrenoid condensates. Strikingly, hyperoxia is also a condition where no starch granules surround pyrenoid, but the number of pyrenoid fraction is not modified [48]. There is limited understanding of the quantitative laws governing solute partitioning into LLPS and adsorption at their interface. Experimental evidence of solute/metabolite partitioning between microcompartments and membranes is also missing [18]. In that context, diffusion-ordered NMR spectroscopy and estimation of diffusion coefficient can provide information on molecules simultaneously present in distinct phases within the cell [73,76,149,173].

Molecular consequences of the composition of the microcompartments

As discussed above, within a photosynthetic cell, many microcompartments derived from phase separation are currently being described at the physicochemical level: the pyrenoid, the thylakoids bilayer membranes that separate two phases: the lumen and the stroma, the LDs, the semicrystalline starch or chrysolaminarin granules, and others that remain more enigmatic such as the plastoglobules or the eyespots. For long, biochemists considered only two phases in cells: the aqueous phase of cytoplasm and organelles and the hydrophobic environment formed by membrane mono or bilayers. The gradual recognition of subcellular environments with distinct or nonaqueous phase has drastically changed the representation of the cell and its functional compartments. Intracellular LD for instance became considered as organelles and not just energy storage aggregates when proteomics revealed the presence of specific proteins and enzymes, and therefore specific functions, on their surface [30]. The more recent identification of intracellular and intraorganellar LLPS mediated by protein–protein or protein–ribonucleic acid transient contacts opened new perspectives. The import of CO₂ is a telling example. CO₂ solubility is poor in water and suffers from hydration equilibrium that is pH dependent. On the contrary, CO₂ solubility increases in organic phases where ionisation and pH are not relevant. The pyrenoid is an aqueous phase, but dense protein packing could modulate CO₂ solvation properties. Indeed, in the *A. thaliana* AP2C3 LLPS, CO₂ molecules contribute to the molecular packing at the origin of the condensation [67]. Metabolite solubility across these different phases is very likely variable [174]. Chemists have been using phase transfer catalysis for decades. Could phase-transfer catalysis also contribute to regulate the carbon metabolism within microalgae? What are the respective solubilities of CO₂, sugar phosphate, glyceric acid, carbohydrate or fatty acids in the different microcompartments mentioned above?

One can also consider the effect of these different phases on the actors of the carbon cycle: the proteins. Most structures of globular enzymes have been solved when the proteins were in crystal phase, and this most likely represents their in-cell overall structure. Nevertheless, the high molecular crowding with large proteins reduces the available space for macromolecules of the same size, this phenomenon is known as excluded volume [175,176]. Excluded volume results in restricted molecular diffusion (viscosity) and is likely

to significantly alter the mobility of proteins. Excluded volume effects were also predicted to have a larger impact on IDP than on globular proteins [177]. The conformational ensemble that adopts an IDP in a dilute phase samples large conformations among others, as demonstrated by their large hydrodynamic radius compared with globular proteins of the same molecular mass [178]. In a crowded environment, these extended conformations may be unfavoured because of the excluded volume effect [177]. Several studies on the effect of molecular crowding on IDPs, either *in vitro* using macromolecular crowders (polyethylene glycol, ficoll, dextran, globular proteins) or in cellular environments (*in vivo* or in cell extracts) suggest that this phenomenon is very complex and each IDP or IDR will behave specifically, depending also on the crowding conditions: While a majority of IDPs will keep their disordered properties in a crowded environment, some of them will partially fold, completely fold, or even sometimes become more disordered [179]. The cell is a complex medium and natural molecular crowding results also favours interactions between molecules, likely to modify the affinity of IDPs with their partners. We have highlighted above several examples of IDP that are key regulators for the condensation of several phases: EPYC1, Cso2 and IM30. In these condensed phases, the concentration of the IDPs can be very high and they can be considered as self-crowders. Recent studies on several such IDPs looking for hypothetical conformational changes upon phase separation suggest that they generally retain their disordered nature, but their dynamics and mobility are slowed down [70–75,77,78]. Key components of the cell may influence these crowding effects and the dynamics of these phase transitions, as ATP, which has been shown when it is highly concentrated in the cell to enhance the solubility of proteins, and thus to act as a crowd controller [179]. Because each compartment has variable constituents, the nature of the molecular crowding is not homogeneous within the cell, the excluded volumes also, and the conformational sampling of dynamic protein is probably specific for each localisation. Besides, the molecular diversity is also restricted in biocondensates that encompass only specific proteins and/or metabolites. Intrinsically disordered proteins have the particularity of interacting with many partners, and compartmentalisation will reduce the presence of several partners to specific localisations [175,176].

These considerations have driven scientists to perform *in cellula* structural investigations of IDPs, and again NMR has proven to be a suitable method. A very complete review of in cell structural investigation

by NMR can be found here [20], and this is a fast expanding field. An attempt to investigate the structural properties of a plant protein within a cell was limited by the requirement of selective isotopic labelling of the target protein against a 'NMR-invisible' background [180]. A first approach is to use total cell lysate that encompasses all the molecular components reorganised by sonication or high pressure to expose them to the isotopically labelled protein [93]. This, however, will not allow to investigate the specificities of distinct microcompartments, which, in contrast, could be approached by the use of purified compartments (purified pyrenoid, purified plastoglobules) or *in vitro* reconstituted LLPS.

Conclusions

In cyanobacteria and in eukaryotic microalgae, carbon acquisition, assimilation and storage involve various metabolic routes that spread across various cellular microcompartments. CO₂ is the substrate of RuBisCO that is located in the carboxysome, a semicrystalline microcompartment in cyanobacteria, or in the chloroplast stroma or in the pyrenoid, a LLPS biocondensate, in eukaryotic microalgae. The existence of distinct microcompartments with distinct pH ranging from 6.9 (lumen) to 7.9 (cytosol, stroma, pyrenoid) separated by boundaries permeable for CO₂ but not HCO₃⁻ (thylakoids), and the presence of CA in each aqueous phase enable the concentration of CO₂ at the vicinity of RuBisCO. The condensation of RuBisCO is also triggered under high O₂ conditions. What is the diffusion of second RuBisCO substrate and the RuBisCO product in and out of these biocondensates? What is RuBisCO catalytic constant in these highly crowded liquid phase? Separated liquid–liquid phases are a fascinating example of supramolecular organisations that can modulate the physiology of a cell. Following the well-studied case of RuBisCO and its substrates, the existence of other distinct supramolecular organisations can be considered. How do enzymes of the CBB cycle organise to optimise both metabolic flux and substrate availability? Several megacomplexes have been proposed in the literature, but are lacking high-resolution structural characterisation. The putative low affinity for these complexes could explain why their reconstitution is challenging. One can also hypothesise the existence of linker proteins that remain to be identified, as this is the case for CP12 that is the linker for the GAPDH-CP12-PRK complex.

CP12 is a conditionally disordered protein with multiple functions but no catalytic activity [12]. A high number of proteins that belong to the IDP family such

as CP12 play a role in the microcompartmentalisation of the cells. They can be scaffold for LLPS demixing (e.g. EPYC1 and CsoS2); sensors to induce the formation of these microcompartments (e.g. AP2C2); or they can contribute to the remodelling of thylakoid membranes (e.g. IM30). Their malleability enables them to adapt to many different phases and interphases that have different physicochemical properties. However, the lack of catalytic activities and their atypical physicochemical properties renders them difficult to identify, and it is likely that more IDPs will be identified in future thanks to advanced omics approaches.

The location of photosynthetic enzymes in a specific organelle, the chloroplast, is known since the middle of the nineteenth century [181,182], and the advantage of physical barriers to confine the toxic O₂ photosynthetic product has been well described. Beyond the membrane-delimited organelles such as the chloroplasts, thylakoid lumen, vacuole, peroxisome, mitochondria or plastoglobules, other intracellular biocondensates such as the pyrenoids or LDs are now emerging. They are not delimited by a lipid bilayer but demix spontaneously due to their chemical composition. Lipid droplets demix because of their lipophilic components and hydrophobic interactions. Protein-rich LLPS demix because of electrostatic interactions and hydrogen bonds. Phase separation, in which solutes self-aggregate but remain in a liquid condensed state, is named coacervation [183]. The biologists' interest for this phenomenon has increased after coacervation was found to occur in cells [32,33], but it has been proposed as one supramolecular organisation as early as the beginning of the twentieth century [184]. Indeed, Oparin hypothesised that life could have emerged under the form of spontaneous coacervates before the occurrence of long amphiphilic lipids [184,185]. Strikingly, we now know that fatty acid synthesis and desaturation involve multiple exchanges in and out of microcompartments of the chloroplast and out of the chloroplast: ER, peroxisomes, mitochondria.

Investigating the structure and function of proteins in such environments is challenging, but it will certainly reveal a novel and better understanding of biological reactions and metabolic pathways. Recent advances in microscopy and spectroscopic methods, and the opportunity to perform molecular scale analysis on living cell by *in-vivo* fluorescent and in-cell spectroscopic methods such as NMR and EPR [20,186] have opened new perspectives. Nuclear magnetic resonance spectroscopy and its various applications appear as the most powerful biophysical methods to investigate *in cellula* and in biomimetic media the structure–

function and ultrastructure–function relationships of enzymes and their cofactors coupled to reaction kinetics and metabolite monitoring. These are exciting objectives that drive research projects in this field.

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Recent work in several fields of science has identified a bias in citation practices such that papers from women and other minority scholars are under-cited relative to the number of such papers in the field [187]. Our list of authors is thus, as many, not representative of the diversity of respected scholars in the fields.

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