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Orchestration of algal metabolism by protein disorder

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Highlights (85 characters)

- IDPs help algae to cope with extreme conditions
- IDPs from algae are under-represented in disordered protein databases
- Only IDPs involved in CO₂ metabolism have been studied so far (EPYC1, CP12, RCA...)
- Disorder is involved in the regulation of enzymes belonging to CO₂ metabolism
- Disorder is also important in algal chaperones

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62 **Abstract**
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65 Intrinsically disordered proteins (IDPs) represent a family of proteins that provide many
66 functional advantages in a large number of metabolic and signaling pathways. Because of
67 their high flexibility that endows them with pressure-, heat- and acid-resistance, IDPs are
68 valuable metabolic regulators that help algae to cope with extreme conditions of pH,
69 temperature, pressure and light. They have, however, been overlooked in these organisms. In
70 this review, we present some well-known algal IDPs, including the conditionally disordered
71 CP12, a protein involved in the regulation of CO₂ assimilation, as probably the best known
72 example, whose disorder content is strongly dependent on the redox conditions, and the
73 essential pyrenoid component 1 that serves as a scaffold for ribulose-1, 5-bisphosphate
74 carboxylase/oxygenase. We also describe how some enzymes are regulated by protein
75 fragments, called intrinsically disordered regions (IDRs), such as ribulose-1, 5-bisphosphate
76 carboxylase/oxygenase activase, the A₂B₂ form of glyceraldehyde-3-phosphate
77 dehydrogenase and the adenylate kinase. Several molecular chaperones, which are crucial for
78 cell proteostasis, also display significant disorder propensities such as the algal heat shock
79 proteins HSP33, HSP70 and HSP90. This review confirms the wide distribution of IDPs in
80 algae but highlights that further studies are needed to uncover their full role in orchestrating
81 algal metabolism.
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106 **Keywords** : Calvin cycle, chaperone, CP12, EPYC, Intrinsically disordered protein, RuBisCO
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109 **Abbreviations**
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111 ADK: adenylate kinase; CBB: Calvin-Benson-Bassham; CCM: CO₂ concentrating
112 mechanism; CD: circular dichroism; CP12: chloroplast protein of 12 kDa; EPYC1: Essential
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121 Pyrenoid component 1; EST: expressed sequence tag; GAPDH: glyceraldehyde-3-phosphate
122 dehydrogenase; HCA: hydrophobic cluster analysis; HSP: heat shock protein; IDP:
123 intrinsically disordered protein; IDR: intrinsically disordered region; LSU: large subunit;
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125 NMR: nuclear magnetic resonance; PGA: phosphoglyceric acid; PRK: phosphoribulokinase;
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127 ROS: reactive oxygen species; PTM: post translational modification; RuBisCO: Ribulose-1,
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129 5-bisphosphate carboxylase/oxygenase; RuBP: Ribulose-1, 5-bisphosphate; RCA: RuBisCO
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131 activase; SAXS: small angle X-ray scattering; SSU: small subunit
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180 **1. Introduction**
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185 ***Regulation of Algal Metabolism***
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188 Algae comprise a hugely diverse, polyphyletic group of photosynthetic organisms including
189 prokaryotic cyanobacteria and eukaryotic algae from the Archaeplastida, Chromalveolata,
190 Cryptophyta, Haptophyta and Dinoflagellata [1]. At least 72,500 algal species are known [2]
191 but this is probably a large underestimate. Algae contribute about 46% of global primary
192 production, mainly in the ocean [3] and also play an important role in global biogeochemical
193 cycles such as oceanic carbon sequestration [4]. Algae are ubiquitous in aquatic and moist
194 environments and some of them are ‘extremophiles’ tolerating, *inter alia*, desiccation [5],
195 high or low temperatures, high or low pH, high salinity, high pressure [6] and high
196 concentrations of heavy metals [7, 8]. Some survival strategies involve the production of
197 novel biochemical compounds and there is a recent impetus towards the valorization of algae
198 metabolic products, such as polyunsaturated fatty acids, fatty acid esters, polysaccharides, and
199 the production of enzymes for various applications [9].
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214 In eukaryotic algae, as in plants, chloroplasts have a central role in metabolism. They
215 all derive from a cyanobacterium, but the number of membranes around a chloroplast varies
216 depending on the number of endosymbioses involved in their evolutionary history [10]. Most
217 of the chloroplastic enzymes involved in the metabolic primary steps are nuclear-encoded [11,
218 12] and their activity can be regulated at the transcription, translation, or protein level.
219 Notably, at the protein level, post-translational modifications, protein-ligand or protein-
220 protein interactions can regulate enzyme activity [13, 14].
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229 Algae comprise a large number of algal species with high biochemical and structural
230 diversity, are ecologically important, and can survive and grow in a large range of different
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239 environments. To understand the basic principles of how algae regulate their metabolism, and
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241 eventually support future biotechnological applications, we need to decipher the mechanisms
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243 of these processes at the molecular level. Among regulatory proteins, a peculiar class of
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245 proteins has emerged, the intrinsically disordered/ductile proteins (IDPs), or the proteins that
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247 contain intrinsically disordered regions (IDRs). In this review, we will discuss their role in the
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249 regulation of algal metabolisms.
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255 ***Reassessment of the protein structure function paradigm***

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257 IDPs escape from the classic structure-function paradigm and are fully functional despite
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259 being fully or partially disordered. The principal feature of these IDPs or IDRs containing
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261 proteins is the absence of a unique stable conformation which results in a high structural
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263 flexibility [15, 16]. Nevertheless, IDPs can also present either small structural motifs, or have
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265 a significant propensity to fold into secondary structural elements [17, 18]. This propensity to
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267 fold is modulated by several factors such as post-translational modifications [19], protein or
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269 ligand binding [20-22], physico-chemical conditions such as redox-conditions [23-27], pH
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271 [28], temperature [29], binding to a membrane, adsorption at an interface [30], etc. Some
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273 IDPs are also named conditionally disordered proteins [31] when their disorder content and
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275 their function is directly modulated by different possible conditions, as redox-conditions. A
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277 large collection of names were found in the literature that describes these proteins, and the
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279 community eventually decided to keep the term IDP [32]. The term will be used in the rest of
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281 this review.
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285 The signatures of IDPs are rather well-established, with a bias in their amino-acid
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287 composition and frequent sequences of low complexity [33, 34]. Based on the sequence
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289 peculiarities of IDPs, a plethora of disorder predictors has been developed during the last two
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291 decades, with increasing prediction accuracy, and are continually updated [35-37]. These
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298 predictors have allowed large scale analyses of an ever growing number of fully sequenced
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300 and annotated genomes in all kingdoms of Life, which revealed the widespread and universal
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302 nature of IDPs. Several databases of IDPs have thus been implemented, which provide pre-
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304 computed disorder predictions of completely-sequenced genomes (D²P²; <http://d2p2.pro/>), of
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306 all UniProt entries (MobiDB; <http://mobidb.bio.unipd.it/>), or which reference only
307
308 experimentally verified IDPs (DisProt, <http://www.disprot.org/>; IDEAL,
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310 <http://idp1.force.cs.is.nagoya-u.ac.jp/IDEAL/>).

313 A range of biophysical approaches are required to characterise experimentally the
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315 disordered properties of IDPs, often advantageously in association with state-of-the-art
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317 modelling tools to comprehend the large ensemble of probable conformational states that
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319 coexist in a given condition [16, 38-41]. IDPs have thus been characterised by small angle X-
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321 ray scattering (SAXS), nuclear magnetic resonance (NMR) spectroscopy, circular dichroism
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323 (CD) spectroscopy, protease sensitivity and many other techniques, to cite but a few (see
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325 Longhi, this issue, [16, 41-43], with each method having advantages and limitations.

328 The functions carried out by IDPs are diverse and complement those of ordered
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330 proteins. They play significant roles in many biological processes, such as control of the cell
331
332 cycle, transcriptional activation, and signalling, and they frequently interact with or function
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334 as central hubs in protein interaction networks [44-48]. Moreover, correlation between
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336 disorder and post-translational modifications (PTMs) such as phosphorylation, methylation,
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338 acylation, glycosylation and ubiquitination has been reported [19, 49]. This is worth noting
339
340 since PTMs can contribute to the diversification and functionality of proteomes by regulating
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342 different properties of proteins (stability, conformation, etc...). Hence IDPs via diverse PTMs
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344 participate in the regulation processes of the cell [19, 46, 50, 51].

347 ***IDP phylogenic distribution***

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357 Analysis of the proteomes using predictors of disorder revealed that the proportion of intrinsic
358 disorder in proteomes is variable in the evolution tree. There is a low proportion of disorder in
359 bacteria and archaea, a higher one in viruses, and a much larger one in eukaryotes, especially
360 in higher vertebrates, including mammals [52-54]. Recently, it was shown that IDPs play a
361 role in the evolution of multi-cellularity and cell type specification [48, 55].
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367 By contrast, the distribution of IDPs among the different kingdoms of Life, that have
368 been experimentally investigated, does not correlate with the distribution of predicted IDPs.
369 Notably, in the experimentally-verified database DisProt [56], IDPs from photosynthetic
370 organisms, and especially from algae, are under-represented while IDPs from bacteria are
371 over-represented, with 19% of all IDPs. In the other databases, this number ranges from 0 to
372 8.1% [56]. This discrepancy in the IDP databases might reflect, in a predominant manner, a
373 biased interest in scientific research for pathogenic organisms that overlooks the wide
374 distribution of disorder in the tree of Life. Another bias might also result from the fact that
375 large scale projects on structural genomics have used *E. coli* as the main expression system
376 for high throughput production and screening of proteins, which naturally limits the efficient
377 and correct production of some eukaryotic proteins.
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392 ***IDP in plants and algae***

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396 A specific feature of IDPs is that they are able to maintain their functional states in a wide
397 range of physiological conditions, unlike well-folded proteins which can be extremely
398 sensitive to variation in their environment. Indeed, IDPs do not aggregate under high
399 temperature or low pH, conditions in which structured proteins usually undergo denaturation
400 and loss-of-function [29, 57, 58]. As mentioned above, algae encounter extreme conditions.
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416 imposing a turgor pressure inside their cell wall [5, 59]. Variation of pressure can be also
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418 detrimental for the conservation of functional cavities in folded enzymes and of
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420 macromolecular scaffolds [60]. For instance, it has been shown that the activity of Calvin-
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422 Benson-Bassham (CBB) cycle enzymes is strongly affected by high pressure [61]. IDPs,
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424 which are devoid of internal cavities, are expected to be more stable upon pressure increase,
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426 and indeed, α -synuclein [62] and the disordered region of titin [63] preserve their disordered
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428 characteristics at high pressure. It is likely that IDPs in general are more stable in these
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430 conditions but this needs further investigations.
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434 Besides, the chloroplast proteome undergoes variations of physico-chemical conditions
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436 with redox switches [64]. The dark-light regulation of the photosynthetic pathway in the
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438 chloroplast is mainly modulated via redox transitions [65-67]. Thanks to their propensity to
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440 receive post-translation modifications that modify their conformational and functional states,
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442 IDPs are particularly suited to sense these changes of physico-chemical conditions, thereby
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444 regulating enzymes activity [68, 69]. For all these reasons, the properties of IDPs are valuable
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446 assets for the regulation of enzymes and metabolic pathways, in the broad range of
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448 environmental conditions that algae withstand.
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452 Up to now, only few IDPs from photosynthetic organisms have been investigated, and
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454 they mainly come from the higher plant *Arabidopsis thaliana*, which has one of the most
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456 accurately annotated sequenced genome thanks to its small size. A recent analysis of 12 plant
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458 genomes revealed that the occurrence of disorder in plants is similar to that in many other
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460 eukaryotes [70, 71] though only few plant IDPs have been experimentally characterised (A.
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462 Covarrubias, this issue). These IDPs or IDR containing proteins are, in the vast majority,
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464 nuclear-encoded proteins, while chloroplast- and mitochondria-encoded proteins have a lower
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466 disorder trend as in Archae and Bacteria, which is in agreement with their phylogenetic
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468 origin.
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475 In the model alga *Chlamydomonas reinhardtii*, a systematic search for disordered
476 protein experimentally showed that this alga contains a large number of non-characterised
477 proteins that have IDP-like properties [72]. Interestingly, the few IDPs that have been
478 biophysically investigated so far are the ones involved in the regulation of their metabolic
479 pathways. This bias arises from the keen interest in the metabolism of algae, which have a
480 high potential in CO₂ assimilation and production of various added-value molecules.
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488 In this review we shine a spotlight on the few known IDPs from algae that help these
489 photosynthetic organisms to adjust their photosynthetic carbon metabolism to the prevailing
490 conditions in their habitat. These IDPs are found in the chloroplast, where the primary step of
491 photosynthesis occurs, and where CO₂ is assimilated and converted into building blocks to
492 produce fatty acids, carbohydrates, proteins and various metabolites. Chaperones, which
493 regulate the proteostasis, and therefore metabolism, through disordered regions, will also be
494 discussed. Finally, we will extend the discussion to other algal predicted IDPs involved in
495 metabolic regulation.
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2. CO₂ acquisition and assimilation

During photosynthetic CO₂ assimilation, chemical energy, conveyed mainly by ATP and NAD(P)H, and supplied by electron transfer reactions through the photosystems I and II, is used by metabolic reactions of the CBB cycle. The efficiency of this assimilation relies on a fine tuning between energy demand and supply. Hence the CBB is highly regulated. This cycle functions in the light and is inactive in the dark. Some enzymes are active only under reducing conditions, corresponding to the light conditions, and are inactive under oxidising conditions corresponding to the dark [13, 65].

RuBisCO activase (RCA)

Ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) is the enzyme that initiates the CBB cycle by converting ribulose-1, 5-bisphosphate (RuBP) and carbon dioxide (CO₂) into two molecules of phosphoglyceric acid (PGA). In addition, RuBisCO catalyses a competitive reaction that converts RuBP in the presence of O₂, into one PGA and one toxic compound, phosphoglycolate [73]. RuBisCO is made up of eight large (LSU, RbcL) and eight small (SSU, RbcS) subunits, in algae and higher plants [74]. In order for RuBisCO to be active, it has long been suggested that a specific lysine residue on the LSU needs to be carbamylated [75]. Today, it is known that the mechanism for regulating RuBisCO is much more complex and involves not only changes in the carbamylation state of this lysine residue, but also conformational changes in the protein. These conformational changes are triggered by a protein called RuBisCO activase (RCA), which is a specific molecular chaperone and an AAA⁺ ATPase [66]. RuBisCO active sites can be locked by sugar phosphate in the close inactive state and RCA prevents this inhibition by promoting ATP-dependent conformational changes that open closed sites, thus facilitating dissociation of inhibitory sugar phosphate

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593 (Figure 1). Furthermore, the presence of RCA allows RuBisCO to be active even at sub-
594 optimal CO₂ concentrations that would not normally permit carbamylation *in vivo* [76, 77].
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598 Some algae in the charophyta phylum have multiple isoforms of RCA: two α -isoform
599 of ~45-48 and 58 kDa, and a β -isoform of ~41-43 kDa [78] (Figure 2B). These isoforms are
600 the product of alternative splicing and differ from each other by a larger and redox-regulated
601 C-terminal extension on the α -isoforms. This C-terminal extension contains three or two
602 regulatory cysteine residues that allow the protein to be activated by light through the action
603 of a small redox protein, the thioredoxin f [78, 79]. Interestingly, RCAs from another phylum
604 of microalgae, the chlorophyta (including *C. reinhardtii*), lack these two cysteine residues and
605 therefore are unlikely to be redox-sensitive.
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615 An *in silico* analysis [80] showed that the C-terminal extension of RCA isoforms is
616 intrinsically disordered (Figures 1B and 2A). Several small but significant differences were
617 observed between the intrinsic disorder propensities in RCAs from two chlorophyta algae,
618 *Tetraselmis* sp. and *Ostreococcus tauri*, probably reflecting a different mode of interaction
619 between their RCAs and RuBisCO [81]. The authors suggest that the difference in the
620 disordered propensities can be ascribed to different evolution pressures on these two algae
621 related to their motility. Indeed, *Tetraselmis* sp. is motile and can actively migrate to optimal
622 habitat, in contrast to *O. tauri*, which is a non-motile microalga. For the latter, therefore, there
623 is a higher evolution pressure on RCA to optimise photosynthesis under non-optimal
624 conditions. However, one cannot exclude that other processes are involved. In other
625 photosynthetic organisms such as in β cyanobacteria, the RCA lacks the N terminal domain
626 necessary for RuBisCO activation found in plants and green algae [66, 78, 82]. In red lineage
627 organisms, there is no RCA gene but it is substituted by another protein, called CbbX. In the
628 purple bacterium *Rhodobacter sphaeroides*, CbbX possesses a short disordered C-terminal
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650 extension (Figures 1C and 2A), the function of this part though, remains unelucidated [83].
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654 The homologues of CbbX from Rhodophyta lack this C-terminal extension (Figure 2C).
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657 ***Essential Pyrenoid component 1 (EPYC1)***

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659 In many algae, RuBisCO is present within a chloroplastic membraneless compartment that
660 concentrates locally proteins involved in carbon fixation, and called a pyrenoid. Pyrenoids are
661 involved in CO₂ concentrating mechanisms (CCMs) that are essential for aquatic organisms,
662 which face low CO₂ concentrations much below the optimal concentration for RuBisCO
663 activity [84, 85]. These CCMs increase CO₂ concentration around RuBisCO and thereby
664 decrease O₂ competition. The pyrenoid is located in the chloroplast, and is surrounded by a
665 starch sheath and traversed by membrane tubules that are continuous with the thylakoid
666 membranes [86]. Recently, it was shown that in the model alga *C. reinhardtii*, RuBisCO
667 accumulation and localization within the pyrenoid is mediated by a disordered repeat nuclear-
668 encoded protein, called Essential Pyrenoid component 1 (EPYC1), previously identified as a
669 low-CO₂ inducible protein (LCI5; Cre10.g436550) [87]. This protein is ubiquitous in
670 pyrenoid-containing algae but is absent in pyrenoid-less algae. In addition to EPYC1,
671 RuBisCO and its chaperone RCA, 135 to 190 putative CCMs proteins including some of their
672 binding partner(s) were also found to be located in the membrane-less pyrenoid, by mass
673 spectrometry analysis [88, 89]. Like many IDPs, EPYC1 is phosphorylated at low CO₂
674 concentration [90]. Furthermore EPYC1 was shown to interact with a predicted
675 serine/threonine protein kinase (KIN4-2; Cre03.g202000) and two 14-3-3 kinases FTT1 and
676 FTT2 [87]. These interactions with kinases probably regulate RuBisCO and EPYC1
677 interactions and activities.
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700 EPYC1 protein displays characteristic features of IDPs. Its CD profile is typical of an
701 IDP (Figure 3A), and it is resistant to denaturing conditions such as boiling for 15 min [91].
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703 Its sequence contains four 60 amino-acids repeats, each domain being predicted to be
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711 disordered (Figure 3C). The role for these tandem domain repetitions remains controversial. It
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713 was hypothesised that these low-complexity repeats allow EPYC1 to bind several RuBisCO
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715 (Figure 3B), which then drives formation of pyrenoid droplets with liquid-like properties [88].
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717 However, it was later shown that a mutant of EPYC1 with a single repeat was still able to
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719 form membrane-less liquid-liquid phase separation droplets upon binding to RuBisCO *in-*
720
721 *vitro*, suggesting that each repeat domain contains several binding sites to RuBisCO. *In-vitro*
722
723 reconstitution of liquid-liquid phase separation droplets indicated that EPYC1 and active
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725 RuBisCO are necessary and sufficient for phase separation, and that the phase separation is
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727 governed by electrostatic interactions [91]. In line with this finding, it is known that proteins
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729 with IDRs and low complexity sequences tend to phase separate and to form membrane-less
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731 organelles [92]. The molecular nature of such membrane-less organelles is under intense
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733 scrutiny [93]. In the current model, the pyrenoid matrix is highly dynamic, in a liquid-phase
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735 that supports efficient molecular diffusion to increase CO₂ concentration and decrease the
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737 oxygenase activity of RuBisCO.
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744 ***CP12 and the regulation of the CBB cycle***

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747 CP12 is a nuclear-encoded chloroplast protein of about 8.5 kDa that is present in many
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749 photosynthetic organisms, including cyanobacteria, algae, and plants [94, 95] as well as in
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751 cyanophages [96]. It has been especially well characterised in some model organisms such as
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753 the higher plant *A. thaliana*, the cyanobacterium *Synechococcus elongatus* and the green alga
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755 *C. reinhardtii* [97]. This small protein is a conditionally disordered protein that has been
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757 characterised by NMR, SAXS, CD, Förster resonance energy transfer, fluorescence
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759 correlation spectroscopy, mass spectrometry and molecular modelling [26, 27, 98-101]. CP12
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761 is involved in the regulation of the CBB cycle but has multiple other faces, such as being a
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763 chaperone. CP12 from *C. reinhardtii* has two disulfide bridges, one at the N-terminus and one
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770 at the C-terminus. While the C-terminal cysteine residues are conserved in all CP12 proteins,
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772 the N-terminal ones may be missing in some photosynthetic organisms, e.g. in rhodophytes,
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774 the glaucophyte *Cyanophora* and in *Synechococcus* [94, 102, 103]. The formation of the two
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776 disulfide bonds under dark or oxidizing conditions allows the algal CP12 to bind two enzymes
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778 from the CBB cycle, namely glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and
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781 phosphoribulokinase (PRK). The formation of this supramolecular ternary edifice results in
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783 the inactivation of these two enzymes and consequently leads to the arrest of the CBB cycle in
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785 the dark. In the light, CP12 is reduced and the breakage of its two disulfide bridges leads to
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787 the dissociation of the ternary complex, with the resulting dissociated forms of PRK and
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789 GAPDH being fully active. CP12 therefore participates in the regulation and in the “work on
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791 demand” of the CBB cycle that is active under light and inactive under dark.
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794 While in the oxidised state, CP12 has some α -helices, in the reduced state it becomes
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796 fully disordered (Figure 4A and 4B) [27, 99, 104, 105]. However, in both reduced and
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798 oxidised states, CP12 is highly mobile. NMR, CD and SAXS showed that the algal oxidised
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800 CP12 is composed of three regions with distinct conformational sampling [27]: its C-terminus
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802 folds in a stable turn, ready for GAPDH binding, it is linked to the N-terminal domain through
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804 a randomly disordered linker, while the N-terminus oscillates on the millisecond timescale
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806 between helical and random states (Figure 4C). The existence of a conformational equilibrium
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808 between two states of the algal CP12 was also shown previously by ion mobility mass
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810 spectrometry [98]. The combined analysis of NMR and SAXS data enabled the quantification
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812 of the relative population of each state (40% random-coil state and 60% helical). Upon binding
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814 to GAPDH, this equilibrium shifts towards more extended conformations (Figure 4D). In
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816 other words CP12 undergoes an induced unfolding. This phenomenon, called cryptic disorder,
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818 which contributes to decreasing the entropy cost of binding, might explain the very high
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820 affinity of CP12 for its partners, with a dissociation constant in the nM range [106]. This
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829 GAPDH/CP12 sub-complex in turn enables the binding of PRK [107]. The idea that two
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831 proteins could form a high-affinity complex while remaining highly dynamic has long been
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833 “nearly heretical”. However, a recent study has brought to light the case of two fully
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835 disordered proteins forming a dynamic complex of very high affinity (pM range), without any
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837 gain of structure in the two proteins [108]. This last example shows that disordered proteins
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839 might interact strongly without anchor sites, nor disorder-to-order transitions, opening a new
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841 concept and avenue in the field of IDPs.
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844 CP12 can bind metal ions, such as Cu^{2+} , Ni^{2+} [109] and Ca^{2+} [110], as well as enzymes
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846 such as the FBP aldolase [111]. Of interest also, CP12 can play the role of a specific
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848 chaperone for GAPDH preventing its aggregation and inactivation by heat treatment [112].
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850 Hence, CP12 is a versatile protein considered as a jack-of-all-trades but master of the CBB
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852 cycle [113], that indirectly regulates the CO_2 assimilation by interacting with PRK and
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854 GAPDH. This regulation by CP12 is conserved in different species, but the molecular
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856 mechanism of the interactions and the strength of binding between the PRK/GAPDH/CP12
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858 complex differ among species. For example, in *A. thaliana* the dissociation constant for
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860 GAPDH/CP12 is in the μM range [114], and CP12 undergoes an induced folding upon
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862 binding to GAPDH, in contrast to *C. reinhardtii* [27]. The broad adaptation to multiple
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864 environmental conditions might explain these divergent molecular mechanisms.
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869 ***GAPDH-B and ADK3, two enzymes with C-terminal extensions homologous to CP12***

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871 There are two main forms of chloroplast GAPDH. The first one, GapA, is present in the
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873 majority of photosynthetic organisms [102, 115] and forms A_4 homotetramers. The second
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875 isoform, GapB, is found in higher plants and in the alga *Ostreococcus*. It is extremely similar
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877 to GapA, but has a C-terminal extension (about 30 amino acids) homologous to CP12 [116,
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879 117]. The GapB subunit forms functional A_2B_2 heterotetramers with the GapA subunit, as
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888 well as inactive A₈B₈ oligomers, although the relevance and the role of these complexes
889 remain disputed. However, it is well established that the disordered "tail" or the C-terminal
890 extension on GapB endows GAPDH with autonomous redox regulation [115].
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893
894 In *C. reinhardtii*, where only GapA exists, this autonomous regulation is
895 impossible. However, as the first steps of glycolysis and the oxidative pathway of phosphate
896 pentoses take place in the chloroplast [118], there is an obligatory need for a fine regulation of
897 the activity of this A₄ isoform. It has been shown that the redox regulation of this chloroplast
898 A₄ GAPDH, depends on its interaction with CP12. Therefore, the regulation of GAPDH
899 relies, as for RuBisCO, on IDRs either on the enzyme itself or on its partner, CP12. Since
900 GAPDH is a moonlighting protein, with many cellular functions [119], fusion with this CP12-
901 like tail may endows it with further other regulatory functions yet unknown and to be
902 explored.
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906 Other important enzymes are the adenylate kinases (ADKs). These enzymes
907 maintain the energetic balance in cellular compartments including chloroplasts and
908 mitochondria, by catalyzing the interconversion of AMP, ATP, and ADP (AMP+ATP↔
909 2ADP). In *C. reinhardtii*, ten genes encoding ADKs are present. Within the chloroplast
910 stroma of photosynthetic organisms, where the ATP:ADP ratio has to be kept at an optimal
911 level to allow a sustainable rate of CO₂ fixation, ADK3 plays a major role in the regulation of
912 concentrations of adenylates [120].
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915
916 ADK3 from *C. reinhardtii* comprises two domains: a canonical domain, homologous
917 to the *Escherichia coli* ADK (44% identity), and a "tail" of 25 residues, very close to the C-
918 terminal portion of CP12 (68% identity) [121]. The kinetic parameters for the wild-type and
919 truncated ADK3 (removing the C terminus, namely ADK3-ΔCP12) are very similar,
920 indicating that the C-terminal extension of ADK3 does not affect the activity and kinetic
921 properties of the enzyme, nor its global secondary structure. Since CP12-like extension
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947 confers GAPDH redox regulation, it was hypothesised that it might be the same with ADK3.
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949 This redox regulation was not observed, but the CP12-like extension confers ADK3 with a
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951 high affinity for the chloroplast GAPDH [122]. Via this disordered region, ADK3 down-
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953 regulates the NADPH-dependent GAPDH activity, therefore stopping the consumption of
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955 NADPH by the CBB cycle. The C-terminal extension on ADK is also found in the sequence
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957 of ADK from other algae and might play the same role.
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960 In addition, the C terminus of ADK3 has a strong effect on the thermal stability of the
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962 enzyme, and this effect is probably the result of its predicted disorder propensity, in
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964 agreement with IDPs heat-resistance [29, 57].
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967 ***Protection against oxidative damage***

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970 The metabolism of microalgae is tightly redox regulated. On the other hand, the transient
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972 accumulation of reactive oxygen species (ROS) causes subsequent oxidative damage, protein
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974 unfolding and aggregation. Chloroplasts are organelles where molecular oxygen is produced
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976 during photosynthesis and where ROS production occurs, therefore protein-thiols protective
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978 mechanisms are active in these organelles. Under conditions causing oxidative stress,
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980 glutathionylation and nitrosylation play roles in enzyme regulation and signalling [123]. A
981
982 proteomic study on *C. reinhardtii* has identified 225 proteins that are potential targets of
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984 glutathionylation, including 10 out of 11 enzymes of the CBB cycle [124], suggesting a
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986 central role of such modification in the global regulation of the carbon assimilation pathway.
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988 The role of CP12 in oxygen photoautotrophs beyond light/dark regulation, includes protection
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990 of PRK and GAPDH from oxidative stress [125] and a role in the glutathionylation of ADK3
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992 that remains to be defined. Indeed, while ADK3 can be glutathionylated, the truncated ADK3-
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994 Δ CP12 cannot, even though the site of glutathionylation is not on the cysteine residues present
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996 in the C-terminal extension on ADK3 [121].
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3. Molecular chaperones

Protein homeostasis, or proteostasis, is controlled by the so-called chaperones, in response to stress, and in normal conditions [126]. These proteins are thus essential for the “orchestration” of metabolism. Several molecular chaperones have now been identified and classified as conditionally disordered proteins [127]. The functions and underlying mechanisms of molecular chaperones are very well studied in bacteria and in the cytosol or endoplasmic reticulum of eukaryotic cells, but their role in the metabolic processes of the chloroplast have been overlooked.

An experimental search for IDPs in *C. reinhardtii* showed that chaperones and ribosomal proteins were the most represented IDPs [72]. Twenty proteins related to chaperone-function were identified, such as the heat-shock proteins HSP33, HSP40, HSP70s, FKBP, HSP90 co-chaperone, The disordered propensities of HSP70, HSP90 and HSP90 co-chaperone (Figure 5) have been shown experimentally in other organisms than algae [128], except for HSP33 that was characterised in *C. reinhardtii* [69].

HSP33 has been well studied in *E. coli* and is a redox chaperone that has a flexible C terminus that contains a conserved cysteine-rich center, Cys-X-Cys-X_n-Cys-X-X-Cys [23, 25]. These cysteine residues bind Zn²⁺ under reducing conditions, thus maintaining the protein in its inactive form. In the oxidised form, the C-terminus unfolds after Zn²⁺ release and the protein becomes active. To be active, this bacterial chaperone therefore needs to become disordered and this internal order-to-disorder transition is required to control substrate binding and release [129]. Interestingly, HSP33 is present in only two clades of eukaryotes: (i) Kinetoplastids and parasitic oomycetes and (ii) photosynthetic organisms, but only in algae and mosses, not in higher land plants [69]. In *C. reinhardtii*, and in general in the green algal lineage, the first cysteine residue of the conserved cysteine-rich center has been lost and

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1064
1065 replaced by a glycine residue. As a result, the C-terminus of HSP33 has lost its ability to bind
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1067 Zn in reducing conditions and is disordered in both oxidised and reduced forms, unlike the
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1069 bacterial protein. Only few structural changes of HSP33 from *C. reinhardtii* have been
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1071 observed experimentally between the oxidised and the reduced forms, as predicted (Figure
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1073 5A), and this was linked to a lower affinity for Zn²⁺ [69]. In *C. reinhardtii*, HSP33 expression
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1075 increases in response to light-induced oxidative stress and appears to participate in the rapid
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1077 response to exposure of the algal cells to oxidizing conditions. This protein is therefore
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1079 essential to algal proteostasis and metabolism.
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1082 In the cytosol of eukaryotic cells, the function of a plethora of proteins involved in
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1084 signal transduction, like hormone receptors and kinases, depends on their folding by the
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1086 HSP90 and HSP70 machineries [130, 131]. The HSP70s family comprises one subset of the
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1088 best-studied chaperones in *C. reinhardtii*. These proteins play a role in all major subcellular
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1090 compartments of the cell, and the HSP70B, HSP70C and HSP70A are targeted to the
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1092 chloroplast, mitochondrion and cytosol, respectively [132]. The chloroplast HSP70B is
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1094 induced either by light, or by heat or cold stress [132, 133]. Interestingly, the algal HSP70B
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1096 that is highly homologous to other HSP70B from different species (see Table 1 in [132])
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1098 exhibits long disorder regions of more than 30 amino acid residues, and in particular a 71-
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1100 amino acid disordered region at its C terminus (Figure 5B). Its flexibility allows binding and
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1102 release of clients, in good agreement with its function. In *C. reinhardtii*, HSP70B and
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1104 HSP90C can form a multimeric complex [134], although this complex has never been
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1106 successfully reconstituted *in vitro* from the two recombinant proteins [135]. The algal
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1108 HSP90C also bears a disordered C-terminus of about 50 aminoacid residues (Figure 5C). Both
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1110 HSP70B and HSP90C were found in the *Chlamydomonas* phosphoproteome [136] indicating
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1112 that they are phosphorylated as many IDPs. Despite their importance in the cell, these heat-
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1124 shock proteins, HSP70B, HSP90C and HSP33 remain understudied in *C. reinhardtii* and
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1126 other algae compared to other organisms [137, 138].
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1129 1130 **Algal IDPs in other cellular processes**

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1132 The experimental search for putative IDPs in *C. reinhardtii* also revealed few enzymes
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1134 with long intrinsically disordered segments, involved in the algal metabolism (Figure 6) that
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1136 are nuclear-encoded and targeted to either the mitochondrion or the chloroplast [72]. These
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1138 IDR-containing enzymes have not been studied so far to our knowledge. Yet, one can
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1140 hypothesise that the IDRs of these enzymes may also play important roles in the metabolism.
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1142 To cite but a few, the magnesium chelatase complex, involved in the light capture in the
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1144 chloroplast, contain an IDR. This enzyme synthesises a precursor of chlorophyll. Downstream
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1146 of the CBB, the synthetic pathway of the storage compound starch also involves an IDR-
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1148 containing protein, the soluble starch synthase III (Figure 6B). Besides, four enzymes with
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1150 long IDRs targeted to the mitochondrion were identified by Zhang *et al.*: the peptide
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1152 methionine sulfoxide reductase, the dihydrolipoamide succinyltransferase oxoglutarate
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1154 dehydrogenase E2 component (DLST-2OGDH) (Figure 6B), the F1F0 ATP-synthetase ϵ -
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1156 subunit and the carbonic anhydrase CA-8, all involved in the respiration process.
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1163 **5. Conclusions**

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1165 The autonomous chloroplastic and mitochondrial genomes encode only a small subset of
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1167 proteins and the majority of the proteins present in these organelles are nuclear-encoded and
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1169 imported after their synthesis in the cytosol. This import process allows the optimization of
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1171 metabolic pathways by IDPs, although the chloroplast and the mitochondrion originate from
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1173 bacteria, which have a lower content in IDPs. Furthermore, IDPs, with their ductile properties,
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1175 are very robust and well-suited to cope with the drastic environmental changes encountered
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1183 by algae (extreme changes in temperature, pressure, pH, light etc.) and tune the metabolic
1184 pathways according to these changes. The data obtained from the experimental study on *C.*
1185 *reinhardtii*, although not exhaustive [72], and the predictions from algal genomes [71]
1186 demonstrate the wide distribution of protein disorder in algae. Remarkably a study comparing
1187 the distribution of disorder in different cell types, from mammals (including *Homo sapiens*),
1188 plants, algae, and bacteria showed that the proteome of *C. reinhardtii* contains the highest
1189 number of residues with a disorder score [55].
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1199 Whereas very few studies have been undertaken hitherto, the growing interest in microalgae
1200 and their metabolic properties will undoubtedly trigger original studies on the role of IDPs in
1201 the regulation of cellular and metabolic processes in these microorganisms.
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1223 **Figure captions**

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1226 **Figure 1: Mechanism of action of RCA. A:** Model of the interaction between RuBisCO and
1227 RCA and of the mechanism of action of RCA activation, figure extracted from [139]. The
1228 disordered C-terminal extension of RCA is indicated by the letter C. **B:** Model structure of
1229 RCA from the higher plant tobacco (*Nicotiana tabacum*), with C-terminal extensions not
1230 resolved in the electron microscopy images, represented by black lines (RCSB entry
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1242 3ZW6.pdb, [140]). **C:** Model structure of the red-type AAA⁺ rubisco activase CbbX from the
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1244 purple bacteria *R. sphaeroides*. The C-terminal extensions not resolved in the negative stain
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1246 images are represented by a black line (RCSB entry 3ZUH.pdb [83]).
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Figure 2: Disorder propensity of the C-terminal extension of RuBisCO activase and CbbX. **A.** Hydrophobic Cluster Analysis (HCA, [141]) plots of the C-terminal extension of rubisco activase and CbbX from the following organisms, from top to bottom: *C. reinhardtii* (accession number XP_001692244.1); *Chlorokybus atmophyticus*, isoform β (EST HO20293, [78]); *Klebsormidium flaccidum*, isoforms α_2 and α_1 (EST HO431775.1, [78]); the purple bacterium *Rhodobacter sphaeroides* (accession number AAC44827.1). **B:** Schematic alignment of RCA from cyanobacteria, chlorophyta and charophyta highlighting their C-terminal extension. Figure extracted from [78]. **C:** Schematic alignment of CbbX from purple bacteria, rhodophyta and heterokonta. Interestingly, and contrary to the green-type viridiplantae, the rhodophyta seem to have lost the C-terminal disordered extensions.
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Figure 3: Disorder in the essential pyrenoid component EPYC1 forming membraneless pyrenoid with RuBisCO. **A:** The circular dichroism spectrum of EPYC1 indicates random-coil structural properties, in contrast to the spectra obtained for RuBisCO and lysozyme. Figure extracted from supplementary figure 1 of [91]. **B:** Schematic of the composition of the pyrenoid, with a ratio of EPYC1: RuBisCO from one to four. Among the 135-190 proteins that are present in the pyrenoid, we chose to represent RCA that is discussed in this review. **C:** Schematic of the EPYC1 protein sequence with its four repeating domains. The HCA plot of the first repeat is shown (*C. reinhardtii* sequence, accession number PNW77439.1).
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1301 **Figure 4: Redox conformational transitions of CP12 governing the light-dark CBB-cycle**
1302 **regulation.** **A:** Schematic of the redox transitions of CP12 and GAPDH-CP12 formation. The
1303 two pairs of cysteine residues of the green-lineage CP12 are redox regulated by the
1304 thioredoxins (Trx) that are reduced (under light) or oxidised (under dark). **B:** Under reducing
1305 conditions, CP12 is fully disordered and highly flexible. **C:** Under oxidizing conditions, the
1306 two pairs of cysteine residues form two disulfide bridges, and CP12 is partially disordered.
1307 The C-terminal bridge is in a stable structure, depicted in blue, while the N-terminal bridge is
1308 in a region that interconverts between two states on a millisecond timescale, depicted in red: a
1309 random-coil state and a helical hairpin. **D:** This latter region unfolds when the C-terminal
1310 region is bound to GAPDH.
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1325 **Figure 5: Three chloroplastic intrinsically disordered chaperons of *C. reinhardtii*.**
1326 HSP33, HSP70b and HSP90C are predicted as containing disordered regions using Ponder-
1327 VLXT (A score higher than 0.5 indicates a propensity for structural disorder)[142]. **A:** Ponder-
1328 VLXT score of *C. reinhardtii* HSP33 (accession number, XP_001700446). The prediction of
1329 the reduced state of HSP33 is obtained using the mutant C274S,C309S,C312S. **B:** Ponder-
1330 VLXT score of *C. reinhardtii* HSP70b (accession number XP_001696432). **C:** Ponder-VLXT
1331 score of *C. reinhardtii* HSP90 (accession number XP_001702984.1).
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1342 **Figure 5: Examples of *C. reinhardtii* metabolic enzymes with long intrinsically**
1343 **disordered regions .** **A:** Schematic of the metabolic pathways which possess enzymes with
1344 long intrinsically disordered regions and that are heat-resistant. These enzymes have been
1345 identified by Zhang *et al.* [72]. Some enzymes are chloroplastic: the magnesium chelatase
1346 complex (Mg²⁺ CC, accession number: XP_001700902.1), the soluble starch synthase III
1347 (SSS III, accession number: AAY42381.1); while others are targeted to the mitochondrion:
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1360 the peptide methionine sulfoxide reductase (PMSR, accession number: XP_001689879.1), the
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1362 carbonic anhydrase 8 (CA-8, accession number: XP_001697606.1), the dihydrolipoamide
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1364 succinyltransferase, the oxoglutarate dehydrogenase E2 component (DLST-2OGDH,
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1366 accession number: XP_001692539.1) and the F1F0 ATP synthetase ϵ subunit (F1F0 ATP ϵ ,
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1368 accession number: XP_001702609.1). Other IDPs described in this review are also included,
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1370 in particular those regulating the CBB cycle, and chaperones. **B:** The IUPred2A [143]
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1372 disorder prediction for the mitochondrial DLST-2OGDH and the chloroplastic soluble starch
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1374 synthase III are given as examples. A score higher than 0.5 indicates a propensity for
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1376 structural disorder.
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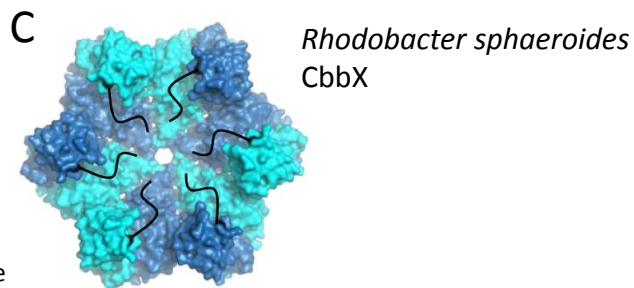
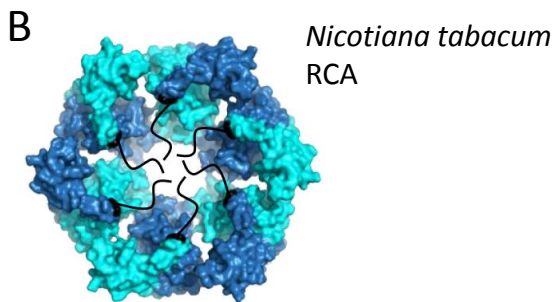
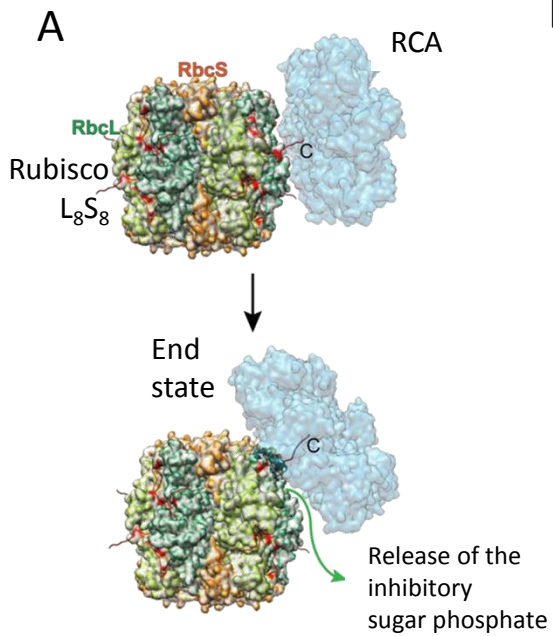
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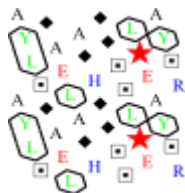
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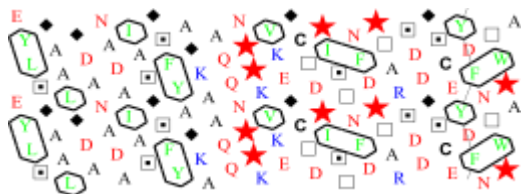
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(*C. reinhardtii*)



Charophyta β C-ter
(*Chlorokybus atmophyticus*)



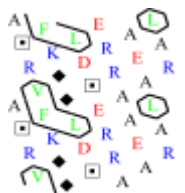
Charophyta $\alpha 2$ C-ter (*Klebsormidium flaccidum*)



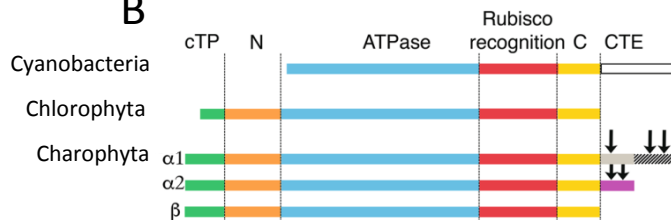
Charophyta RCA $\alpha 1$ C-ter (*K. flaccidum*)



Purple bacterium CbbX C-ter
(*Rhodobacter sphaeroides*)



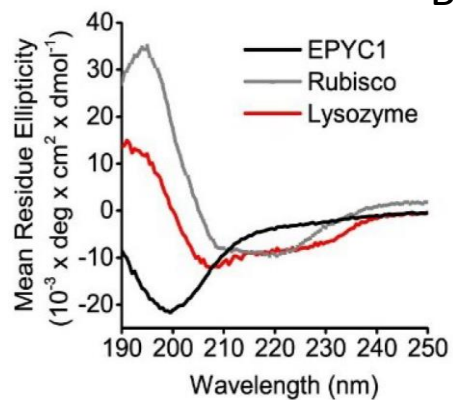
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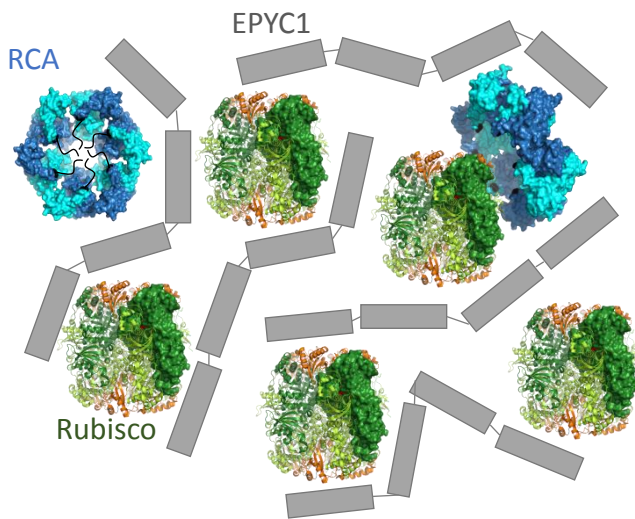
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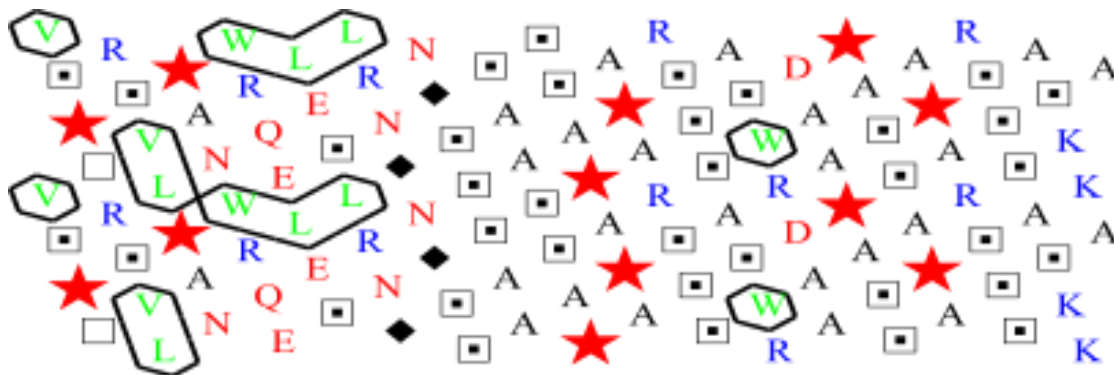


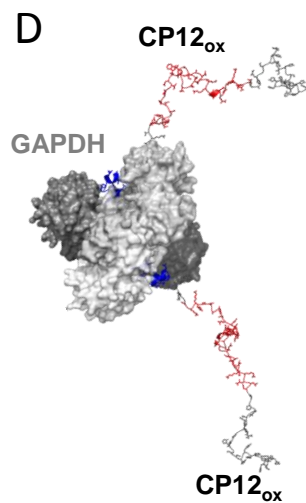
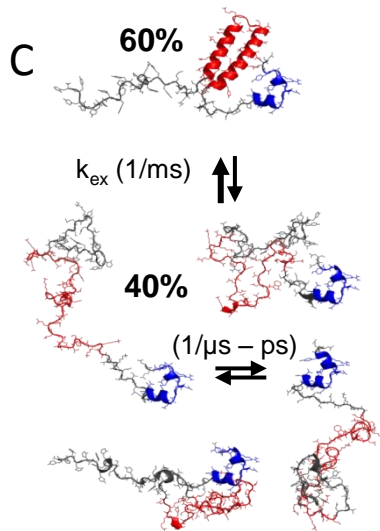
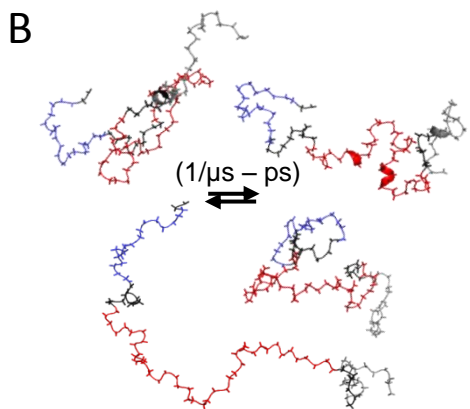
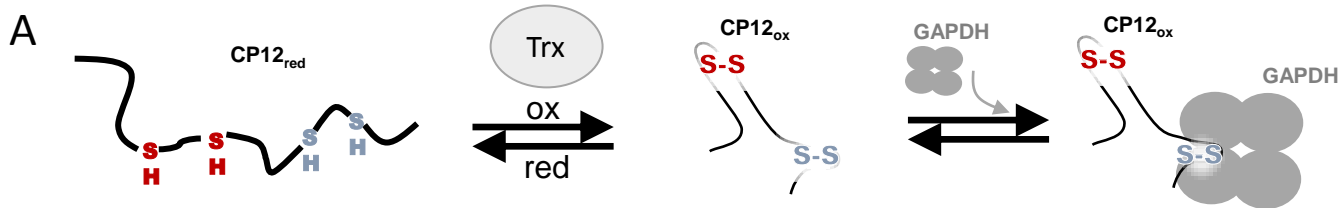
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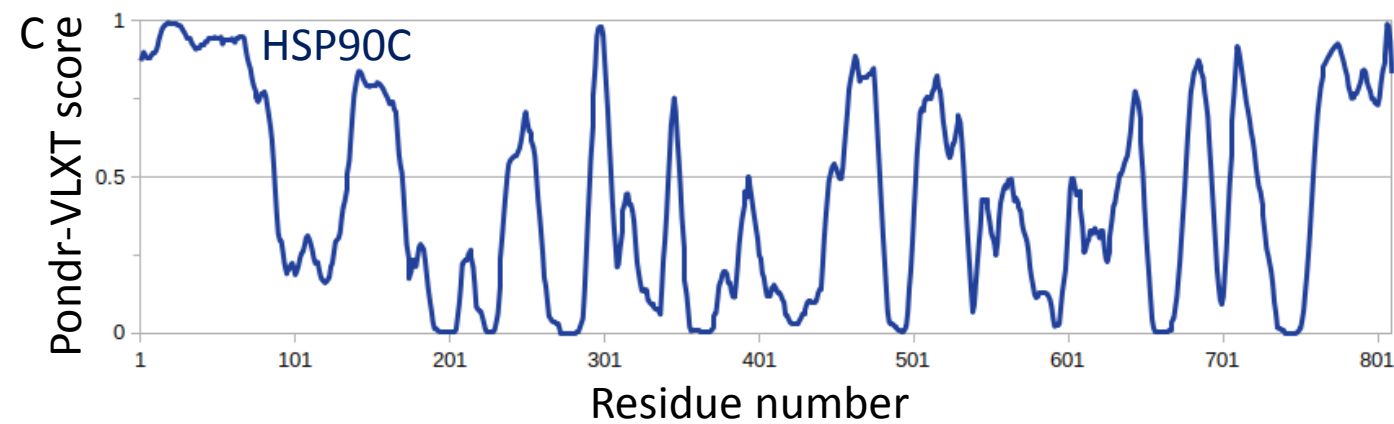
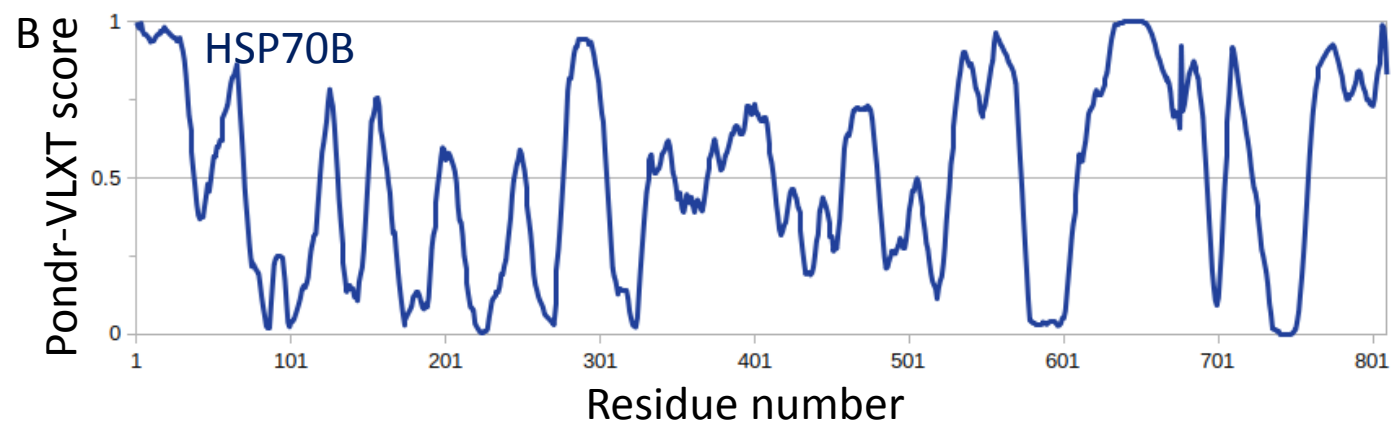
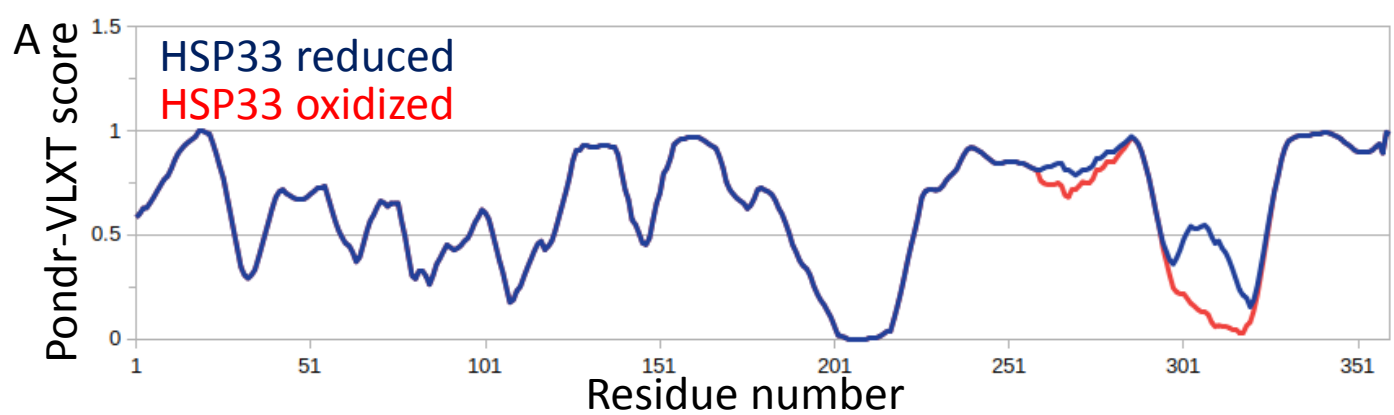
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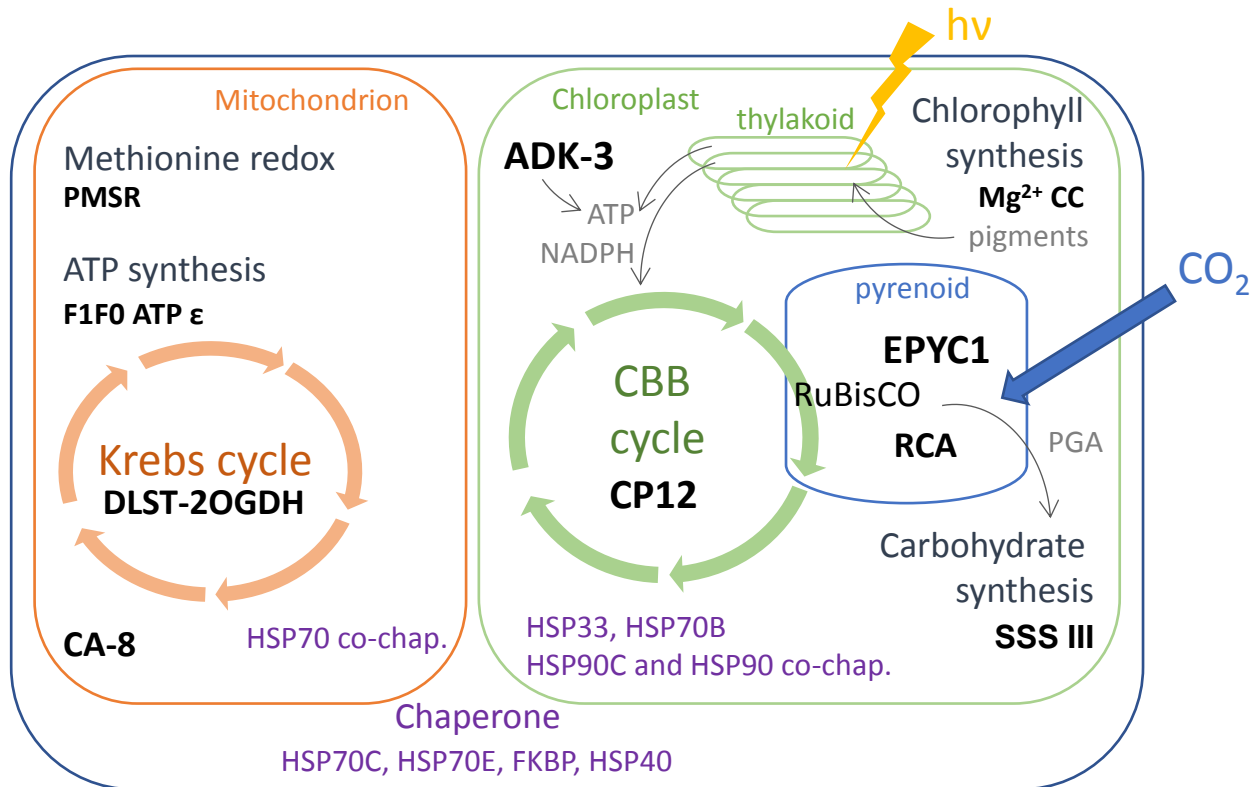
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