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Calvin-Benson cycle regulation is getting complex

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(Article begins on next page)

1 **HIGHLIGHTS**

2

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

8

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

13

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

17

18 **TITLE**

19 **CALVIN-BENSON CYCLE REGULATION IS GETTING COMPLEX**

20

21 **AUTHOR NAMES**

22 Libero Gurrieri¹, Simona Fermani^{2,3}, Mirko Zaffagnini¹, Francesca Sparla¹, Paolo Trost¹

23

24 ¹Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy, I-40126

25 ²Department of Chemistry Giacomo Ciamician, University of Bologna, Bologna, Italy, I-40126

26 ³CIRI Health Sciences & Technologies (HST), University of Bologna, Bologna, Italy, I-40126

27

28 Corresponding author: paolo.trost@unibo.it (P. Trost);

29 [https://fabit.unibo.it/en/research/research-groups/research-group-molecular-plant-](https://fabit.unibo.it/en/research/research-groups/research-group-molecular-plant-physiology-coordinator-trost)
30 [physiology-coordinator-trost](https://fabit.unibo.it/en/research/research-groups/research-group-molecular-plant-physiology-coordinator-trost); twitter @paotrost

31

32 **KEYWORDS**

33 Redox regulation, protein complexes, photosynthesis, metabolism

34

35 **ABSTRACT**

36

37 Oxygenic phototrophs use the Calvin-Benson cycle to fix CO₂ during photosynthesis. In the
38 dark, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase
39 (PRK), two enzymes of the Calvin-Benson cycle, form an inactive complex with the
40 regulatory protein CP12, mainly under the control of thioredoxins and pyridine nucleotides.
41 In the light, complex dissociation allows GAPDH and PRK reactivation. GAPDH/CP12/PRK
42 complexes are conserved from cyanobacteria to angiosperms and coexist, in land plant
43 species, with auto-assembling GAPDH complexes that are analogously regulated. The
44 recently described three-dimensional structures of all the elements of this ubiquitous
45 regulatory system, together with novel genome editing techniques, opens a new avenue
46 for understanding the regulatory potential of photosynthetic carbon fixation by *in vivo* site-
47 specific mutagenesis.

48

49 **MAIN TEXT**

50

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

53 Photosynthesis is fundamentally a redox process. Fixation of one C atom from CO₂
54 (oxidation state +4) into a C atom of a sugar (average oxidation state 0) requires four
55 electrons that in oxygenic photosynthetic organisms are provided by the oxidation of two
56 water molecules by the Oxygen Evolving Complex (OEC) of Photosystem II. In essence,
57 oxygenic photosynthesis thus transfers electrons from water to carbon through a light-driven
58 electron transport chain that starts with the OEC and ends up with an oxidoreductase, that
59 is NAD(P)H-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (**Figure 1**).
60 GAPDH does not reduce CO₂ directly, but it catalyzes the reaction that gives to the Calvin-
61 Benson cycle its alternative names of photosynthetic carbon reduction cycle or reductive
62 carbon fixation pathway [1,2] that underline the redox nature of this metabolism.

63

64 By mounting a CO₂ molecule on a sugar, Rubisco generates the organic acid that GAPDH
65 will reduce to sugar again, thereby creating the conditions to reconstitute the substrate of
66 Rubisco and set aside the fixed carbon in the form of sugar-phosphates (**Figure 1**). The
67 Rubisco reaction is exergonic but the whole cycle requires three ATP per CO₂: two ATPs are
68 used by phosphoglycerate kinase (PGK) to activate the products of Rubisco prior to
69 reduction by GAPDH, and one ATP is used for the regeneration of the substrate of Rubisco

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

73

74 Four consecutive reactions of the cycle depend on external inputs: phosphoribulokinase
75 (ATP), Rubisco (CO₂), phosphoglycerate kinase (ATP) and GAPDH (NADPH). The first and last
76 enzyme of this metabolic sequence are redox-regulated and can assemble into an inactive
77 complex with a third partner protein known as CP12 (**Figure 1**). Reversible complex
78 formation and modulation of GAPDH and PRK activities are mainly controlled by the
79 thioredoxins redox state, and by tris-phosphorylated (NADP⁺ and NADPH) versus bis-
80 phosphorylated pyridine nucleotides (NAD⁺ and NADH). Both parameters are perturbed by
81 light/dark or stress conditions and provide a link between Calvin-Benson cycle regulation
82 and environmental cues [3-6].

83

84 From cyanobacteria to land plants, all oxygenic photosynthetic organisms use the Calvin-
85 Benson cycle to fix CO₂, and with a few exceptions, all contain CP12 (**Box 1**). Non-oxygenic
86 phototrophic bacteria may contain a Calvin-Benson cycle based on deeply divergent
87 enzymes in respect to cyanobacteria, but they do not have CP12 [7-10]. In this review, we
88 provide a view of the regulation of the Calvin-Benson cycle, based on both functional
89 studies and the recently solved atomic structures of all components and complexes of the
90 CP12-regulatory system in different oxygenic phototrophs.

91

92 **Phosphoribulokinase, the kinase that prepares the substrate for Rubisco**

93 PRK belongs to the nucleoside/nucleotide kinase superfamily [11] and exists in three types:
94 plant-type, found in most cyanobacteria, plants (Plantae) and photosynthetic protists;
95 archeal-type and bacterial-type, the latter in proteobacteria and in oceanic a-
96 cyanobacteria that contain bacterial-type Rubisco form 1A and no canonical CP12 genes
97 [12-14]. Plant and archeal- types are dimeric and share a similar structure [14-17] while
98 bacterial PRK is octameric with monomers showing limited structural similarity to
99 plant/archeal types [18]. Among the three types, only plant-type PRK contain regulatory
100 cysteines whose redox state is controlled by thioredoxins (TRXs) and coexists with CP12,
101 which provides a further level of regulation (Box 1).

102

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

105 surrounded by additional secondary structures (**Figure 2**). The N-terminal regions, at the two
106 ends of the β -sheet, show the characteristic nucleoside/nucleotide kinase superfamily fold
107 and harbor the active sites [14]. Dimerization between C-terminal regions involves a single
108 β -strand for each monomer and a dimer interface of about 550 Å², smaller than in both
109 archeal and bacterial PRKs (~ 1,550 Å², [15,18]) and indicative of structural flexibility (**Figure**
110 **3**) [16]. C-terminal regions underwent extensive rearrangements along evolution of the
111 three PRK types and are structurally more variable than N-terminal regions [14-16,18].

112

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

120

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

127

128 Together with CP12, PRK is part of the minimal redox toolkit that controls the carbon flux in
129 day/night cycles in cyanobacteria [21]. PRK regulation, based on disulfides that can form
130 in either PRK or CP12, is conserved in cyanobacteria and along the green lineage [10] (**Box**
131 **1**). Regulatory cysteines of the N-terminal region (Cys15/Cys54 in arabidopsis (*Arabidopsis*
132 *thaliana*) and Cys16/Cys55 in *Chlamydomonas reinhardtii* [16]) are responsible for the
133 oxidative inactivation of PRK itself [22] (**Figure 2D**). Cys15 (arabidopsis numbering in the
134 following) is contained in the P-loop which flips to allow the formation of the disulfide with
135 Cys54 [14,17,19]. The reactivity of Cys15 is ensured by a conserved molecular environment
136 within the P-loop [16,17]. Plant-type PRKs may form a second disulfide in the C-terminal
137 region [14,16,23] (**Figure 2A**) which is found when the enzyme is in complex with GAPDH and
138 CP12 [17,19]. The C-terminal disulfide does not affect the activity but appears necessary for
139 GAPDH/CP12/PRK assembly in *Chlamydomonas reinhardtii* [23].

140

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

150

151 **Glyceraldehyde-3-phosphate dehydrogenase, the enzyme of the photosynthetic carbon** 152 **reduction**

153

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

158

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

167

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

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

181

182 The catalytic domain contains two anion recognition sites, named P_i and P_s, which harbour
183 the phosphate groups of BPGA (**Figure 3**). In the P_s site, the thiolate of Cys149, activated by
184 His176, makes a covalent bond with the substrate prior to its reduction. As detailed below,
185 GAPDH may participate in different types of complexes (e.g. A₄-GAPDH/CP12/PRK, A₈B₈-
186 GAPDH) without significantly changing the overall structure of the tetramer which appears
187 remarkably rigid (**Figures 3,4**). Flexible partners need to adapt to GAPDH, not viceversa, a
188 concept that is probably valid also for the manifold interactions that glycolytic C₄-GAPDH
189 displays in its moonlighting functions [32,33].

190

191 **CP12, the PRK/GAPDH regulatory protein**

192

193 CP12 is an intrinsically disordered protein of about 80 amino acids [8,9,34-36] that adopts a
194 defined tridimensional structure after binding to its partners GAPDH and PRK. Canonical
195 CP12 is characterized by four conserved cysteines able to form two disulfide bridges and
196 the consensus sequence AWD_VEEL. The N-terminal region of CP12 contains one of the two
197 cysteine pairs followed by the consensus sequence which is involved in PRK binding. The C-
198 terminal region contains the second cysteine pair and binds GAPDH. Genes coding for
199 canonical CP12 are present in almost all genomes of organisms that fix CO₂ via the Calvin-
200 Benson cycle, in particular cyanobacteria, green algae and land plants [9,8] (**Box 1**).
201 Notable exceptions include oceanic α -cyanobacteria which contain only CP12 genes with
202 neither the N-terminal disulfide nor the consensus sequence [13], and prasinophycean
203 green algae like *Ostreococcus tauri* which contain no CP12 genes at all [37]. On the other
204 hand, *Ostreococcus* species are the only green algae (Chlorophyta) known to contain
205 redox-regulated B subunits of GAPDH, which are typical of Streptophyta (land plants and
206 charophycean algal ancestors) [38].

207

208 The disulfide bridges of CP12 are formed in the presence of oxidized TRX [24] or other
209 oxidants (GSSG, H₂O₂)[39], and the C-terminal disulfide requires less oxidizing power than

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

213

214 Although oxidized CP12 remains essentially disordered, a local structural motif in the C-
215 terminal domain is stabilized by its disulfide bridge, allowing the interaction with GAPDH and
216 thus the initiation of the ternary complex assembly [41,42]. Based on this property, CP12 has
217 been defined as a conditionally rather than intrinsically disordered protein [42]. The oxidized
218 N-terminal domain folds into a stable two-helix bundle only after CP12 has bound to GAPDH
219 [19,43] or to other protein domains like in cyanobacterial fusion proteins with cystathionine
220 β -synthase domains (CP12-CBS; **Box 1**) [13,44]. In any case, the N- and C-terminal domains
221 of CP12 are connected by a flexible linker that remains flexible even when the rest of CP12
222 is fully folded [17,19] (**Figure 2**).

223

224 **GAPDH/CP12, the intermediate complex that recruits PRK**

225

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

231

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

245

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

247

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

255

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

262

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

273

274 Ternary complex structures clearly show that 100% of PRK- and 50% of GAPDH-active sites
275 are blocked by CP12, and that CP12 interferes with coenzyme binding in the remaining
276 GAPDH subunits (**Figure 3**). Since PRK in the complex can exist in different redox states
277 [51,52], CP12 provides a way to inhibit PRK activity independently from PRK redox regulation.
278 Moreover, CP12 is the only way to regulate A₄-GAPDH. Experimentally measured, both PRK
279 and GAPDH activities of the complex are strongly inhibited but total inhibition is never

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

289
290 Besides the canonical binary (GAPDH/CP12₂) and ternary complexes here described
291 (GAPDH₂/CP12₄/PRK₂), further possibilities may exist. For instance, GAPDH-CP12₄ complexes
292 were obtained *in vitro* with cyanobacteria proteins at high CP12:GAPDH ratios [19, 46] and
293 reconstituted GAPDH/CP12/PRK complexes of *Chlamydomonas reinhardtii* were found to
294 bind aldolase *in vitro* with strong affinity (K_D 55 nM) [53]. Whether these complexes do exist
295 *in vivo* in chlamydomonas or other organisms is currently unknown. In higher plants at least,
296 the CP12-complexes that could be extracted from tobacco plants did not contain any
297 other protein than GAPDH (A and B subunits) and PRK [54].

298
299 **The A₂B₂-GAPDH isoform of land plants has acquired from CP12 its regulatory properties**

300
301 In land plants and charophycean algal ancestors (Streptophytes) CP12 coexists with A₂B₂-
302 GAPDH, which is a regulated version of A₄-GAPDH [37, 55-57] (**Box 1**). All regulatory
303 properties of A₂B₂-GAPDH depend on the C-terminal extension (CTE) of B-subunits that is
304 homologous to the C-terminal domain of CP12 [56,58]. Similar to A₄-GAPDH, fully active A₂B₂-
305 GAPDH shows a marked kinetic preference for NADPH over NADH based on both higher
306 V_{max} (1.5-fold) and 5-fold lower K_m [30,31]. The kinetic preference is based on proper
307 coenzyme recognition and abolished in oxidized A₂B₂-GAPDH, that carries a disulfide bridge
308 in the CTE and uses NADPH and NADH with similar and low efficiency [58].

309
310 When the CTE, which is negatively charged, is oxidized, it is attracted by the positive cleft
311 between A and B subunits, the same cleft occupied by CP12 in GAPDH/CP12 complexes
312 (**Figure 3**). However, the position of the CTE is less deep within the cleft and its C-terminus
313 does not reach the P-sites like CP12 [17,19,46,59]. Nevertheless the NADP(H)-dependent
314 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 (A₄-like), or an A₄-GAPDH (behaving like
325 reduced/activated A₂B₂-GAPDH) into an oxidized/inhibited A₂B₂-like GAPDH [30,31,59].

326

327 Understanding the regulation of A₂B₂-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 A₈B₈, but also A₄B₄ and others,
330 [55,58,60,61]). Inhibited complexes of A₂B₂ 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 A₂B₂-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 A₂B₂-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

350 acceptor [65-68] (**Figure 4**). *In vitro*, hydrogen peroxide may directly induce disulfide bridges
351 formation in CP12, and A₄-GAPDH, whose catalytic cysteine is highly sensitive to H₂O₂-
352 oxidation to sulfinic acid, is fully protected when assembled in ternary complex with CP12
353 [39]. Whether this protective mechanism is operative also *in vivo* is currently unknown.

354

355 Activities of both PRK and GAPDH rapidly drop in the dark in wild type arabidopsis plants
356 but much more slowly in 2CP-knock out mutants [65,66]. Dark-inactivation takes few minutes
357 and correlate with the sequestration of GAPDH and PRK in CP12-complexes [52,61] and
358 A₈B₈-GAPDH [60,62], although not in all species and with all techniques dark-complexes are
359 easily detected [61,69]. In most species, PRK is found fully sequestered in dark-complexes
360 but some GAPDH remains as free tetramers (A₂B₂; A₄) [52,61]. Recent quantifications of
361 Calvin-Benson cycle enzymes in *Chlamydomonas reinhardtii* consistently showed that A-
362 subunits of GAPDH exceed PRK subunits by a factor of 4 [70]. Since the GAPDH/PRK subunit
363 ratio of the ternary complex is invariably 2 [17,19,52] (**Figure 3**) we argue that, in
364 *Chlamydomonas* at least, there's no enough PRK to sequestrate all GAPDH in dark-
365 complexes.

366

367 The existence of ternary complexes implies that also CP12 must be oxidized in the dark *in*
368 *vivo*, in agreement with *in vitro* redox potential determinations [24]. It is also known from *in*
369 *vitro* studies that GAPDH/CP12/PRK and A₈B₈-GAPDH are stabilized by low NADP(H)/NAD(H)
370 ratios that seem to be established in darkened chloroplasts [71,4] and in cyanobacteria
371 [48,72] thereby favoring dark-complexes stability (**Figure 4**).

372

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

379

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

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

401

402 In higher plants, the physiological role of CP12 is also complex and not yet fully understood.
403 *Arabidopsis thaliana* contains three CP12 paralogues with tissue-specific expression
404 [29,9,75,76]. CP12-1 and CP12-2 are closely related and represent a single CP12 form found
405 throughout the higher plants, while CP12-3 represents a divergent CP12 form not universally
406 present in higher plants [8]. In arabidopsis, CP12-1 and CP12-2 are highly expressed in leaves
407 but no phenotypic alterations were observed in single CP12 mutants. On the contrary,
408 transgenic lines with strongly reduced levels of both CP12-1 and CP12-2 were negatively
409 affected in photosynthetic capacity and biomass yield [77]. Intriguingly, the amount of PRK,
410 but not its transcript level, was dramatically reduced in CP12-double mutants, suggesting
411 that the stability of PRK might be under control of CP12 with functional consequence on
412 photosynthetic capacity and growth. While opening a new perspective on a possible role
413 of CP12-complexes in preserving PRK from degradation in the night, these results prevent
414 an easy demonstration of CP12 function in higher plants based on classical reverse genetic
415 approaches.

416

417 **Concluding remarks and Future perspectives**

418

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

427

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

436

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668 OUTSTANDING QUESTIONS

- 669 • Which is the dynamics of diphosphorylated (NAD) versus triphosphorylated (NADP)
670 pyridine nucleotides in higher plants chloroplasts in light/dark cycles and fluctuating
671 light conditions?
- 672 • Are dark-complexes (GAPDH/CP12/PRK and A₈B₈-GAPDH) of land plants important
673 for adaptation to fluctuating light?
- 674 • Is NTRC (NADPH-thioredoxin C) important for dark-complexes dissociation?
- 675 • Is the recently described electron chain based on atypical thioredoxins ACHT/TRXL-
676 2 and 2-cys peroxiredoxins important for dark-complexes association?
- 677 • How the CP12-derived C-terminal extension of subunits B makes A₂B₂-GAPDH of
678 land plants able to auto-assemble in A₈B₈-GAPDH and other types of GAPDH-
679 complexes? Complexes of AB-GAPDH are the only elements of the regulatory
680 system of GAPDH and PRK in land plants whose tridimensional structure is not yet
681 know.
- 682 • How the dynamics of dark-complexes formation and dissociation could be
683 detected *in vivo* in real time?
- 684 • How do complexes relate to stress conditions *in vivo*? Reactive oxygen species are
685 produced in several types of stress and, *in vitro*, hydrogen peroxide oxidizes CP12
686 and induces aggregation/protection of GAPDH and PRK, but whether this
687 mechanism is relevant *in vivo* is not yet known.

- 688
- Can the effect of CP12 in protecting PRK from proteolysis in *Arabidopsis thaliana* be
689 extended to other species and/or other types of complexes like A₈B₈-GAPDH?
690 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
691 at the level of GAPDH and PRK, with apparently overlapping functions? AB-GAPDH
692 and PRK are both redox-regulated, both individually and through CP12; AB-GAPDH
693 and A₄-GAPDH/PRK are both able to form inactive complexes under similar control
694 (thioredoxins and pyridine nucleotides).
695

696

697 LEGENDS TO FIGURES

698 **Figure 1. Photosynthesis is a redox-regulated redox process.** (a) The oxidation state of C in
699 CO₂ is (+4). The average oxidation state of C atoms in sugars is (0). Rubisco adds a CO₂ (+4)
700 to RuBP (0) generating two molecules of PGA (+2 x 2). PGK adds ATP to PGA, and GAPDH
701 uses four electrons (e⁻) from NADPH to reduce BPGA (+2 x 2) to G3P (0). NADPH is derived
702 from light reactions of photosynthesis. The electron transport is coupled to trans-thylakoidal
703 proton motive force that generates ATP via ATP-synthase. G3P regenerates Ru5P in a series
704 of reactions with P-sugar intermediates (*not shown*). Two irreversible phosphatases drive the
705 Ru5P regeneration from G3P. PRK and PGK restore the phosphates lost by the cycle. (b) In
706 oxygenic phototrophs, PRK and GAPDH can form a regulatory complex with CP12. The
707 hierarchical process of aggregation is structurally characterized. GAPDH [27,28] forms a
708 binary complex with CP12 when NAD(H) substitutes NADP(H) in GAPDH and CP12 is oxidized
709 (4 electrons) and bears two disulfides [52,58]. PRK is redox-regulated [14,16,17] and both PRK
710 forms can bind the GAPDH/CP12 binary complex to form the ternary complex
711 GAPDH₂/CP12₄/PRK₂ in which both enzymes are strongly inhibited [17,19]. Complex
712 dissociation and enzyme reactivation is obtained by reduced thioredoxins or NADP(H) or
713 BPGA or ATP, with different efficiency [79]. Abbreviations: BPGA, 1,3-bisphosphoglyceric
714 acids; CET, cyclic electron transport; G3P, glyceraldehyde-3-phosphate; GAPDH,
715 glyceraldehyde-3-phosphate dehydrogenase; LET, linear electron transport; OEC, oxygen
716 evolving complex; PGA, 3-phosphoglyceric acid; PGK, phosphoglycerate kinase; PQ,
717 plastoquinone; PRK, phosphoribulokinase; PSI, photosystem I; PSII, photosystem II; Ru5P,
718 ribulose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; TRX, thioredoxin.

719

720 **Figure 2. Phosphoribulokinase: structure and regulation.** (a) Dimer of algal PRK
721 (*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* GAPDH₂-CP12₄-PRK₂ 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 A₄-GAPDH/CP12 binary complex.** (a)
744 Typical GAPDH tetramer with subunits in different colours (crystal structure of GAPDH from
745 *Spinacia oleracea* [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 $\leq 4 \text{ \AA}$ [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 A₄-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

757 (yellow) subunit [41]. (c) In A₂B₂-GAPDH, oxidized CTE (green) occupies the cleft between B
758 (light-green) and A (sand) subunits, similarly to CP12 in the binary complex. However, CTE
759 does not occupy the P-sites but causes the 2'-phosphate of NADP(H) of the B-subunit (light-
760 green) to lose crucial interactions with T33, R77 and S188, which are responsible for the
761 high NADP(H)-dependent activity of the enzyme [59]. Atom colour codes: oxygen red;
762 nitrogen blue; sulphur yellow.

763

764 **Figure 4. Current view of GAPDH/PRK regulation based on combined *in vivo* and *in vitro***

765 **evidence.** The model is based on data collected from different organisms and its general
766 principles apply to cyanobacteria, green algae and land plants, although AB-GAPDHs are
767 only found in land plants [58]. From left to right: at variable light conditions, redox-sensitive
768 proteins CP12, PRK and A₂B₂-GAPDH equilibrate with thioredoxins and tend to be reduced,
769 enzymatically active and free from complexes [79]. In light-to-dark transitions, atypical
770 thioredoxins (ACHT, TRXL-2) with less negative redox potential than thioredoxins *f* and *m* [68],
771 convey electrons from reduced targets to hydrogen peroxide via 2-cys peroxiredoxins [66-
772 69]. Chloroplast NADP(H)/NAD(H) ratio tend to decrease in the dark [4,72], favouring the
773 substitution of NADP(H) with NAD(H) in bispecific GAPDHs. Oxidation of CP12 and C-terminal
774 extension (CTE) of GAPDH B-subunits, and low NADP(H)/NAD(H) ratios, favour the formation
775 of dark-complexes of inactive enzymes (GAPDH₂CP12₄PRK₂ and A₈B₈-GAPDH)[17,19,48,55].
776 In dark-to-light transitions and then in full light, dark complexes are dissociated and enzyme
777 activities fully recovered. Thioredoxins *f* and *m*, reduced by ferredoxin-thioredoxin
778 reductase at the onset of light reduce CP12 and CTE disulfides, causing dark-complexes
779 dissociation [24,55]. NADPH-thioredoxin C (NTRC) may contribute to the same effect [64,65].
780 Complexes dissociation is aided by NADPH, BPGA and ATP, all able to bind in different
781 positions and cause CP12 or CTE displacement from their binding sites [45,48,59]. Dashed
782 arrows indicate interactions that are not fully proven yet. Abbreviations: 2CP, 2-cys-
783 peroxyredoxins; BPGA, 1,3-bisphosphoglyceric acids; CTE; C-terminal extension of GAPDH
784 B-subunits; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NTRC, NADPH-
785 thioredoxin C; PRK, phosphoribulokinase; TRX, thioredoxin.

786

787 **TEXT BOXES**

788

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

790

791 *Early times: before primary endosymbiosis*

792 Early eukaryotes contained a NAD-specific GAPDH isoform of eubacterial origin, localized
793 in the cytosol and involved in glycolysis. This prototypical GAPDH, named C₄-GAPDH and
794 coded by GAPC genes, is ubiquitously expressed in present eukaryotes [51] (Figure I).
795 Cyanobacteria, early and present ones, contain a C₄-like GAPDH, coded by GAP1 genes,
796 but also a GAPDH of the A₄-type coded by GAP2 genes [80]. A₄-GAPDH is characterized by
797 the double specificity for pyridine nucleotides and the role in the Calvin-Benson cycle. Early
798 cyanobacteria, also contained PRK and canonical CP12 with conserved N- and C-terminal
799 disulfides and central consensus sequence [73], hence potentially able to bind A₄-GAPDH
800 and PRK [10].

801

802

803 *Origin of photosynthetic eukaryotes*

804 Early photosynthetic eukaryotes formed by primary endosymbiosis contained C₄-GAPDH
805 derived from the eukaryotic host and A₄-GAPDH of cyanobacterial origin located in
806 chloroplasts, together with PRK and canonical CP12. GAP1 genes coding for
807 cyanobacterial C₄-GAPDH were apparently lost after endosymbiosis. The set of genes
808 coding for GAPDH (GAPC and GAPA), CP12 and PRK in early photosynthetic eukaryotes is
809 typically found conserved in green algae and land plants of present days, possibly with
810 paralogues derived from whole genome duplications [10,73,51]. Unicellular
811 prasinophycean algae (an early diverging group of green algae with members that are
812 prominent in the oceanic picoplankton) like *Ostreococcus tauri* represent an exception in
813 having no CP12 in their genome[37].

814

815 *CP12 and PRK biodiversity in cyanobacteria*

816 Besides canonical CP12 genes, cyanobacteria may also contain genes for CP12 variants
817 that are not found in green photosynthetic eukaryotes [73]. These include CP12 isoforms
818 with no C-terminal cysteines and/or N-terminal cysteines and/or central consensus
819 sequence. Oceanic species included in genera *Prochlorococcus* and *Synechococcus* and
820 belonging to the phylogenetic α -group of cyanobacteria (Rae 2010)[82] are an exception
821 in having no canonical CP12 genes but only variants without consensus sequence and thus
822 unable to bind PRK [73]. Oceanic α -cyanobacteria are an exception also for PRK, that is a
823 bacterial-type (octameric) [18]. Several species of cyanobacteria outside α -cyanobacteria
824 may also contain, besides canonical CP12, cystathionine beta-synthase (CBS)-CP12 fusion
825 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 (A₂B₂-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. A₂B₂-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 Cp₄-GAPDH in plastids, particularly heterotrophic ones, derived from
837 GAPC [81]. Land plants thus contain four GAPDH isoforms (cytosolic C₄; chloroplastic A₄ and
838 A₂B₂; plastidial Cp₄), together with PRK and canonical CP12, able to build complexes with
839 PRK and GAPDH (A₄ or A₂B₂) 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

843

844

845









