Calvin–Benson cycle regulation is getting complex

HIGHLIGHTS

- Two enzymes of the Calvin-Benson cycle, GAPDH and PRK, together with the regulatory protein CP12, can assemble into an inactive multimeric complex. With the recent characterization of the structures of free PRK and GAPDH/CP12/PRK ternary complexes, the hierarchical process of aggregation can be described at molecular definition.

- CP12-complexes are conserved in oxygenic phototrophs, but land plants also contain an auto-assembling GAPDH isoform, evolutionary derived from CP12. Both types of complexes form in the dark and dissociate in light, mainly under the control of thioredoxins and pyridine nucleotides.

- CP12 is a major light/dark regulator of the Calvin-Benson cycle in cyanobacteria and contributes to the more sophisticated regulation of the cycle in land plants, where dark-complexes may play an additional role in protecting enzymes from proteolysis.

TITLE

CALVIN-BENSON CYCLE REGULATION IS GETTING COMPLEX

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KEYWORDS

Redox regulation, protein complexes, photosynthesis, metabolism

ABSTRACT

Oxygenic phototrophs use the Calvin-Benson cycle to fix CO_2 during photosynthesis. In the dark, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK), two enzymes of the Calvin-Benson cycle, form an inactive complex with the regulatory protein CP12, mainly under the control of thioredoxins and pyridine nucleotides. In the light, complex dissociation allows GAPDH and PRK reactivation. GAPDH/CP12/PRK complexes are conserved from cyanobacteria to angiosperms and coexist, in land plant species, with auto-assembling GAPDH complexes that are analogously regulated. The recently described three-dimensional structures of all the elements of this ubiquitous regulatory system, together with novel genome editing techniques, opens a new avenue for understanding the regulatory potential of photosynthetic carbon fixation by *in vivo* site-specific mutagenesis.

MAIN TEXT

The Calvin-Benson cycle is the photosynthetic carbon reduction cycle of oxygenic phototrophs

Photosynthesis is fundamentally a redox process. Fixation of one C atom from CO_2 (oxidation state +4) into a C atom of a sugar (average oxidation state 0) requires four electrons that in oxygenic photosynthetic organisms are provided by the oxidation of two water molecules by the Oxygen Evolving Complex (OEC) of Photosystem II. In essence, oxygenic photosynthesis thus transfers electrons from water to carbon through a light-driven electron transport chain that starts with the OEC and ends up with an oxidoreductase, that is NAD(P)H-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (**Figure 1**). GAPDH does not reduce CO_2 directly, but it catalyzes the reaction that gives to the Calvin-Benson cycle its alternative names of photosynthetic carbon reduction cycle or reductive carbon fixation pathway [1,2] that underline the redox nature of this metabolism.

By mounting a CO_2 molecule on a sugar, Rubisco generates the organic acid that GAPDH will reduce to sugar again, thereby creating the conditions to reconstitute the substrate of Rubisco and set aside the fixed carbon in the form of sugar-phosphates (**Figure 1**). The Rubisco reaction is exergonic but the whole cycle requires three ATP per CO_2: two ATPs are used by phosphoglycerate kinase (PGK) to activate the products of Rubisco prior to reduction by GAPDH, and one ATP is used for the regeneration of the substrate of Rubisco

by phosphoribulokinase (PRK) (**Figure 1**). The two kinases together with three dephosphorylation steps allow the cycle to proceed spontaneously in one-way direction if sufficient ATP is made available by light reactions of photosynthesis.

Four consecutive reactions of the cycle depend on external inputs: phosphoribulokinase (ATP), Rubisco (CO_2), phosphoglycerate kinase (ATP) and GAPDH (NADPH). The first and last enzyme of this metabolic sequence are redox-regulated and can assemble into an inactive complex with a third partner protein known as CP12 (**Figure 1**). Reversible complex formation and modulation of GAPDH and PRK activities are mainly controlled by the thioredoxins redox state, and by tris-phosphorylated ($NADP^+$ and NADPH) versus bis-phosphorylated pyridine nucleotides (NAD^+ and NADH). Both parameters are perturbed by light/dark or stress conditions and provide a link between Calvin-Benson cycle regulation and environmental cues [3-6].

From cyanobacteria to land plants, all oxygenic photosynthetic organisms use the Calvin-Benson cycle to fix CO_2, and with a few exceptions, all contain CP12 (**Box 1**). Non-oxygenic phototrophic bacteria may contain a Calvin-Benson cycle based on deeply divergent enzymes in respect to cyanobacteria, but they do not have CP12 [7-10]. In this review, we provide a view of the regulation of the Calvin-Benson cycle, based on both functional studies and the recently solved atomic structures of all components and complexes of the CP12-regulatory system in different oxygenic phototrophs.

Phosphoribulokinase, the kinase that prepares the substrate for Rubisco

PRK belongs to the nucleoside/nucleotide kinase superfamily [11] and exists in three types: plant-type, found in most cyanobacteria, plants (Plantae) and photosynthetic protists; archeal-type and bacterial-type, the latter in proteobacteria and in oceanic a-cyanobacteria that contain bacterial-type Rubisco form 1A and no canonical CP12 genes [12-14]. Plant and archeal- types are dimeric and share a similar structure [14-17] while bacterial PRK is octameric with monomers showing limited structural similarity to plant/archeal types [18]. Among the three types, only plant-type PRK contain regulatory cysteines whose redox state is controlled by thioredoxins (TRXs) and coexists with CP12, which provides a further level of regulation (Box 1).

The structure of plant-type PRKs became available only in 2019 [14,16,17,19]. The core structure of the dimer is an extended mixed β-sheet formed by nine β-strands per subunit,

surrounded by additional secondary structures (**Figure 2**). The N-terminal regions, at the two ends of the β-sheet, show the characteristic nucleoside/nucleotide kinase superfamily fold and harbor the active sites [14]. Dimerization between C-terminal regions involves a single β-strand for each monomer and a dimer interface of about 550 $Å^2$, smaller than in both archeal and bacterial PRKs (~ 1,550 $Å^2$, [15,18]) and indicative of structural flexibility (**Figure 3**) [16]. C-terminal regions underwent extensive rearrangements along evolution of the three PRK types and are structurally more variable than N-terminal regions [14-16,18].

The active site includes a positive groove that provides complementary charges for the phosphate groups of ribulose-5-phosphate (Ru5P) and ATP (**Figure 2**) [14,16,17]. The characteristic P-loop of nucleotide-binding proteins contributes to the bonding network that places the ATP γ-phosphate near the carbon-1 of Ru5P. Between them Asp58 and His106 act as a catalytic base that activates the carbon-1 of Ru5P thereby favoring the attack of the γ-phosphate of ATP (**Figure 2**). No phospho-enzyme intermediate is necessary during catalysis [17].

Binding assays pointed out an obligate sequence for substrates entry. ATP is bound first and causes a conformational change that permits Ru5P to fit in its pocket [17]. A structural study on a kinase of the same superfamily (uridine-cytidine kinase; [20]) suggests that the movement of a helical hairpin usually referred as the lid determines the order of substrate binding [14]. When PRK is bound to CP12 in the ternary complex, the lid is fixed in open conformation by CP12 itself (**Figure 2E**).

Together with CP12, PRK is part of the minimal redox toolkit that controls the carbon flux in day/night cycles in cyanobacteria [21]. PRK regulation, based on disulfides that can form in either PRK or CP12, is conserved in cyanobacteria and along the green lineage [10] (**Box 1**). Regulatory cysteines of the N-terminal region (Cys15/Cys54 in arabidopsis (*Arabidopsis thaliana*) and Cys16/Cys55 in *Chlamydomonas reinhardtii* [16]) are responsible for the oxidative inactivation of PRK itself [22] (**Figure 2D**). Cys15 (arabidopsis numbering in the following) is contained in the P-loop which flips to allow the formation of the disulfide with Cys54 [14,17,19]. The reactivity of Cys15 is ensured by a conserved molecular environment within the P-loop [16,17]. Plant-type PRKs may form a second disulfide in the C-terminal region [14,16,23](**Figure 2A**) which is found when the enzyme is in complex with GAPDH and CP12 [17,19]. The C-terminal disulfide does not affect the activity but appears necessary for GAPDH/CP12/PRK assembly in *Chlamydomonas reinhardtii* [23].

TRX *f* is the most efficient reductant of PRK N-terminal disulfide [16,22,24]. The interaction between TRX *f* and PRK was proposed to be mediated by complementary charges around Cys54 in PRK and active site cysteines in TRX *f* [16,25]. In an alternative model, a conformational swing of the flexible clamp loop of PRK would be required to expose Cys54 to the TRX attack [17]. The clamp loop is found in algae and plants (that contain TRX *f*), but not in cyanobacteria that do not contain TRX *f* [10,16,17,26]. Notwithstanding the still debated role of the clamp loop, the presence of flexible elements in plant-type PRKs (dimer interface, lid, clamp loop) confers conformational adaptability to more rigid partners like TRXs and GAPDH.

Glyceraldehyde-3-phosphate dehydrogenase, the enzyme of the photosynthetic carbon reduction

Land plants genomes typically contain four different types of GAPDH genes (*GAPA, GAPB, GAPC, GAPCp*) whose protein products form four tetrameric isoforms (A_4 and A_2B_2 in chloroplasts; C_4 in the cytosol; Cp_4 in heterotrophic plastids). Green algae and cyanobacteria contain only A_4- and C_4-GAPDH isoforms (**Box 1**).

The 3D structure is known and well conserved in all GAPDH isoforms (**Figure 3**). Each subunit contains a catalytic domain that binds the substrate, and a cofactor-binding domain that binds, in the same position, either NAD(H) or NADP(H). An S-loop belonging to the adjacent subunit contributes to cofactor stabilization and recognition [27,28]. Specificity toward pyridine nucleotides varies from glycolytic GAPDH isoforms C_4 and Cp_4, that are NAD(H)-specific, to A_4 and A_2B_2-GAPDH of the Calvin-Benson cycle that can use both NADP(H) and NAD(H) (**Box 1**). Bispecific GAPDHs (A_4, A_2B_2) are typically regulated by interaction with CP12 and PRK, but A_2B_2-GAPDH is also specifically regulated in a CP12-independent manner [29].

In NADPH, the ribose closest to the adenine is 2'-phosphorylated compared with NADH that carries a hydroxyl group in the same position. In GAPDH isoforms that bind NAD(H), the 2'-hydroxyl of NAD(H) makes a hydrogen bond with Asp32, whose fixed position prevents NADP(H) from binding [28]. Bispecific GAPDHs (A_4 and A_2B_2) can substitute NAD(H) with NADP(H) by letting Asp32 to rotate away from the extra-phosphate. Residues Arg77, Thr33 and Ser188 (*Spinacia oleracea* numbering [30]) interact with the NADP(H) 2'-phosphate (**Figure 3**). NADP(H) recognition results in faster NADP(H) vs. NAD(H)-dependent catalysis, in

spite of the identical redox properties of the two coenzymes. Replacement of specific NADP(H)-interacting residues with non-interacting ones results in GAPDH mutants with decreased NADP(H)-dependent activity, but similar NADH-dependent activity [30,31]. Understanding coenzyme recognition is important for understanding GAPDH regulation because it specifically affects the NADP(H)-dependent activity, leaving the NAD(H)-dependent one unaffected [29].

The catalytic domain contains two anion recognition sites, named P_i and P_s, which harbour the phosphate groups of BPGA (**Figure 3**). In the P_s site, the thiolate of Cys149, activated by His176, makes a covalent bond with the substrate prior to its reduction. As detailed below, GAPDH may participate in different types of complexes (e.g. A_4-GAPDH/CP12/PRK, A_8B_8-GAPDH) without significantly changing the overall structure of the tetramer which appears remarkably rigid (**Figures 3,4**). Flexible partners need to adapt to GAPDH, not viceversa, a concept that is probably valid also for the manifold interactions that glycolytic C_4-GAPDH displays in its moonlighting functions [32,33].

CP12, the PRK/GAPDH regulatory protein

CP12 is an intrinsically disordered protein of about 80 amino acids [8,9,34-36] that adopts a defined tridimensional structure after binding to its partners GAPDH and PRK. Canonical CP12 is characterized by four conserved cysteines able to form two disulfide bridges and the consensus sequence AWD_VEEL. The N-terminal region of CP12 contains one of the two cysteine pairs followed by the consensus sequence which is involved in PRK binding. The C-terminal region contains the second cysteine pair and binds GAPDH. Genes coding for canonical CP12 are present in almost all genomes of organisms that fix CO_2 via the Calvin-Benson cycle, in particular cyanobacteria, green algae and land plants [9,8] (**Box 1**). Notable exceptions include oceanic α-cyanobacteria which contain only CP12 genes with neither the N-terminal disulfide nor the consensus sequence [13], and prasinophycean green algae like *Ostreococcus tauri* which contain no CP12 genes at all [37]. On the other hand, *Ostreococcus* species are the only green algae (Chlorophyta) known to contain redox-regulated B subunits of GAPDH, which are typical of Streptophyta (land plants and charophycean algal ancestors) [38].

The disulfide bridges of CP12 are formed in the presence of oxidized TRX [24] or other oxidants (GSSG, H_2O_2)[39], and the C-terminal disulfide requires less oxidizing power than

the N-terminal one ($E_{m,7.9}$ -352 and -326 mV, respectively, in arabidopsis CP12-2)[40]. The N-terminal region of CP12, from either arabidopsis or *Anabaena*, was recently exploited to develop FRET-based sensors detecting the thioredoxin redox state *in vivo* [5].

Although oxidized CP12 remains essentially disordered, a local structural motif in the C-terminal domain is stabilized by its disulfide bridge, allowing the interaction with GAPDH and thus the initiation of the ternary complex assembly [41,42]. Based on this property, CP12 has been defined as a conditionally rather than intrinsically disorderd protein [42]. The oxidized N-terminal domain folds into a stable two-helix bundle only after CP12 has bound to GAPDH [19,43] or to other protein domains like in cyanobacterial fusion proteins with cystathionine β-synthase domains (CP12-CBS; **Box 1**)[13,44]. In any case, the N- and C-terminal domains of CP12 are connected by a flexible linker that remains flexible even when the rest of CP12 is fully folded [17,19](**Figure 2**).

GAPDH/CP12, the intermediate complex that recruits PRK

The GAPDH/CP12 association is the first obligatory step of the hierarchical assembly of the ternary complex with PRK [40,45](**Figure 1**). Two prerequisites allow the interaction to occur: GAPDH has to be loaded with NAD(H), rather than NADP(H), and CP12 has to bear the C-terminal disulfide. The structure of the GAPDH/CP12 complex has been solved from arabidopsis [41] and two different cyanobacteria [19,46].

The binding of disordered CP12 to rigid GAPDH implies an entropic penalty (5 kcal mol^{-1}) as CP12 becomes ordered, that has to be counteracted by enthalpy (energy released by novel interactions, -15 kcal mol^{-1}; 40]. The mechanism of formation of the GAPDH/CP12 binary complex of arabidopsis was proposed to involve a conformational selection step (GAPDH binds a defined local conformation of CP12) followed by an induced folding step, in which CP12 slips into the active site of GAPDH where it is stabilized by an extensive network of hydrogen bonds [41]. As a result, P-sites of GAPDH are fully occupied by CP12, and Glu72 of CP12 occupies the binding site for the 2'-phosphate group of NADP(H) [41,46](**Figure 3**). For this structural reason, the GAPDH/CP12 complex is stable in the presence of NAD(H) while NADP(H) can disrupt the complex and counteract the inhibition of the NADP(H)-dependent activity of GAPDH [45,47,48]. In the binary complex, the NAD(H)-dependent activity is instead inhibited because the binary complex is stable and only two active sites out of four can perform the catalysis [19] (**Figure 3**).

GAPDH/CP12/PRK, the complex of temporarily inactivated enzymes

The ternary complex consist of two GAPDH tetramers and two PRK dimers linked by four oxidized CP12 and results from the capability of GAPDH/CP12 binary complexes to bind PRK dimers [35,39,45,49](**Figure 3**). Similar atomic structures of GAPDH/CP12/PRK complexes were recently obtained by cryo-EM from the cyanobacterium *Thermosynechococcus elongatus* [19] and X-ray crystallography from *Arabidopsis thaliana* [17]. The ternary complex shows a spindle-shape with a hole in the middle, GAPDH tetramers at the two tips and two CP12/PRK/CP12 elements connecting them (**Figure 1 and 3**).

Once formed the GAPDH/CP12 complex, the oxidized N-terminal domain of CP12 folds into a two-helical bundle [19] that may plug into the PRK active site [17](**Figure 2**). Positive residues of PRK, mainly belonging to the P-loop and lid, and normally involved in phosphates binding, interact with the negative consensus sequence of CP12 (AWD_VEEL)[14,16,17,19]. Accommodation of CP12-helical bundle in the catalytic groove of PRK fixes the lid in open conformation [19].

Whether the redox state of PRK disulfides is relevant for complex formation is not yet clear. Oxidation of C-terminal cysteines may fix PRK in a favorable conformation for complex assembly [23] and indeed, the C-terminal cysteines of ternary complex PRK are usually, but not always, engaged in a disulfide [17,19]. Conversely, the N-terminal disulfide is probably dispensable as even in the complex, the P-loop of PRK can flip and promote the formation of the disulfide without clashing with CP12 [17]. Thanks to their flexibility [16], complexed PRKs can assume a twisted conformation compared to free PRKs [17,19] and CP12/PRK binding is slightly different in different species [17]. In general, the young components of the systems (in evolutionary terms, PRK and CP12, [50]) seem to adjust their conformations to the firm structure of old GAPDH tetramers, and not viceversa.

Ternary complex structures clearly show that 100% of PRK- and 50% of GAPDH-active sites are blocked by CP12, and that CP12 interferes with coenzyme binding in the remaining GAPDH subunits (**Figure 3**). Since PRK in the complex can exist in different redox states [51,52], CP12 provides a way to inhibit PRK activity independently from PRK redox regulation. Moreover, CP12 is the only way to regulate A_4-GAPDH. Experimentally measured, both PRK and GAPDH activities of the complex are strongly inhibited but total inhibition is never

observed because the substrates of the reactions (ATP, BPGA, NADPH) may all contribute to complex dissociation by competing with CP12-binding sites [43,47,48,52](**Figure 4**). *In vitro* at least, the higher plant complex is dissociated in the minutes time scale ($t_{1/2}$ 0.3-0.6 min)[24] upon reduction of the N-terminal disulfide of CP12 by TRX *f* [17,24]. Interestingly, PRK reactivation by TRXs is faster when PRK is part of the ternary complex than free oxidized form, suggesting that CP12 represent a quicker way to control PRK activity [24]. Overall CP12 acts as an entropy-driven redox switch that triggers complex dissociation when it is reduced and the resulting increase in entropy is the driving force for prompt reactivation of enzymes activities [40].

Besides the canonical binary (GAPDH/$CP12_2$) and ternary complexes here described ($GAPDH_2$/$CP12_4$/PRK_2), further possibilities may exist. For instance, GAPDH-$CP12_4$ complexes were obtained *in vitro* with cyanobacteria proteins at high CP12:GAPDH ratios [19, 46] and reconstituted GAPDH/CP12/PRK complexes of *Chlamydomonas reinhardtii* were found to bind aldolase *in vitro* with strong affinity (K_D 55 nM) [53]. Whether these complexes do exist *in vivo* in chlamydomonas or other organisms is currently unknown. In higher plants at least, the CP12-complexes that could be extracted from tobacco plants did not contain any other protein than GAPDH (A and B subunits) and PRK [54].

The A_2B_2-GAPDH isoform of land plants has acquired from CP12 its regulatory properties

In land plants and charophycean algal ancestors (Streptophytes) CP12 coexists with A_2B_2-GAPDH, which is a regulated version of A_4-GAPDH [37, 55-57](**Box 1**). All regulatory properties of A_2B_2-GAPDH depend on the C-terminal extension (CTE) of B-subunits that is homologous to the C-terminal domain of CP12 [56,58]. Similar to A_4-GAPDH, fully active A_2B_2-GAPDH shows a marked kinetic preference for NADPH over NADH based on both higher V_{max} (1.5-fold) and 5-fold lower K_m [30,31]. The kinetic preference is based on proper coenzyme recognition and abolished in oxidized A_2B_2-GAPDH, that carries a disulfide bridge in the CTE and uses NADPH and NADH with similar and low efficiency [58].

When the CTE, which is negatively charged, is oxidized, it is attracted by the positive cleft between A and B subunits, the same cleft occupied by CP12 in GAPDH/CP12 complexes (**Figure 3**). However, the position of the CTE is less deep within the cleft and its C-terminus does not reach the P-sites like CP12 [17,19,46,59]. Nevertheless the NADP(H)-dependent activity is inhibited because none of the residues involved in NADP(H) recognition (Ser188;

315 Thr33; Arg77) are correctly in place [59]. Arg77 is displaced from its normal position, possibly
316 because attracted by negative charges of the CTE. Ser188 and Thr33 do not interact with
317 $NADP^+$ either (**Figure 3**). As a result, the 2'- phosphate of NADP(H) is either completely free
318 (in subunits B) or (in subunits A) loosely interacting with the last residue of the CTE (Glu362),
319 itself engaged in a salt bridge that anchors the CTE to the cleft [59]. Consistent with the
320 concept that the kinetic preference for NADP(H) depends on the capability to recognize
321 NADP(H) from NAD(H), the NADPH-activity is down-regulated to the level of the constitutive
322 NADH-dependent one [31,58]. Mutants analyses were consistent with this model: by
323 introducing single mutations it was possible to convert a redox-sensitive GAPDH (with B-
324 subunits) into a redox-insensitive GAPDH (A_4-like), or an A_4-GAPDH (behaving like
325 reduced/activated A_2B_2-GAPDH) into an oxidized/inhibited A_2B_2-like GAPDH [30,31,59].
326
327 Understanding the regulation of A_2B_2-GAPDH is complicated by the fact that when bound
328 NADP(H) is substituted by NAD(H), the oxidation of the CTE causes the aggregation of the
329 tetramers in inhibited multimeric complexes (mainly A_8B_8, but also A_4B_4 and others,
330 [55,58,60,61]). Inhibited complexes of A_2B_2 are the only members of the GAPDH/PRK
331 regulatory system whose atomic structure has not been yet described.
332
333 **Relevance and *in vivo* evidence of CP12-dependent and independent regulation of the**
334 **Calvin-Benson cycle**
335
336 Both PRK, CP12 and A_2B_2-GAPDH contain cysteines able to form disulfide bridges with
337 negative effects on enzyme activities and positive effects on complex formation (**Figure 4**).
338 *In vitro*, such disulfides are efficiently reduced by TRXs *f* and *m* [24] whose redox states, *in*
339 *vivo*, tend to be reduced in the light [5,62]. Under same conditions, PRK and A_2B_2-GAPDH
340 are also found reduced, enzymatically active and free from complexes in different higher
341 plant species [52,54,60,62-66]. The reduction/activation/dissociation state of both GAPDH
342 and PRK correlate with light intensity in the minute-time scale and is blocked by inhibitors of
343 the photosynthetic electron transport chain required to reduce TRXs [52,62]. Besides TRXs,
344 NTRC does also contribute to reduction/activation of the system, possibly by direct
345 interaction with PRK [63,66].
346
347 In the dark, PRK and GAPDH (and TRXs *f*/*m*) are conversely detected in oxidized form [63-
348 67]. Electrons derived from disulfide bridge formation are shuttled to 2-cys peroxiredoxins
349 (2CP) by atypical TRXs ACHT and TRXL2, with hydrogen peroxide acting as final electron

acceptor [65-68](**Figure 4**). *In vitro*, hydrogen peroxide may directly induce disulfide bridges formation in CP12, and A_4-GAPDH, whose catalytic cysteine is highly sensitive to H_2O_2-oxidation to sulfinic acid, is fully protected when assembled in ternary complex with CP12 [39]. Whether this protective mechanism is operative also *in vivo* is currently unknown.

Activities of both PRK and GAPDH rapidly drop in the dark in wild type arabidopsis plants but much more slowly in 2CP-knock out mutants [65,66]. Dark-inactivation takes few minutes and correlate with the sequestration of GAPDH and PRK in CP12-complexes [52,61] and A_8B_8-GAPDH [60,62], although not in all species and with all techniques dark-complexes are easily detected [61,69]. In most species, PRK is found fully sequestered in dark-complexes but some GAPDH remains as free tetramers (A_2B_2; A_4) [52,61]. Recent quantifications of Calvin-Benson cycle enzymes in *Chlamydomonas reinhardtii* consistently showed that A-subunits of GAPDH exceed PRK subunits by a factor of 4 [70]. Since the GAPDH/PRK subunit ratio of the ternary complex is invariably 2 [17,19,52] (**Figure 3**) we argue that, in *Chlamydomonas* at least, there's no enough PRK to sequestrate all GAPDH in dark-complexes.

The existence of ternary complexes implies that also CP12 must be oxidized in the dark *in vivo*, in agreement with *in vitro* redox potential determinations [24]. It is also known from *in vitro* studies that GAPDH/CP12/PRK and A_8B_8-GAPDH are stabilized by low NADP(H)/NAD(H) ratios that seem to be established in darkened chloroplasts [71,4] and in cyanobacteria [48,72] thereby favoring dark-complexes stability (**Figure 4**).

Altogether, plenty of *in vivo* evidence support the notion that oxygen phototrophs temporarily store PRK and GAPDH in inactive complexes of known atomic structure under low photosynthetic conditions or in the dark, and dissociate such complexes to release the active enzymes during illumination. The relative importance of thioredoxin redox state, NAD(H)/NADP(H) ratios or activating metabolites like BPGA and ATP in the equilibrium between ternary complex and free enzymes *in vivo* remains to be understood.

Further studies demonstrated, particularly in cyanobacteria, that the CP12-complex is important for regulating the Calvin-Benson cycle under diel light/dark cycles. A mutant strain of *Synechococcus* PCC7942 lacking a canonical CP12, grew normally in continuous light but slower than wild type cells in 12h/12h light/dark cycle, indicating that dark-inactivation of GAPDH and PRK is not dispensable [48]. Marine a-cyanobacteria like

Prochlorococcus which do not contain a canonical CP12 gene in their genome [73](**Box 1**) can be infected by cyanophages (bacteriophages that infect cyanobacteria) that do contain a *CP12* gene in their genome of only 24 genes [72]. Optimal reproduction for these parasites requires ATP and NADPH produced by light reactions of photosynthesis. The expression of phage CP12 in infected cells is induced in the light and the resulting inhibition of the Calvin-Benson cycle from PRK to GAPDH diverts photosynthetically produced ATP and NADPH from carbon fixation to phage replication [73](**Figure 1**). The concept that CP12 is a master regulator of the Calvin-Benson cycle in cyanobacteria in light/dark conditions was recently exploited in a successful biotechnological strategy aimed in rewiring carbon metabolism in glucose-fed *Synechococcus elongatus* PCC7942 cells. Deletion of CP12 combined with overexpression of PRK, allowed the PRK-to-GAPDH portion of the Calvin-Benson cycle to function in the dark in these cells [74], further confirming the role of CP12 in regulating the Calvin-Benson cycle according to light availability. Moreover, many cyanobacterial species contain CP12-CBS proteins in which CP12 is fused to two cystathionine β-synthase domains (**Box 1**). CP12-CBS do not bind GAPDH but can bind and inhibit PRK in an AMP-dependent manner [44].

In higher plants, the physiological role of CP12 is also complex and not yet fully understood. *Arabidopsis thaliana* contains three CP12 paralogues with tissue-specific expression [29,9,75,76]. CP12-1 and CP12-2 are closely related and represent a single CP12 form found throughout the higher plants, while CP12-3 represents a divergent CP12 form not universally present in higher plants [8]. In arabidopsis, CP12-1 and CP12-2 are highly expressed in leaves but no phenotypic alterations were observed in single CP12 mutants. On the contrary, transgenic lines with strongly reduced levels of both CP12-1 and CP12-2 were negatively affected in photosynthetic capacity and biomass yield [77]. Intriguingly, the amount of PRK, but not its transcript level, was dramatically reduced in CP12-double mutants, suggesting that the stability of PRK might be under control of CP12 with functional consequence on photosynthetic capacity and growth. While opening a new perspective on a possible role of CP12-complexes in preserving PRK from degradation in the night, these results prevent an easy demonstration of CP12 function in higher plants based on classical reverse genetic approaches.

Concluding remarks and Future perspectives

GAPDH and PRK, two essential enzymes of the Calvin-Benson cycle are stored as inactive complexes in the dark in most oxygenic phototrophs. Complex assembly depends on the regulatory protein CP12 and, in land plants, also on the CP12-derived C-terminal extension of GAPDH B-subunits. An overarching control on the regulatory system is mediated by the TRXs redox state, pyridine nucleotides and metabolites (ATP and BPGA), allowing inactivated complexes to release active enzymes and viceversa, depending on light conditions and photosynthetic activity. Structure/function relationships in GAPDH and PRK regulation through complex formation are beginning to be understood.

Reverse genetics, the default approach for studying gene function, is of little use for studying regulatory mechanisms based on post-translational modifications and protein-protein interactions like the CP12-(in)dependent regulation of GAPDH and PRK of the Calvin-Benson cycle. To address these questions, specific amino acids mutations are more informative than gene knockouts. This approach requires detailed knowledge of protein structures/function relationships and sophisticated genome editing techniques [78], but we believe that protein-based *in vivo* studies will soon integrate traditional genomic approaches in plant physiology.

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OUTSTANDING QUESTIONS

- Which is the dynamics of diphosphorylated (NAD) versus triphosphorylated (NADP) pyridine nucleotides in higher plants chloroplasts in light/dark cycles and fluctuating light conditions?
- Are dark-complexes (GAPDH/CP12/PRK and A_8B_8-GAPDH) of land plants important for adaptation to fluctuating light?
- Is NTRC (NADPH-thioredoxin C) important for dark-complexes dissociation?
- Is the recently described electron chain based on atypical thioredoxins ACHT/TRXL-2 and 2-cys peroxiredoxins important for dark-complexes association?
- How the CP12-derived C-terminal extension of subunits B makes A_2B_2-GAPDH of land plants able to auto-assemble in A_8B_8-GAPDH and other types of GAPDH-complexes? Complexes of AB-GAPDH are the only elements of the regulatory system of GAPDH and PRK in land plants whose tridimensional structure is not yet know.
- How the dynamics of dark-complexes formation and dissociation could be detected *in vivo* in real time?
- How do complexes relate to stress conditions *in vivo*? Reactive oxygen species are produced in several types of stress and, *in vitro*, hydrogen peroxide oxidizes CP12 and induces aggregation/protection of GAPDH and PRK, but whether this mechanism is relevant *in vivo* is not yet known.

- Can the effect of CP12 in protecting PRK from proteolysis in *Arabidopsis thaliana* be extended to other species and/or other types of complexes like A_8B_8-GAPDH? Whether dark-complexes may have a general role in proteostasis is not yet known.
- Why land plants have so many regulatory mechanisms of the Calvin-Benson cycle at the level of GAPDH and PRK, with apparently overlapping functions? AB-GAPDH and PRK are both redox-regulated, both individually and through CP12; AB-GAPDH and A_4-GAPDH/PRK are both able to form inactive complexes under similar control (thioredoxins and pyridine nucleotides).

LEGENDS TO FIGURES

Figure 1. Photosynthesis is a redox-regulated redox process. (a) The oxidation state of C in CO_2 is (+4). The average oxidation state of C atoms in sugars is (0). Rubisco adds a CO_2 (+4) to RuBP (0) generating two molecules of PGA (+2 x 2). PGK adds ATP to PGA, and GAPDH uses four electrons (e^-) from NADPH to reduce BPGA (+2 x 2) to G3P (0). NADPH is derived from light reactions of photosynthesis. The electron transport is coupled to trans-thylakoidal proton motive force that generates ATP via ATP-synthase. G3P regenerates Ru5P in a series of reactions with P-sugar intermediates (*not shown*). Two irreversible phosphatases drive the Ru5P regeneration from G3P. PRK and PGK restore the phosphates lost by the cycle. (b) In oxygenic phototrophs, PRK and GAPDH can form a regulatory complex with CP12. The hierarchical process of aggregation is structurally characterized. GAPDH [27,28] forms a binary complex with CP12 when NAD(H) substitutes NADP(H) in GAPDH and CP12 is oxidized (4 electrons) and bears two disulfides [52,58]. PRK is redox-regulated [14,16,17] and both PRK forms can bind the GAPDH/CP12 binary complex to form the ternary complex $GAPDH_2/CP12_4/PRK_2$ in which both enzymes are strongly inhibited [17,19]. Complex dissociation and enzyme reactivation is obtained by reduced thioredoxins or NADP(H) or BPGA or ATP, with different efficiency [79]. Abbreviations: BPGA, 1,3-bisphosphoglyceric acids; CET, cyclic electron transport; G3P, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LET, linear electron transport; OEC, oxygen evolving complex; PGA, 3-phosphoglyceric acid; PGK, phosphoglycerate kinase; PQ, plastoquinone; PRK, phosphoribulokinase; PSI, photosystem I; PSII, photosystem II; Ru5P, ribulose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; TRX, thioredoxin.

Figure 2. Phosphoribulokinase: structure and regulation. (a) Dimer of algal PRK (*Chlamydomonas reinhardtii*, [16]), the left subunit is represented as surface potential (red,

722 negative; blue, positive), the right subunit as cartoon with cysteines as yellow spheres. The
723 large positive area between C16 and C55 corresponds to the active site. The C16-C55
724 disulfide inhibits PRK activity. The C243-C249 disulfide has no effects on activity but is
725 commonly found in ternary complexes. The lid (green) is suspected to move during catalysis.
726 Correspondence between *A. thaliana* and *C. reinhardtii* Cys numbering is shown on top of
727 the panel. (b) Algal PRK overview with the central 18-strands β-sheet highlighted in colours.
728 The small dimer interface is represented by two short β-strands (red). (c) Cyanobacterial PRK
729 active site with substrate analogues (*Synechococcus elongatus*, [17]). G6P and ADP are
730 located in Ru5P and ATP sites, respectively. The positive surface of the binding sites is
731 complementary to the negative phosphate groups. Histidine-106 (H106) and aspartate-58
732 (D58) form the catalytic base activating carbon-1 of Ru5P. (d) Reduce/active (blue) and
733 oxidized/ inactive (red) plant PRK (*Arabidopsis thaliana*, [17]). The movement of the P-loop
734 represents the only relevant structural difference between reduced and oxidized PRK. After
735 P-loop flipping, C15 and C54 are close enough to form a disulfide that distorts the active
736 site and inhibits PRK activity. (e) CP12 (yellow cartoon) inhibits PRK (surface potential)
737 occupying its active site (detail from the arabidopsis $GAPDH_2$-$CP12_4$-PRK_2 complex [17],
738 after digital removal of GAPDH). The CP12 consensus sequence (AWD_VEEL, red) interacts
739 with positive patches of the Ru5P binding site. The approximate positions of the binding sites
740 of ATP and Ru5P are indicated. The lid is open and CP12 prevents it to close. Abbreviations:
741 G6P, glucose-6-phosphate; Ru5P, ribulose-5-phosphate.
742
743 **Figure 3. Crystal structures of GAPDH isoforms and A_4-GAPDH/CP12 binary complex.** (a)
744 Typical GAPDH tetramer with subunits in different colours (crystal structure of GAPDH from
745 *Spinacia oleracia* [30]). In the upper-left subunit, the cofactor-binding domain (salmon) that
746 can bind either NAD(H) or NADP(H) is differentiated from the catalytic domain (red) that
747 binds the substrate. Upper magnification: the 2'-hydroxyl group of NAD(H) (yellow) is
748 stabilized by D32 (yellow), while the 2'-phosphate of NADP(H) (cyan) interacts with R77 and
749 T33 (cyan), and S188 of the S-loop of the adjacent subunit (light-blue). In bispecific GAPDHs
750 the binding of NADP(H) is made possible by the rotation of D32 (cyan) away from the 2'-
751 phosphate of NADP(H). Dashed lines indicate interactions $\leq$ 4 Å [28,30]. Lower
752 magnification: the active site contains the C149-H176 catalytic pair. The P_i and P_s sites
753 allocate BPGA phosphate groups (substituted by sulphate ions in the crystal structure) [30].
754 (b) In the A_4-GAPDH/CP12 binary complex, the C-terminus of CP12 (cyan) fits into the cleft
755 separating two A-GAPDH subunits, with Y76 and N78 occupying the P-sites of the light-blue
756 subunit, preventing substrate binding, while E72 prevents NADP(H)-binding to the opposite

(yellow) subunit [41]. (c) In A_2B_2-GAPDH, oxidized CTE (green) occupies the cleft between B (light-green) and A (sand) subunits, similarly to CP12 in the binary complex. However, CTE does not occupy the P-sites but causes the 2'-phosphate of NADP(H) of the B-subunit (light-green) to loose crucial interactions with T33, R77 and S188, which are responsible for the high NADP(H)-dependent activity of the enzyme [59]. Atom colour codes: oxygen red; nitrogen blue; sulphur yellow.

Figure 4. Current view of GAPDH/PRK regulation based on combined *in vivo* and *in vitro* evidence. The model is based on data collected from different organisms and its general principles apply to cyanobacteria, green algae and land plants, although AB-GAPDHs are only found in land plants [58]. From left to right: at variable light conditions, redox-sensitive proteins CP12, PRK and A_2B_2-GAPDH equilibrate with thioredoxins and tend to be reduced, enzymatically active and free from complexes [79]. In light-to-dark transitions, atypical thioredoxins (ACHT, TRXL-2) with less negative redox potential than thioredoxins *f* and *m* [68], convey electrons from reduced targets to hydrogen peroxide via 2-cys peroxiredoxins [66-69]. Chloroplast NADP(H)/NAD(H) ratio tend to decrease in the dark [4,72], favouring the substitution of NADP(H) with NAD(H) in bispecific GAPDHs. Oxidation of CP12 and C-terminal extension (CTE) of GAPDH B-subunits, and low NADP(H)/NAD(H) ratios, favour the formation of dark-complexes of inactive enzymes ($GAPDH_2CP12_4PRK_2$ and A_8B_8-GAPDH)[17,19,48,55]. In dark-to-light transitions and then in full light, dark complexes are dissociated and enzyme activities fully recovered. Thioredoxins *f* and *m*, reduced by ferredoxin-thioredoxin reductase at the onset of light reduce CP12 and CTE disulfides, causing dark-complexes dissociation [24,55]. NADPH-thioredoxin C (NTRC) may contribute to the same effect [64,65]. Complexes dissociation is aided by NADPH, BPGA and ATP, all able to bind in different positions and cause CP12 or CTE displacement from their binding sites [45,48,59]. Dashed arrows indicate interactions that are not fully proven yet. Abbreviations: 2CP, 2-cys-peroxyredoxins; BPGA, 1,3-bisphosphoglyceric acids; CTE; C-terminal extension of GAPDH B-subunits; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NTRC, NADPH-thioredoxin C; PRK, phosphoribulokinase; TRX, thioredoxin.

TEXT BOXES

Box 1 - Evolution and biodiversity of GAPDH, PRK and CP12 along the green lineage

Early times: before primary endosymbiosis

Early eukaryotes contained a NAD-specific GAPDH isoform of eubacterial origin, localized in the cytosol and involved in glycolysis. This prototypical GAPDH, named C_4-GAPDH and coded by GAPC genes, is ubiquitously expressed in present eukaryotes [51] (Figure I). Cyanobacteria, early and present ones, contain a C_4-like GAPDH, coded by GAP1 genes, but also a GAPDH of the A_4-type coded by GAP2 genes [80]. A_4-GAPDH is characterized by the double specificity for pyridine nucleotides and the role in the Calvin-Benson cycle. Early cyanobacteria, also contained PRK and canonical CP12 with conserved N- and C-terminal disulfides and central consensus sequence [73], hence potentially able to bind A_4-GAPDH and PRK [10].

Origin of photosynthetic eukaryotes

Early photosynthetic eukaryotes formed by primary endosymbiosis contained C_4-GAPDH derived from the eukaryotic host and A_4-GAPDH of cyanobacterial origin located in chloroplasts, together with PRK and canonical CP12. GAP1 genes coding for cyanobacterial C_4-GAPDH were apparently lost after endosymbiosis. The set of genes coding for GAPDH (GAPC and GAPA), CP12 and PRK in early photosynthetic eukaryotes is typically found conserved in green algae and land plants of present days, possibly with paralogues derived from whole genome duplications [10,73,51]. Unicellular prasinophycean algae (an early diverging group of green algae with members that are prominent in the oceanic picoplankton) like *Ostreococcus tauri* represent an exception in having no CP12 in their genome[37].

CP12 and PRK biodiversity in cyanobacteria

Besides canonical CP12 genes, cyanobacteria may also contain genes for CP12 variants that are not found in green photosynthetic eukaryotes [73]. These include CP12 isoforms with no C-terminal cysteines and/or N-terminal cysteines and/or central consensus sequence. Oceanic species included in genera *Prochlorococcus* and *Synechococcus* and belonging to the phylogenetic a-group of cyanobacteria (Rae 2010)[82] are an exception in having no canonical CP12 genes but only variants without consensus sequence and thus unable to bind PRK [73]. Oceanic a-cyanobacteria are an exception also for PRK, that is a bacterial-type (octameric) [18]. Several species of cyanobacteria outside a-cyanobacteria may also contain, besides canonical CP12, cystathionine beta-synthase (CBS)-CP12 fusion proteins in which two CBS domains are fused to a canonical CP12 and form hexamers that

826 bind/inhibit PRK in an AMP-dependent manner, thereby potentially interfering with PRK
827 redox activation [44].
828
829 *Land plants*
830 At the origin of land plants evolution another GAPDH isoform (A_2B_2-GAPDH) appeared as a
831 result of a gene fusion between a duplicated *GAPA* gene and the C-terminal domain of
832 CP12 [58] that gave rise to *GAPB* genes. A_2B_2-GAPDH shows CP12-derived regulatory
833 properties. Green algae *Ostreococcus tauri* and related species, are the only known
834 examples of organisms outside Streptophyta (land plants and charophytes) to have both
835 *GAPA* and *GAPB* genes, the latter possibly recruited by horizontal gene transfer [37]. Land
836 plants also contain Cp_4-GAPDH in plastids, particularly heterotrophic ones, derived from
837 *GAPC* [81]. Land plants thus contain four GAPDH isoforms (cytosolic C_4; chloroplastic A_4 and
838 A_2B_2; plastidial Cp_4), together with PRK and canonical CP12, able to build complexes with
839 PRK and GAPDH (A_4 or A_2B_2) but not with Cp4-GAPDH [9].
840
841 **Figure I** – Schematic representation of the evolution and biodiversity of GAPDH, PRK and
842 CP12 along the green lineage and in cyanobacteria
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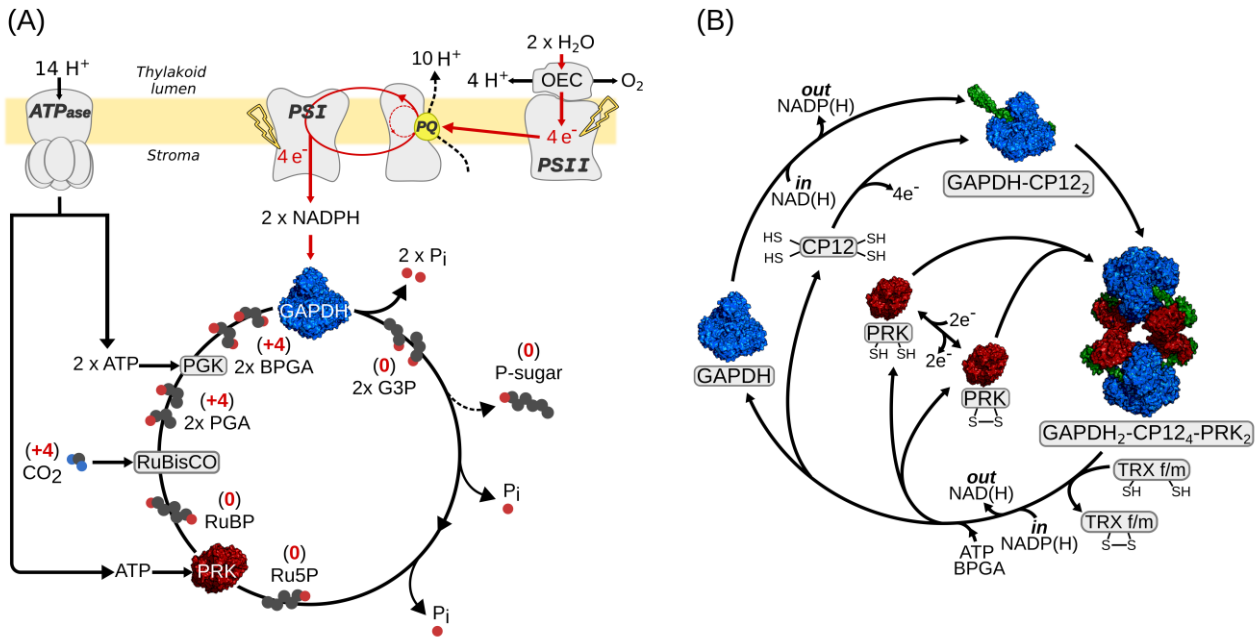
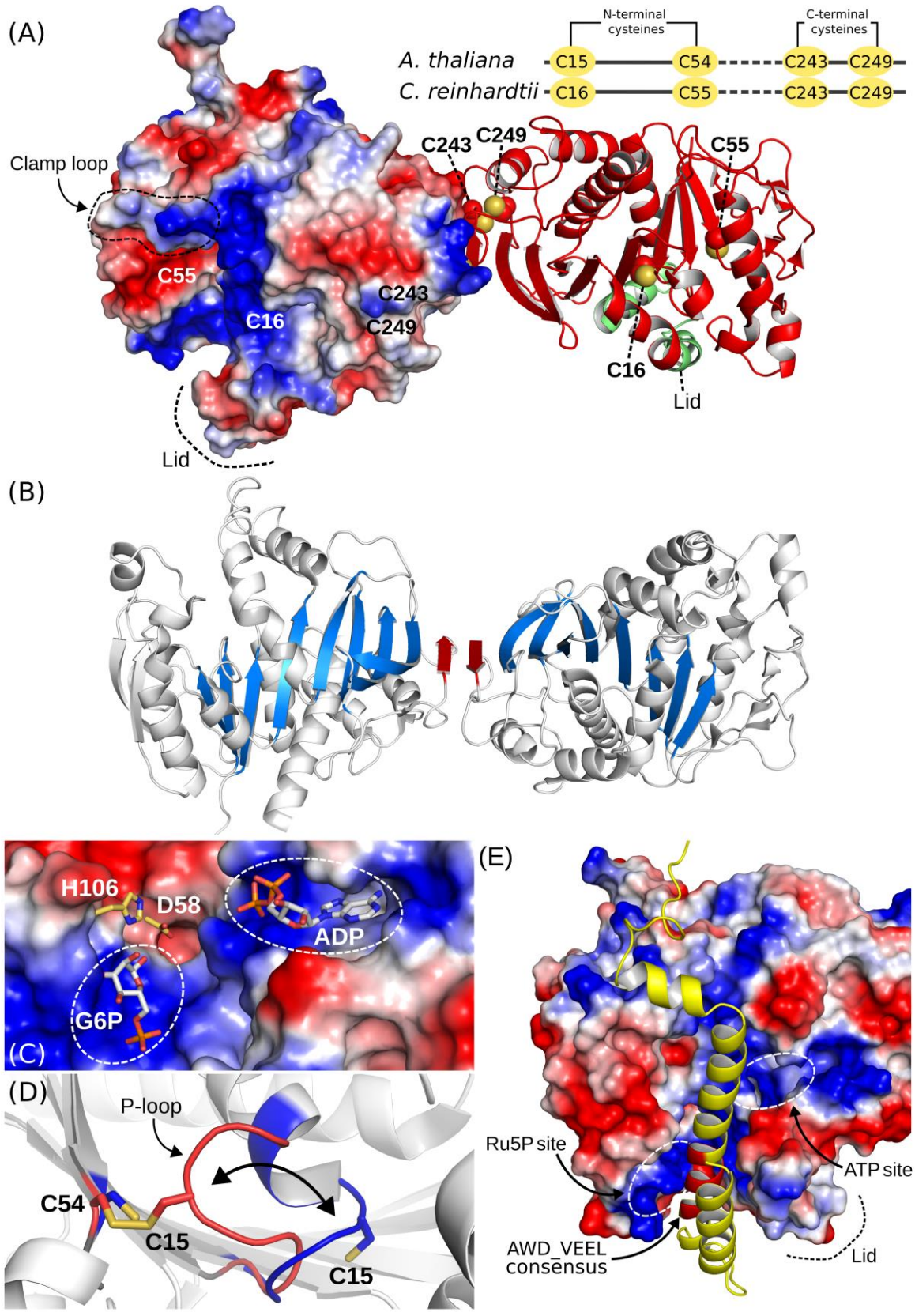
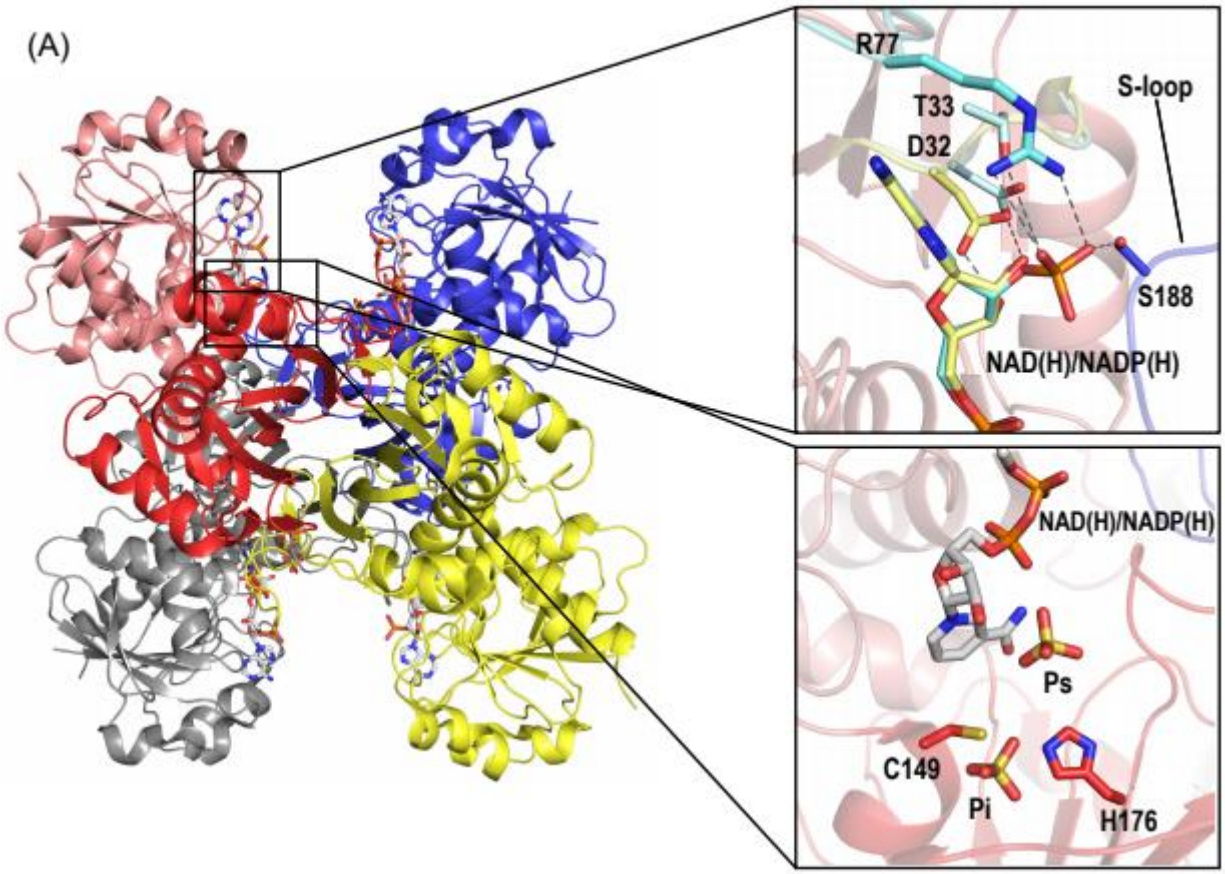
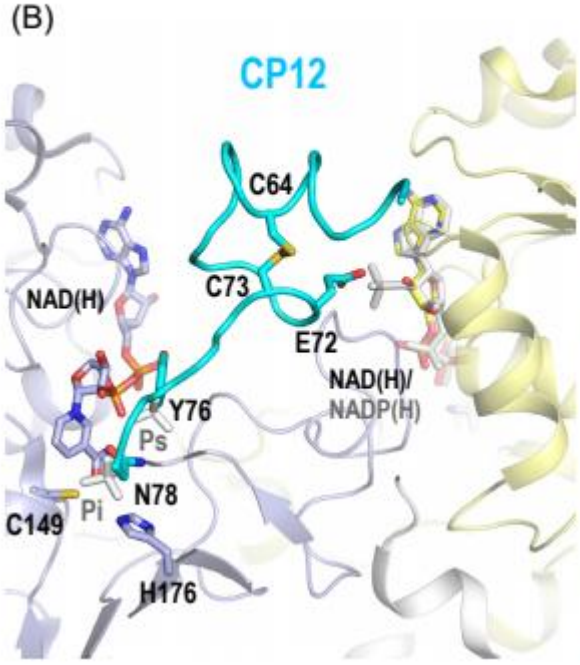
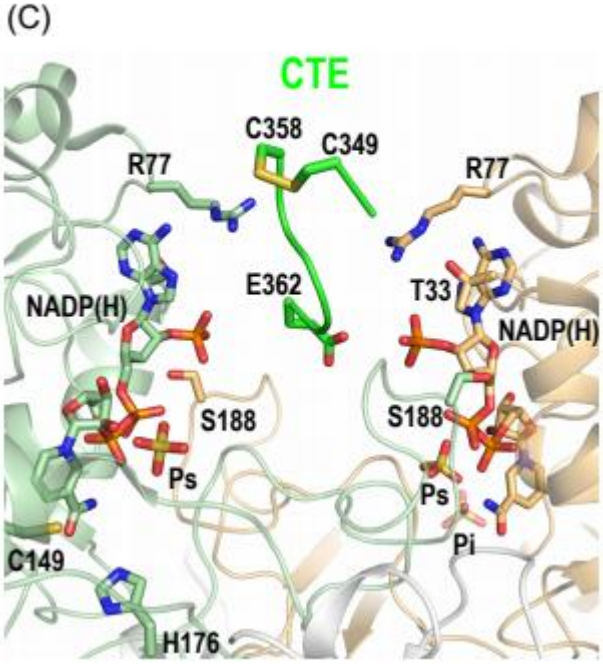
844
845

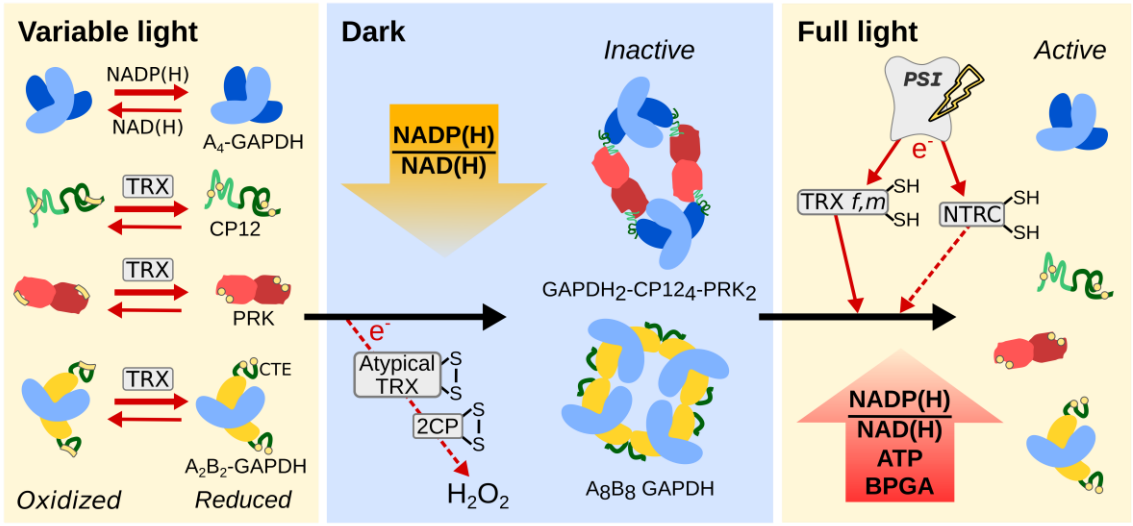

(A)
14 H+
Thylakoid lumen
ATPase
Stroma
PSI
PQ
PSII
10 H+
4 H+
OEC
2 x H2O
O2
4 e-
4 e-
2 x NADPH
2 x Pi
GAPDH
(+4)
2x BPGA
(0)
2x G3P
(0)
P-sugar
2 x ATP
PGK
(+4)
2x PGA
(+4)
CO2
RuBisCO
(0)
RuBP
Pi
ATP
PRK
(0)
Ru5P
Pi
(B)
out
NADP(H)
in
NAD(H)
4e-
GAPDH-CP12₂
HS
HS
CP12
SH
SH
PRK
SH SH
2e-
2e-
PRK
S—S
GAPDH
GAPDH₂-CP12₄-PRK₂
TRX f/m
SH SH
TRX f/m
S—S
out
NAD(H)
in
NADP(H)
ATP
BPGA

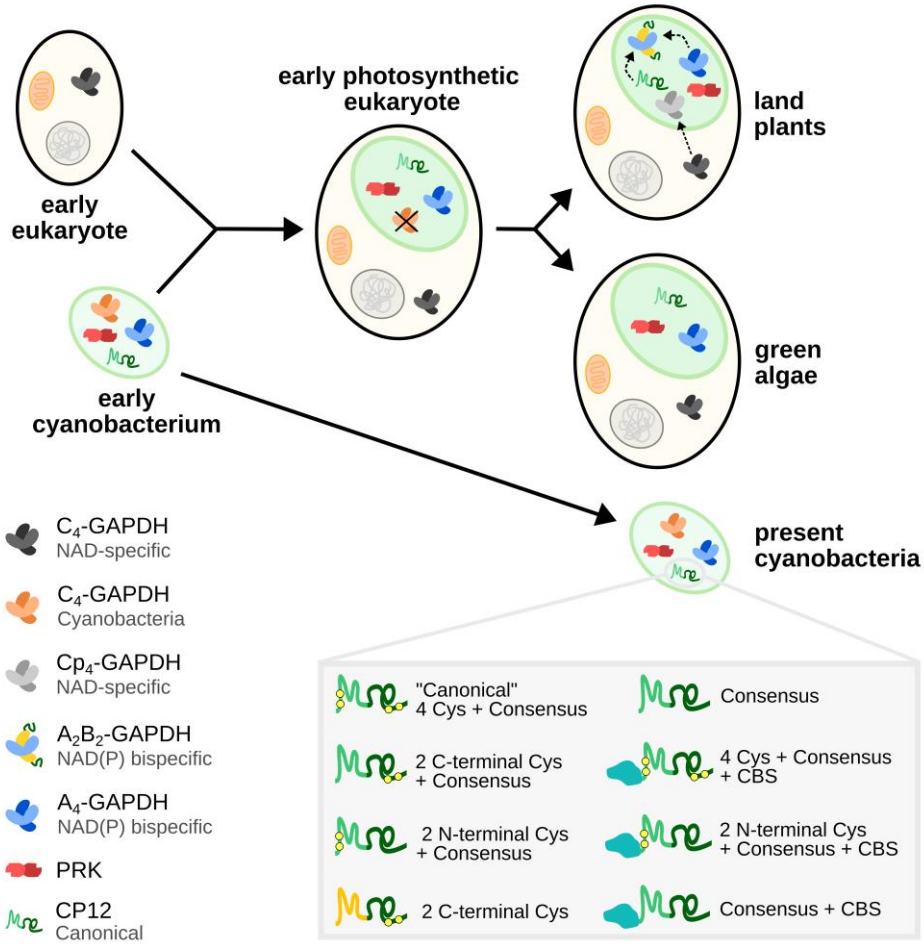

(A)
N-terminal cysteines
C-terminal cysteines
A. thaliana
C15
C54
C243
C249
C. reinhardtii
C16
C55
C243
C249
Clamp loop
C55
C16
C243
C249
Lid
C243
C249
C55
C16
Lid
(B)
H106
D58
ADP
G6P
(C)
(D)
P-loop
C54
C15
C15
(E)
Ru5P site
ATP site
AWD_VEEL consensus
Lid


(A)
R77
S-loop
T33
D32
S188
NAD(H)/NADP(H)
NAD(H)/NADP(H)
Ps
C149
Pi
H176


(B)
CP12
C64
C73
NAD(H)
E72
NAD(H)/
NADP(H)
Y76
Ps
N78
Pi
C149
H176


(C)
CTE
C358
C349
R77
R77
E362
T33
NADP(H)
NADP(H)
S188
S188
Ps
Ps
Pi
C149
H176


Variable light
NADP(H)
NAD(H)
A4-GAPDH
TRX
CP12
TRX
PRK
TRX
CTE
A2B2-GAPDH
Oxidized
Reduced
Dark
NADP(H)
NAD(H)
Inactive
GAPDH2-CP124-PRK2
e-
Atypical TRX
S
S
2CP
S
S
H2O2
A8B8 GAPDH
Full light
Active
PSI
e-
TRX f,m
SH
SH
NTRC
SH
SH
NADP(H)
NAD(H)
ATP
BPGA


early eukaryote
early cyanobacterium
early photosynthetic eukaryote
land plants
green algae
present cyanobacteria
C4-GAPDH
NAD-specific
C4-GAPDH
Cyanobacteria
Cp4-GAPDH
NAD-specific
A2B2-GAPDH
NAD(P) bispecific
A4-GAPDH
NAD(P) bispecific
PRK
CP12
Canonical
"Canonical"
4 Cys + Consensus
Consensus
2 C-terminal Cys
+ Consensus
4 Cys + Consensus
+ CBS
2 N-terminal Cys
+ Consensus
2 N-terminal Cys
+ Consensus + CBS
2 C-terminal Cys
Consensus + CBS