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REVIEW

Thioredoxin-dependent regulation of photosynthetic glyceraldehyde-3-phosphate dehydrogenase: autonomous vs. CP12-dependent mechanisms

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Abstract Regulation of the Calvin–Benson cycle under varying light/dark conditions is a common property of oxygenic photosynthetic organisms and photosynthetic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the targets of this complex regulatory system. In cyanobacteria and most algae, photosynthetic GAPDH is a homotetramer of GapA subunits which do not contain regulatory domains. In these organisms, dark-inhibition of the Calvin–Benson cycle involves the formation of a kinetically inhibited supramolecular complex between GAPDH, the regulatory peptide CP12 and phosphoribulokinase. Conditions prevailing in the dark, i.e. oxidation of thioredoxins and low NADP(H)/NAD(H) ratio promote aggregation. Although this regulatory system has been inherited in higher plants, these phototrophs contain in addition a second type of GAPDH subunits (GapB) resulting from the fusion of GapA with the C-terminal half of CP12. Heterotetrameric A_2B_2-GAPDH constitutes the major photosynthetic GAPDH isoform of higher plants chloroplasts and coexists with CP12 and A_4-GAPDH. GapB subunits of A_2B_2-GAPDH have inherited from CP12 a regulatory domain (CTE for C-terminal extension) which makes the enzyme sensitive to thioredoxins and pyridine nucleotides, resembling the GAPDH/CP12/PRK system. The two systems are similar in other respects: oxidizing conditions and low NADP(H)/NAD(H) ratios promote aggregation of A_2B_2-GAPDH into strongly inactivated A_8B_8-GAPDH hexadecamers, and both CP12 and CTE specifically affect the NADPH-dependent activity of GAPDH. The alternative, lower activity with NADH is always unaffected. Based on the crystal structure of spinach A_4-GAPDH and the analysis of site-specific mutants, a model of the autonomous (CP12-independent) regulatory mechanism of A_2B_2-GAPDH is proposed. Both CP12 and CTE seem to regulate different photosynthetic GAPDH isoforms according to a common and ancient molecular mechanism.

Keywords Calvin-Benson cycle · Disulfide · Light/dark regulation · Pyridine nucleotides · Supramolecular complex · Thioredoxin

Abbreviations

BPGA	1,3-bisphosphoglycerate
CTE	C-terminal extension of subunits GapB
GapA and GapB	subunits A and B, respectively, of photosynthetic glyceraldehyde-3-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
NADP-MDH	NADP-dependent malate dehydrogenase
PRK	phosphoribulokinase

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Introduction: light-regulation of photosynthetic glyceraldehyde-3-phosphate dehydrogenase

Chloroplast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the eleven enzymes of the Calvin–Benson cycle and, together with phosphoglycerate kinase, catalyzes the reduction of 3-phosphoglycerate, the product of Rubisco reaction, into glyceraldehyde-3-phosphate, the first phosphorylated sugar produced in the cycle (Arnon et al. 1954). Chloroplast GAPDH has been the first light-regulated enzyme to be discovered in plants (Ziegler and Ziegler 1965). After GAPDH, other enzymes of the Calvin–Benson cycle were found to be regulated in vivo under light/dark conditions (fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, phosphoribulokinase, Rubisco) strongly supporting the view that plants possess a flexible system to finely modulate the Calvin–Benson cycle during the day and entirely suppress it during the night (Buchanan 1980; Wolosiuk et al. 1993).

Plants are sessile photosynthetic organisms living under continuously changing light regime and challenging environmental conditions, and the Calvin–Benson pathway of CO_2 assimilation into carbohydrates is a major route for the chemical conversion of light energy in all photosynthetic eukaryotes and in cyanobacteria, often accounting for > 90% of total photosynthetic output. Therefore, matching the rate of photosynthetic carbon assimilation with the rate of photosynthetic production of ATP and NADPH is an absolute priority for plants. To achieve this, plants possess a complex regulatory network involving a considerable variety of proteins and molecular signals.

Conditions in chloroplasts change dramatically in light vs. darkness. At the onset of illumination, stromal pH increases as protons are pumped into thylakoids (Kramer et al. 1999) and most intermediates of the Calvin–Benson cycle strongly increase concomitant with activation of photosynthetic metabolism (Gerhardt et al. 1987). The energy charge of adenylates increases in chloroplasts in the light and pyridine nucleotides become more reduced (Stitt et al. 1982; Heineke et al. 1991). Moreover, the pool of diphosphorylated pyridine nucleotides (NAD and NADH) decreases in the light while tri-phosphorylated NADP and NADPH symmetrically increase (Muto et al. 1981, Heineke et al. 1991), possibly as a result of a light-modulated NAD-kinase (Delumeau et al. 2000; Chai et al. 2005). The redox state of chloroplast thioredoxins is also affected by light/dark conditions via photosystem I and ferredoxin:thioredoxin reductase. Thioredoxin *f* in particular can both reductively activate Calvin–Benson cycle enzymes in the light and promote enzyme inactivation in the dark, when the thioredoxin pool becomes oxidized (Buchanan and Balmer 2005).

Light-regulated Calvin–Benson cycle enzymes are generally affected by several, mutually interacting, light-sensitive chloroplast parameters (Wolosiuk et al. 1993). The activity of chloroplast GAPDH is activated, for example, by mildly alkaline pH, Calvin–Benson cycle intermediates (BPGA, 3-phosphoglycerate), pyridine nucleotides (NADP and NADPH) and reduced thioredoxins, while it is inhibited by NAD(H) and oxidized thioredoxins (Pupillo and Giuliani Piccari 1975; Wolosiuk and Buchanan 1976, 1978; Trost et al. 1993; Baalmann et al. 1995; Sparla et al. 2002).

Although chloroplast GAPDH isoforms involved in the Calvin–Benson cycle are bispecific dehydrogenases able to accept either NADPH or NADH as electron donors, nonetheless their whole regulatory mechanism does only affect the NADPH-dependent reaction (or the $NADP^+$-dependent reverse reaction). The NAD(H)-reaction, which might possibly play a role in chloroplast dark metabolism, is constitutive and insensitive to any kind of regulation. This distinct biochemical feature (Pupillo and Giuliani Piccari 1973) has been exploited in recent studies aimed at elucidating the molecular basis of chloroplast GAPDH regulation (Sparla et al. 2004, 2005).

This review is focussed on the thioredoxin-dependent regulation of chloroplast GAPDH, an aspect of GAPDH regulation which underlies the whole regulatory system of this enzyme (Baalmann et al. 1995). The issue is complicated by the co-existence of different GAPDH subunits and isoforms in chloroplasts of higher plants which markedly differ in regulatory properties. In spite of these differences, it will shortly become apparent that any GAPDH isoform potentially involved in the Calvin–Benson cycle is directly or indirectly controlled by the redox state of thioredoxins and that different regulatory mechanisms do actually stem from a common type of redox regulation which originally appeared in cyanobacteria.

Diversity and evolution of GAPDH subunits and isozymes

GAPDHs constitute a large and diverse family of dehydrogenases universally represented in living organisms. The family can be divided into two classes: members of the class II have only been found in archaebacteria and show very limited sequence similarities to class I GAPDHs encompassing all eubacterial

and eukaryotic GAPDH genes (Figge et al. 1999). Class I is itself divided in two major groups exemplified by genes *Gap1* and *Gap2* of cyanobacteria (Martin and Schnarrenberger 1997); a small third group with members only present in prokaryotes will not be dealt with in this review.

The first group includes the best-known glycolytic GAPDHs (GapC in eukaryotes, Gap1 in eubacteria) which are normally NAD-specific and cytosolic. Besides GapC, land plants (i.e. embryophytes, including bryophytes and higher (vascular) plants) contain a second type of glycolytic GAPDH targeted to plastids (GapCp, Petersen et al. 2003). Both GapC and GapCp form NAD-specific homotetramers *in vivo* and are not subject to complex regulatory mechanisms.

The second group of Class I GAPDH is specifically present in organisms displaying oxygenic photosynthesis and catalyzing the unique reductive step of the Calvin–Benson cycle. In cyanobacteria, Calvin–Benson cycle GAPDH is encoded by *Gap2* genes which correspond to *GapA* genes in eukaryotes (Figge et al. 1999). Both Gap2 and GapA subunits form homotetrameric enzymes, but differ from glycolytic GAPDH (Gap1, GapC, GapCp) by their ability to use both NADPH and NADH as cofactors, with a marked kinetic preference for NADPH (Koksharova et al. 1998; Falini et al. 2003). Similar to cyanobacteria, primitive photosynthetic eukaryotes including *Cyanophora*, red and green algae (except charophytes and related organisms) appear to contain a single type of photosynthetic GAPDH encoded by *GapA* genes (Petersen et al. 2006). Although this type of photosynthetic GAPDH is not regulated per se (it is not affected by metabolites or pyridine nucleotides or thioredoxins unlike the higher plants enzyme), it is dark-inactivated in vivo through the interaction with CP12, a small regulatory protein widespread in oxygenic photosynthetic organisms, and the Calvin–Benson enzyme PRK (Wedel and Soll 1998). Thioredoxins and pyridine nucleotides in concert with metabolites cooperate in the modulation of this regulatory process (Wedel and Soll 1998; Graciet et al. 2004a; Tamoi et al. 2005).

A further contribution to enzyme diversity within the group of photosynthetic GAPDHs derived from a gene duplication event which apparently occurred near the origin of Streptophyta (which include charophytes and land plants, Petersen et al. 2006) giving rise to a novel GAPDH subunit named GapB. This protein appears to be a construct of a GapA moiety fused at the C-terminus with the C-terminal half of CP12 (Pohlmeyer et al. 1996). The portion of CP12 acquired by GapB subunits confers original regulatory properties to GapB-containing GAPDH isozymes. These "modern" GAPDH isozymes are made up of GapA and GapB subunits in stoichiometric ratio and are directly regulated by thioredoxins, pyridine nucleotides and metabolites (Pupillo and Giuliani Piccari 1975; Wolosiuk and Buchanan 1976, 1978; Trost et al. 1993; Baalmann et al. 1995; Sparla et al. 2002). Moreover AB-GAPDH isoforms have a peculiar propensity to form kinetically inefficient hexadecamers (A_8B_8) under conditions prevailing in chloroplasts in the dark (i.e. the NADPH-dependent activity is decreased to a lower level than the NADH-dependent one), and to dissociate into fully active tetramers (A_2B_2) under photosynthetic and hence activating conditions (Pupillo and Giuliani Piccari 1973, 1975; Scagliarini et al. 1993; Baalmann et al. 1994). This recently evolved type of autonomous GAPDH regulation coexists in land plants with the ancient, CP12-based regulatory mechanism (Scheibe et al. 2002).

In land plants the family of GAPDH is thus typically represented by four types of genes: *GapC* and *GapCp* (glycolytic, NAD-specific), *GapA* and *GapB* (photosynthetic, NAD(P)H-dependent). Due to further, highly conservative duplications, the actual number of GAPDH genes in a given plant may be higher. *Arabidopsis thaliana* for instance contains seven *Gap* genes, i.e. one *GapB* and three pairs each of *GapA*, *GapC* and *GapCp* genes (Marri et al. 2005a).

A_4-GAPDH: the simplest isoform of photosynthetic GAPDH as a paradigm of the fully active enzyme

The nature of the non-regulatory A_4-GAPDH homotetramer in higher plants is somewhat controversial. Transcripts for GapA are prevailing in dark-grown seedlings, but a strong increase of both GapA and GapB-transcripts is observed during de-etiolation (Cerff and Kloppstech 1982; Dewdney et al. 1993). A_4-GAPDH may thus be the default GAPDH isoform of etiolated tissues, while AB-GAPDH would prevail in green tissues. Affimetrix ATH1 GeneChip analysis of Arabidopsis transcripts (Schmid et al. 2005) shows that the sum of GapA-1 and GapA-2 transcripts (93% identical at the amino acid level) invariably exceeds the amount of GapB in leaves, flowers and developing seeds, suggesting the existence of GapA homotetramers in these organs (Fig. 1). On the other hand, self-reassembly of A-subunits from degraded A_nB_n isozymes has been also suggested. B-subunits tend to be proteolyzed during storage (Scagliarini et al. 1998), and according to one extreme view all NAD(P)-GAPDH forms including the stable A_4 isoform could derive from A_nB_n precursors (Brinkmann et al. 1989; Scheibe et al. 1996).

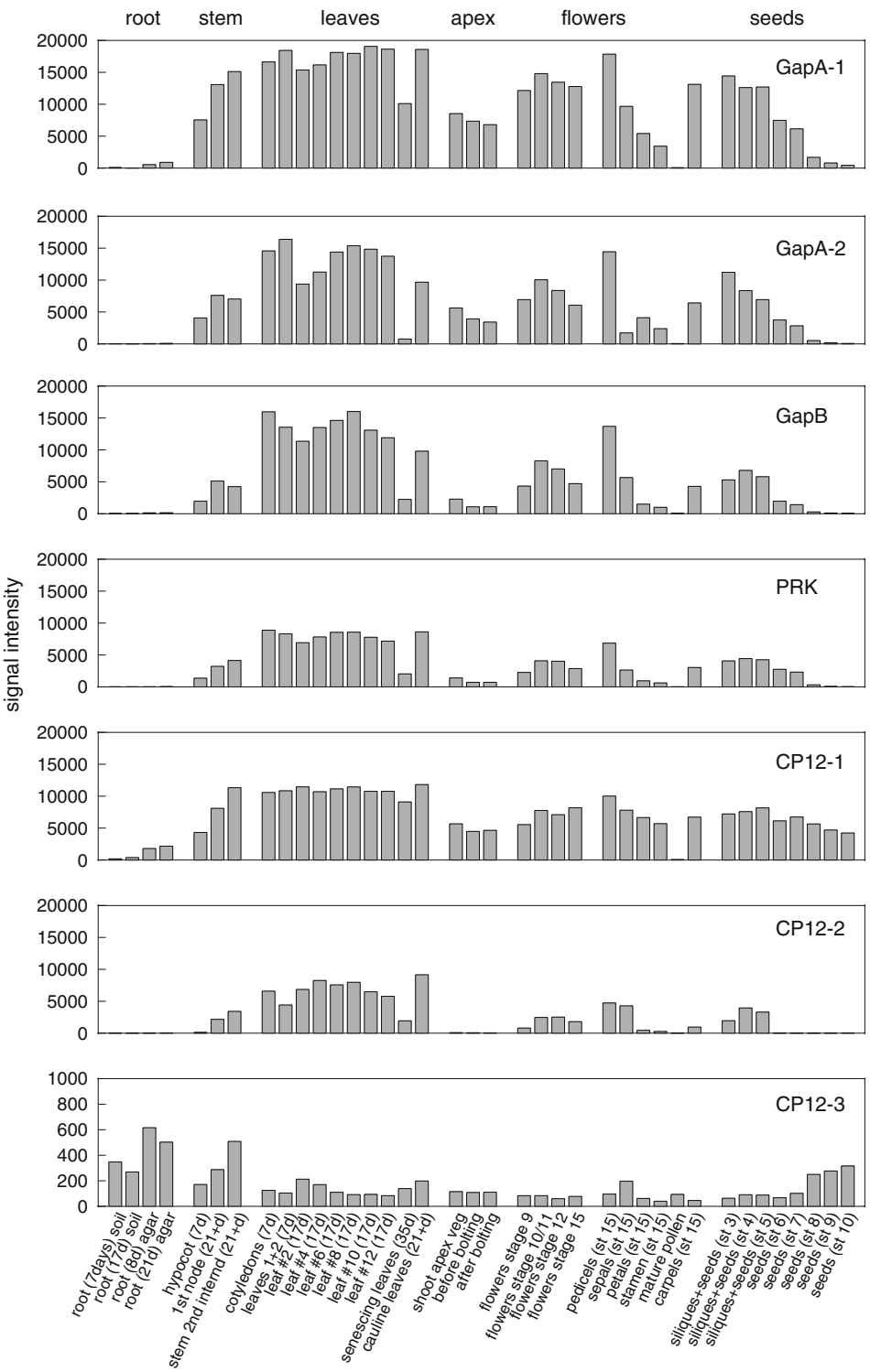

Fig. 1 Expression of *GapA-1* (At3g26650), *GapA-2* (At1g12900), *GapB* (At1g42970), *CP12-1* (At2g47400), *CP12-2* (At3g62410), *CP12-3* (At1g76560) and *PRK* (At1g32060) in different organs and growth stages of *Arabidopsis thaliana*. Note that in the CP12-3 panel the scale of the y-axis is 20-fold smaller than in other panels. Selected microarray data from the AtGenExpress developmental series (Schmid et al. 2005). Original data from triplicate arrays (Arabidopsis ATH1 chip, Affimetrix) are available at www.weigelworld.org. All RNA samples were extracted from wild type *Arabidopsis thaliana*, Columbia ecotype. A concise description of RNA samples is given in the figure. A complete description of RNA samples and experimental procedures can be found in Weigelworld website (www.weigelworld.org)

In any event, very large amounts of A_4-GAPDH were obtained from spinach chloroplasts to grow crystals for structural analysis (Sabatino et al. 1999) and the tridimensional structure of native A_4-GAPDH from spinach is still the only one available among photosynthetic GAPDHs (Fermani et al. 2001). The overall structure (Fig. 2A) is similar to glycolytic GAPDH determined from many animal and bacterial

sources (e.g. Skarzinsky et al. 1987; Song et al. 1998). Each subunit of the tetramer is constituted by two distinct domains, an N-terminal coenzyme-binding domain and a C-terminal catalytic domain which includes the binding site for the substrates (BPGA in the photosynthetic reductive reaction, Fig. 2B). A long and flexible loop of the catalytic domain known as the S-loop protrudes toward the coenzyme-binding domain of the adjacent subunit thereby contributing to formation of the coenzyme binding site (Figs. 2A and 3A, Fermani et al. 2001).

The two alternative coenzymes, NADPH or NADH, interact with each subunit at one and the same binding site. The coenzyme adopts an extended conformation with the nicotinamide group pointing toward the active site, where catalytic Cys-149 (Fig. 2B) makes a transient thioester bond with C-1 of the substrate which is then reduced by NAD(P)H. Although NADPH and NADH both bind to the enzyme in the same position, the kinetic parameters of the NADPH-dependent reaction are different from the NADH-dependent ones. The K_m of A_4-GAPDH for NADPH is 5–10-fold lower than for NADH, and the V_{max} of the NADPH-dependent reaction is about twice as high as with NADH (Scagliarini et al. 1998; Graciet et al. 2003a, Sparla et al. 2004). These kinetic parameters are similar to those of activated A_2B_2-GAPDH (Cerff 1978; Scagliarini et al. 1998; Sparla et al. 2004), supporting the view that A_4-GAPDH corresponds to a GAPDH isoform fixed in a fully active conformation.

Defining the structural basis of coenzyme specificity in GAPDH is necessary for understanding its regulation, as the latter process is strictly NADPH-specific no matter whether it is mediated by the C-terminal extension of GapB or by CP12. At the biochemical level, the hallmark difference between NADPH and NADH is given by the specific 2′-phosphate group of NADPH. The high resolution structure of recombinant A_4-GAPDH from spinach shows that the 2′-phosphate of NADP is kept in place by a salt bridge with Arg-77 and a hydrogen bond with Ser-188, the latter belonging to the S-loop of the opposite subunit (Fig. 3A, Sparla et al. 2004). When NAD substitutes for NADP, the side chain of Asp-32 stabilizes the 2'- and 3'-hydroxyls of NAD with its terminal carboxylate (Fig. 3B, Falini et al. 2003). The capability of Asp-32 to rotate away from NADP, or close to NAD, depending on which type of coenzyme is bound to GAPDH is apparently a crucial feature of photosynthetic NAD(P)-dependent GAPDHs which evolved from a strictly NAD-specific ancestral form (Falini et al. 2003).

Based on these structural data we postulated that any regulatory mechanism able to specifically down-regulate the NADPH-dependent activity of GAPDH would imply a specific role for residues Arg-77 and Ser-188, the committed residues of coenzyme specificity in photosynthetic GAPDH.

CP12 and the ancient regulatory system of photosynthetic GAPDH

Similar to higher plants, also in cyanobacteria and lower photosynthetic eukaryotes the Calvin–Benson cycle is suppressed in the dark (Buchanan 1992), and NAD(P)H-dependent GAPDH is light-modulated in these organisms despite the absence of regulatory GapB subunits (Figge et al. 1999; Wedel and Soll 1998; Lebreton et al. 2003). In fact, regulation of homotetrameric GAPDH in lower photosynthetic organisms requires interaction with other partner proteins, namely CP12 and PRK (Wedel and Soll 1998; Graciet et al. 2004a). Essentially the same type of regulation has been conserved in higher plants where it co-exists with the autonomous regulation of AB-GAPDH (Scheibe et al. 2002).

CP12 is universally distributed in oxygenic photosynthetic organisms and apparently absent in heterotrophs. Lower photosynthetic organisms generally contain a single copy gene for CP12 whereas CP12 proteins in seed plants are coded by small gene families (e.g. three members in Arabidopsis, all predicted to code for plastid proteins). In the case of Arabidopsis, CP12-1 and CP12-2 are 86% identical in amino acid sequence and the expression of *CP12-1* and *CP12-2* genes is generally coordinated to the expression of *GapA-1*, *GapA-2*, *GapB* and *PRK* in different organs and growth stages (Fig. 1), although *CP12-1* is the only gene of this group which is not down-regulated in leaves upon prolonged darkening (Marri et al. 2005a). On the other hand CP12-3 is less than 50% identical to CP12-1 and CP12-2, its expression is extremely low and follows a different pattern, suggesting unrelated and still undefined physiological roles (Fig. 1).

CP12 are small proteins of about 80 amino acids which invariably include a pair of conserved cysteines in their C-terminal portion (Pohlmeyer et al. 1996). A second pair of N-terminal cysteines is conserved in most CP12 proteins, but may be missing in some photosynthetic organisms, e.g. in rhodophytes, *Cyanophora* (Petersen et al. 2006) and in *Synechococcus* PCC7942 (Tamoi et al. 2005). Both cysteine couples are subjected to dithiol/disulfide equilibria under the control of thioredoxins (Wedel and Soll 1998; Scheibe et al. 2002; Graciet et al. 2003b; Marri et al. 2005b). Thioredoxins are ubiquitous, too, in oxygenic photosynthetic organisms (Buchanan 1992).

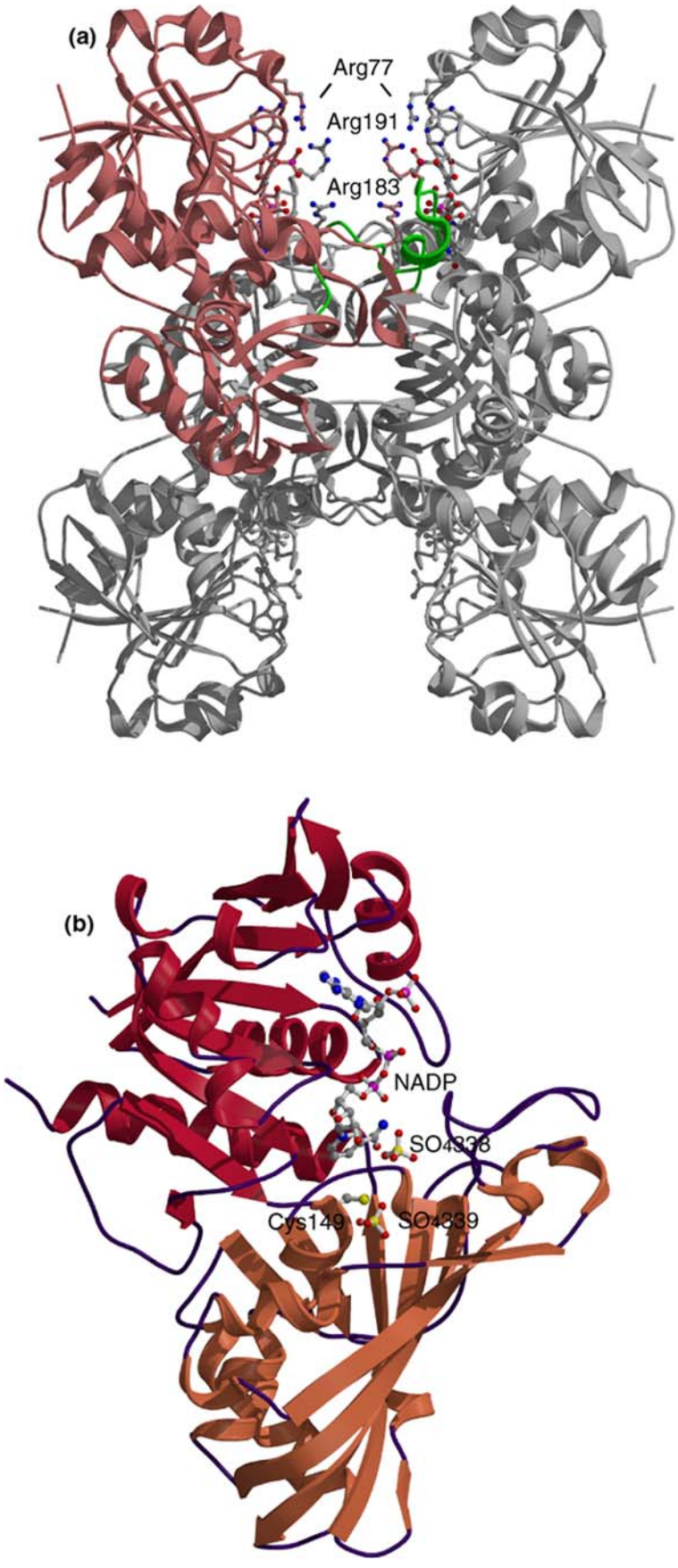

Fig. 2 Ribbon model of photosynthetic A_4-GAPDH from spinach (recombinant enzyme, Sparla et al. 2004). (**a**) Each subunit of the tetramer binds one NADP (represented as balls and sticks). The upper left subunit is coloured light salmon and the S-loop of this subunit is coloured green; the other three subunits are coloured grey. The side chains of Arg77, Arg183 and Arg191 of the upper left subunit (light salmon) and the upper right subunit (grey) are represented as balls and sticks. These basic residues are suggested to be involved in the interaction with acidic CTE (and possibly CP12) leading to enzyme regulation. Symmetrical residues in the lower subunits are not shown. (b) Overall fold of a single subunit of photosynthetic A_4-GAPDH (Sparla et al. 2004). The subunit is similarly oriented as in the tetramer, the coenzyme binding domain is coloured crimson and the catalytic domain is coloured salmon. NADP, sulphate ions and the side chain of catalytic Cys149 are represented as balls and sticks. Sulphate ions co-crystallize with the protein and are believed to mark the position of phosphates of the substrate BPGA in the catalytic site

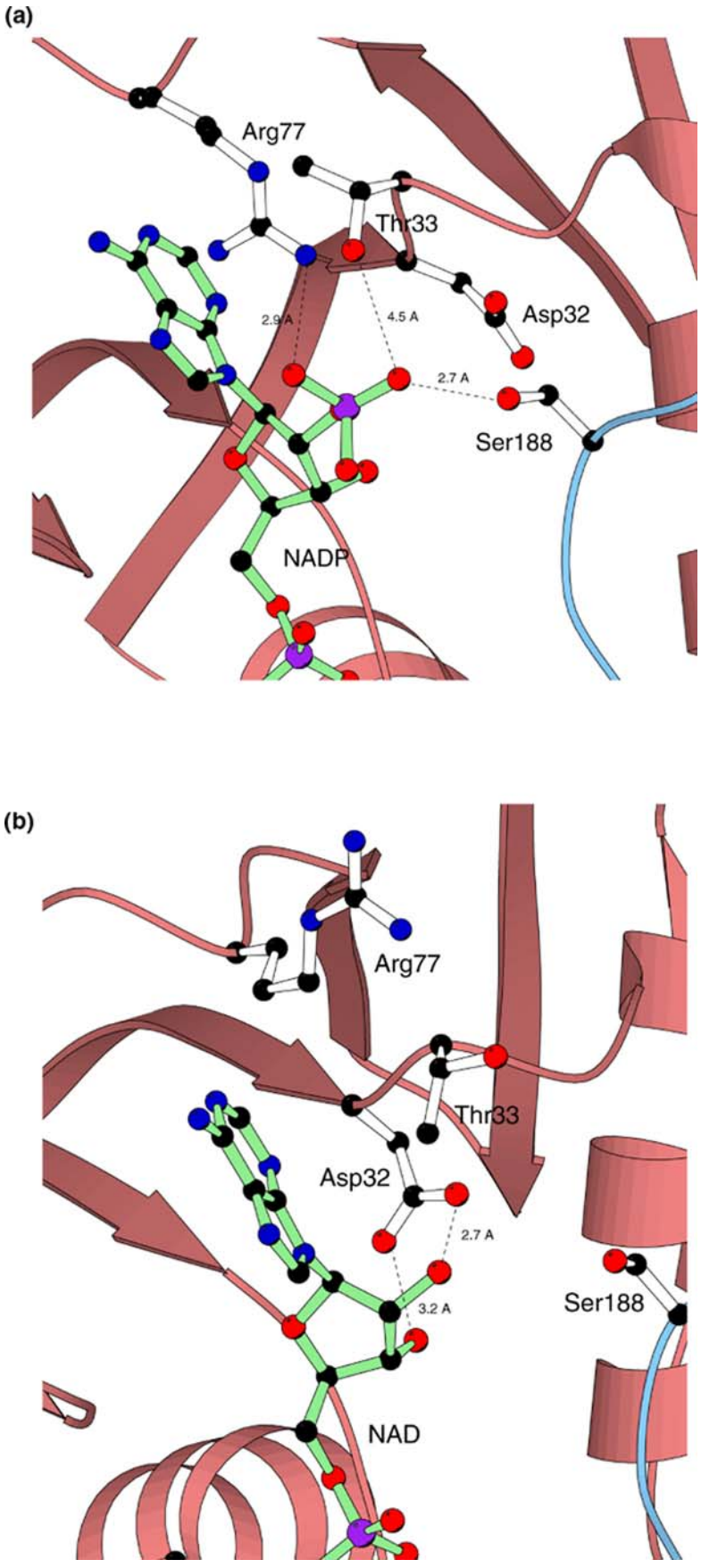

Fig. 3 Molecular basis of coenzyme recognition in photosynthetic GAPDH. Detailed view of the interactions between (**a**) the 2′-phosphate of NADP and (**b**) 2′- and 3′-hydroxyl groups of NAD with recombinant A_4-GAPDH from spinach (Falini et al. 2003; Sparla et al. 2004). Only relevant amino acids and interactions shorter than 5 Å are reported. Note that the coenzyme is bound to the subunit coloured light salmon, while Ser188 belongs to the adjacent subunit (light blue). Atom colour code: C black, N blue, O red, P magenta

The amino acid composition of CP12 proteins diverges from the average composition of globular proteins in the relative abundance of charged amino acids (especially Glu) and paucity of hydrophobic ones (especially Tyr, Phe, Ile and Leu). This is a feature of intrinsically unstructured proteins (IUP, Dyson and Wright, 2005). Softwares developed for predicting structural disorganization in proteins (e.g. PONDR, Peng et al. 2005) calculate that large portions of CP12 may be disordered. Consistent with the prediction, CP12 of the green algae *Chlamydomonas reinhardtii* was shown by NMR to be disordered in solution (Graciet et al. 2003b). Under oxidizing conditions the formation of two internal disulfides reduced the overall disorder of the protein and the content in α-helices increased, but still oxidized CP12 remained very flexible and its structure was not amenable to being solved by conventional methods (Graciet et al. 2003b). Recently, a bioinformatic approach was adopted to design a structural model of oxidized CP12 (Gardebien et al. 2006). Genes coding for IUPs are predicted to represent a significant portion of eukaryotic genomes, but are much less common in prokaryotes (Ward et al. 2004) and CP12 is one of a few known in plants and cyanobacteria. Due to their inherent flexibility, IUPs are typically involved in protein–protein interactions and often promote the formation of supramolecular complexes (Dyson and Wright 2005; Tompa et al. 2005). The functioning of CP12 in chloroplasts does also seem to follow these general principles.

The involvement of CP12 as a scaffold protein in supramolecular complexes containing GAPDH and PRK has been demonstrated in several photosynthetic organisms including cyanobacteria (*Synechocystis* PCC6803, Wedel and Soll, 1998; *Synechococcus* PC7942, Tamoi et al. 2005), *Chlamydomonas reinhardtii* (Wedel and Soll 1998; Graciet et al. 2003b) and higher plants (spinach, Wedel et al. 1997; Scheibe et al. 2002; Arabidopsis, Marri et al. 2005b). Reports on GAPDH/PRK interactions in bean leaves (Wara-Aswapati et al. 1980) and in *Scenedesmus obliquus*, another green unicellular alga (Nicholson et al. 1987), published long before the discovery of CP12 (Pohlmeyer et al. 1996) can also be interpreted as CP12-dependent effects. Recurring features of GAPDH/CP12/PRK supramolecular complexes in different species include size of the complex (published values range between 460 and 640 kDa) and the inhibition of both GAPDH and PRK when embedded in the complex. Moreover, in most cases it was shown that complex formation was promoted by oxidizing conditions and by NAD(H), while reducing conditions and NADP(H) tend to dissociate the complex thereby activating the released enzymes. Consequently, the complex in chloroplasts or cyanobacteria is predicted to prevail *in vivo* under darkness while being dissociated in the light (Fig. 4). Interestingly, a *Synechococcus* PC7942 mutant expressing no CP12 grew normally under continuous light but was impaired in growth under light/dark cycle (12 h/12 h), strongly suggesting that CP12 was required for a fine modulation of the Calvin–Benson cycle under variable light regime (Tamoi et al. 2005).

The GAPDH/CP12/PRK system has been investigated more in depth in *Chlamydomonas* (Graciet et al. 2004a) but the system works similarly also in cyanobacteria or higher plants (Wedel and Soll 1998; Scheibe et al. 2002; Marri et al. 2005b). A_4-GAPDH can bind CP12 only in the presence of NAD(H) and when CP12 is oxidized (cysteines linked by disulfides). In *Chlamydomonas* (Graciet et al. 2003b) the interaction between A_4-GAPDH(NAD) and oxidized CP12 is quite strong (K_D 0.4 nM) and GAPDH activity is inhibited by CP12 (mostly a k_{cat} effect, Graciet et al. 2003a). However no major kinetic effects of CP12 on Arabidopsis GAPDH can be observed (Marri et al. 2005b). CP12 has little affinity for PRK, but PRK avidly binds the GAPDH/CP12 binary complex (K_D 60 nM in *Chlamydomonas*, Graciet et al. 2003b). The role of CP12 is crucial in this context because the affinity of PRK for GAPDH is poor in the absence of CP12 (Graciet et al. 2003b; Scheibe et al. 2002; Marri et al. 2005b). Both in *Chlamydomonas* (Lebreton et al. 2003) and *Arabidopsis* (Marri et al. 2005b), PRK is itself regulated by thioredoxins and we have found that Arabidopsis PRK does bind the GAPDH/CP12 binary complex only when it is previously oxidized. Although PRK activity is substantially inhibited by oxidation (Marri et al. 2005b), complexation with GAPDH/CP12 has a much stronger inhibitory effect. The NADPH-dependent activity of A_4-GAPDH, in turn, is also strongly inhibited as soon as PRK joins the complex (Fig. 4). Inhibition of catalytic activities by complex formation is a reversible process and different conditions (reduced thioredoxin, NADP(H), ATP, BPGA) are able to dissociate the complex and release free enzymes (Wedel and Soll 1998; Scheibe et al. 2002; Graciet et al. 2004a; Marri et al. 2005b; Tamoi et al. 2005). Interestingly neither for PRK nor for GAPDH does complex dissociation necessarily implicate full enzyme activation (see Marri et al. 2005b, for the *Arabidopsis* system and Graciet et al. 2004a for *Chlamydomonas*), in some cases it is only a step towards full kinetic competence. In the *Arabidopsis* system for instance, free A_4-GAPDH

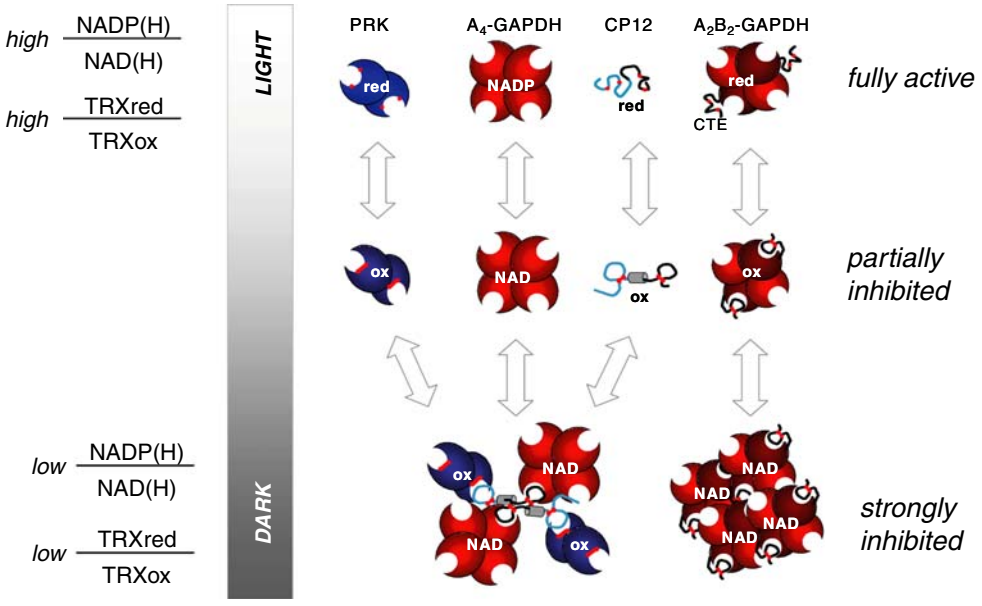

Fig. 4 A schematic view of A_4-GAPDH, CP12, PRK and AB-GAPDH interactions and regulation. ''Light conditions'' promote the dissociation of supramolecular complexes. Reduced PRK, A_4-GAPDH in the NADP-binding conformation and reduced A_2B_2-GAPDH are fully active while reduced CP12 does not interact with partner proteins. ''Dark conditions'' promote disulfide formation in PRK, CP12 and A_2B_2-GAPDH while A_4-GAPDH shifts to a NAD-binding conformation. These conditions promote the formation of supramolecular complexes A_4-GAPDH/CP12/PRK and A_8B_8-GAPDH. Within these complexes both NADPH–GAPDH and PRK activities are strongly inhibited

released by dissociation of the complex by ATP or NADP or reduced thioredoxin is partially inactive and recovers full activity only after incubation with BPGA (Marri et al. 2005b). This effect is peculiar since purified A_4-GAPDH is known to be insensitive to the substrate/activator BPGA (Scagliarini et al. 1998). It is therefore apparent that free A_4-GAPDH, after release from the complex, preserves a conformational memory of its previous inactive state. In this view, BPGA seems to reset this conformational memory and promote the stabilization of a fully active conformation. Similar conformational effects were observed in the *Chlamydomonas* system (Graciet et al. 2004a).

In conclusion, CP12 provides a means for a robust light-regulation of GAPDH and PRK in oxygenic photosynthetic organisms. In cyanobacteria and lower photosynthetic eukaryotes, which do not contain autonomously regulated GAPDH based on GapB subunits, this is apparently the only possibility to decrease NADPH-dependent GAPDH activity in the dark. Higher plants have inherited this ancient regulatory mechanism which coexists here with a more recently developed, autonomously regulated AB-GAPDH as the major GAPDH isoform of advanced photosynthetic organisms.

CP12-dependent vs. CTE-mediated regulation of GAPDH: alternative regulatory systems based on the same molecular mechanism?

Upon evolution of GAPDH from unicellular green algae to higher plants, characterized by the acquisition of GapB chimeric subunits, redox regulation of photosynthetic GAPDH has become a highly sophisticated process. GapB subunits contain a predominant glyceraldehyde-3-phosphate dehydrogenase moiety which is nearly 80% identical to GapA subunits of the same species, and a minor C-terminal extension (CTE) of about 30 amino acids clearly related to the C-terminal end of CP12 (Pohlmeyer et al. 1996). As a result, GapB subunits become autonomously redox-sensitive. These subunits are associated in vivo with GapA to form the major GAPDH isoform of higher plants chloroplasts, consisting of GapA and GapB in stoichiometric ratio (AB-GAPDH; Cerff and Chambers 1979; Brinkmann et al. 1989; Ferri et al. 1990).

At variance with A_4-GAPDH, the activity of purified AB-GAPDH is strongly redox regulated such that the NADPH-dependent activity of this isozyme can be titrated by varying the ambient redox potential in the presence of thioredoxin *f* (Sparla et al. 2002). Under reducing (i.e. activating) conditions, the catalytic

activity (k_{cat}) and the affinity (K_m) for the substrates of AB-GAPDH are similar to those of A_4-GAPDH (Sparla et al. 2004). However, under oxidizing conditions AB-GAPDH displays a specific drop in k_{cat} of the NADPH-dependent reaction, while K_m values remain fairly constant. The redox-potential vs. activity plot was characterized by a midpoint potential around –355 mV at pH 7.9 (Hutchinson et al. 2000; Sparla et al. 2002), slightly more reducing than thioredoxin *f* (Hirasawa et al. 1999).

The redox regulation of native AB-GAPDH is closely mimicked by an artificial recombinant isozymes comprised of GapB subunits alone (Baalmann et al. 1996; Li and Anderson 1997; Sparla et al. 2002). By using GapB as a model system, it was shown that the drop in $k_{cat(NADPH)}$ depended on the formation of an internal disulfide between the two cysteines of the CTE (Sparla et al. 2002). Interestingly, the CTE could be artificially transferred from a GapB to a GapA subunit with the result of generating a redox insensitive GapB (*min*CTE) (similar to GapA) and a redox-sensitive GapA(*plus*CTE) (similar to GapB, Sparla et al. 2005). These redox-sensitive GAPDH isoforms are also prone to aggregate in the presence of NAD(H) giving rise to multimers with low NADPH-dependent activity (Pupillo and Giuliani Piccari 1975; Baalmann et al. 1996; Li and Anderson 1997; Sparla et al. 2002, 2005). Redox-insensitive GapB mutants or GapA, on the contrary, are also insensitive to pyridine nucleotide regulation, strongly suggesting that redox regulation (depending on the CTE) and NAD(P)(H)-regulation (depending on the coenzyme binding site) are two strictly interacting mechanisms (Baalmann et al. 1996; Sparla et al. 2002).

Given the 80% sequence identity between GapA and GapB (excluding the CTE) it was speculated that A_4-GAPDH may represent a structural model for A_2B_2-GAPDH under reductive (non-inhibited) conditions. Preliminary results on the crystallographic structure of A_2B_2-GAPDH support this assumption (Fermani S and Sparla F, unpublished). As mentioned above, Arg-77 and Ser-188 (fully conserved in both GapA and GapB) are supposed to be responsible for the kinetic preference of reduced A_2B_2-GAPDH for NADPH over NADH (Fig. 3; Carugo and Argos 1997). Indeed, mutation of Arg77 into Ala in GapB resulted in the loss of kinetic preference for NADPH (Sparla et al. 2005) and mutation of Ser-188 into Ala had a similar effect in GapA (Sparla et al. 2004). These mutants are thus reminiscent of oxidized AB-GAPDH and suggest that redox regulation of GAPDH isozymes that are directly regulated by thioredoxins depends on the ability of oxidized CTE to interfere with the recognition of bound NADPH mediated by Arg-77 and Ser-188. In this view oxidized AB-GAPDH would be lowly active with NADPH because this coenzyme, though occupying the coenzyme binding site, is not properly recognized and the enzyme would adopt a kinetically inefficient conformation characterized by a $k_{cat(NADPH)}$ similar to $k_{cat(NADH)}$ (Sparla et al. 2004). Interestingly, the overall conformation of both mutant S188A(GapA) (Sparla et al. 2004) and oxidized A_2B_2-GAPDH (Fermani S and Sparla F, unpublished) are slightly different from the conformation of wild type A_4-GAPDH, suggesting that subtle conformational changes may be the cause of the observed kinetic inhibition.

How oxidized CTE would cause this effect is not yet clear. The CTE is rich in charged amino acids and bears a net negative charge (6 Glu, 2 Asp and 2 Arg among the last 20 residues). In neighbourhood of the coenzyme binding site of A_4-GAPDH, two arginines belonging to the S-loop (Arg-183 and Arg-191, Fig. 2A) are exposed to the medium and represent likely candidates to be involved in electrostatic interactions with negative charges of the CTE. In principle, the stability of interactions between the 2'-phosphate of NADP and the side chains of Arg-77 (salt bridge) and Ser-188 (hydrogen bond, Fig. 3A) may also be affected by the close proximity of the negatively charged CTE. As a support to this hypothesis, the redox regulation of recombinant GapB was completely abolished in a mutant having Glu-362 (the last residue of the CTE) exchanged into Gln (Sparla et al. 2005), indicating a crucial role for this single negative residue of the CTE in the whole regulatory process.

Although we do not know yet the exact mechanism by which oxidized CTE, through its terminal Glu-362, would specifically inhibit the NADPH-reaction of AB-GAPDH, it is interesting to observe that light-regulated NADP-malate dehydrogenase (NADP-MDH) shows some analogies with AB-GAPDH (Miginiac-Maslow and Lancelin 2002). In NADP-MDH of higher plants, redox regulation is accomplished by the action of two regulatory domains (an N-terminal and a C-terminal extension) each of them containing a couple of redox-active cysteines. The C-terminal extension of NADP-MDH is reminiscent of the C-terminal extension of GAPDH in that it bears glutamate as the penultimate residue of the sequence. As shown by the crystal structures of oxidized NADP-MDH from *Sorghum* (Johansson et al. 1999) and *Flaveria* (Carr et al. 1999), the side chain of the penultimate glutamate docks the tip of the oxidized C-terminal extension of NADP-MDH into the active site, thereby preventing the access of the substrate. As a major difference with GAPDH, however, NADP-MDH is specific for NADP and is completely inactivated when oxidized (Miginiac-

Maslow and Lancelin 2002). Since oxidized GAPDH is still active with NADH and NADPH at potentially significant rates the CTE cannot simply prevent the access of BPGA, otherwise both activities would be similarly regulated. The peculiarity of GAPDH is that redox-inhibition converts an active NADPH-preferring enzyme into a catalytically inefficient dehydrogenase with no marked coenzyme preference.

The high similarity between the CTE and the C-terminal end of CP12 includes a strict conservation of most of the charged amino acids (including the C-terminal Glu of the CTE which corresponds to a penultimate Glu or Asp in most CP12 proteins, Petersen et al. 2006) suggesting that the function of CTE may resemble that of CP12 at the molecular level. In *Chlamydomonas*, binding of CP12 to GAPDH is prevented when Arg-197 of GAPDH (belonging to the S-loop and corresponding to Arg-191 in spinach, Fig. 2A) is mutated into a glutamate (Graciet et al. 2004b), in full agreement with the model of a negative CTE (or CP12) interacting with a positive S-loop in the GAPDH core.

Both CP12-dependent and CTE-dependent regulatory systems of GAPDH will eventually result in the formation of large complexes stabilized by NAD(H). It is tempting to speculate that interaction of CP12/CTE with the core of GAPDH induces similar conformational effects such that novel protein/protein interactions are triggered. In the case of GapA/CP12 the final supramolecular complex recruits PRK with a possible stoichiometry of $(A_4\text{-GAPDH})_2\text{-CP12}_2\text{-PRK}_2$ (Wedel and Soll 1998; Graciet et al. 2004a), while in the case of AB-GAPDH the ''nocturnal'' complex is a hexadecamer (A_8B_8-GAPDH, Fig. 4; Pupillo and Giuliani Piccari 1975; Wolosiuk and Buchanan 1976).

Hopefully forthcoming tridimensional structures and the analysis of further mutants will soon clarify whether the autonomous regulation of AB-GAPDH by CTE and the CP12-dependent regulation of A_4-GAPDH are based on a similar molecular mechanism, as evolutionary considerations and experimental data would suggest.

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