An intrinsically disordered protein, CP12: jack of all trades and master of the Calvin cycle

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Abstract
Many proteins contain disordered regions under physiological conditions and lack a specific three-dimensional structure. These are referred to as IDPs (intrinsically disordered proteins). CP12 is a chloroplast protein of approximately 80 amino acids and has a molecular mass of approximately 8.2–8.5 kDa. It is enriched in charged amino acids and has a small number of hydrophobic residues. It has a high proportion of disorder-promoting residues, but has at least two (often four) cysteine residues forming one (or two) disulfide bridge(s) under oxidizing conditions that confers some order. However, CP12 behaves like an IDP. It appears to be universally distributed in oxygenic photosynthetic organisms and has recently been detected in a cyanophage. The best studied role of CP12 is its regulation of the Calvin cycle responsible for CO2 assimilation. Oxidized CP12 forms a supramolecular complex with two key Calvin cycle enzymes, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and PRK (phosphoribulokinase), down-regulating their activity. Association–dissociation of this complex, induced by the redox state of CP12, allows the Calvin cycle to be inactive in the dark and active in the light. CP12 is promiscuous and interacts with other enzymes such as aldolase and malate dehydrogenase. It also plays other roles in plant metabolism such as protecting GAPDH from inactivation and scavenging metal ions such as copper and nickel, and it is also linked to stress responses. Thus CP12 seems to be involved in many functions in photosynthetic cells and behaves like a jack of all trades as well as being a master of the Calvin cycle.

Introduction
For many years, it had been thought that the ability of a protein to fulfil its cellular function depends on a well-defined three-dimensional structure. In the last decade, however, evidence has accumulated from all kingdoms of life showing that many proteins involved in regulation, signal transduction, transcription etc. contain disordered regions under physiological conditions and lack a specific three-dimensional structure. These proteins are referred to as IDPs (intrinsically disordered proteins) [1–6]. IDPs have been less studied in plants than in some other kingdoms, but examples include plant stress proteins, dehydrins, such as late embryogenesis abundant proteins [7] and DSP16 (desiccation stress protein 16) [8], and an MSP (manganese-stabilizing protein) of 33 kDa which is part of Photosystem II [9].

CP12, a small chloroplast protein, is also an IDP [10]. It was originally described in spinach, pea and tobacco leaves and shown to interact with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in the chloroplast [11]. Subsequently, it was shown to form a supramolecular complex with GAPDH and PRK (phosphoribulokinase) [12,13]. The term CP12 originated from the behaviour of this protein and means ‘chloroplast protein of 12 kDa’ because using electrophoresis under denaturing conditions, CP12 migrates as a 12 kDa protein, even though it has approximately 80 amino acids and a theoretical molecular mass of approximately 8.2–8.5 kDa, depending on the species. In higher plants and algae, CP12 is encoded in the nucleus and transported into the chloroplast, where it is involved in regulating the Calvin cycle.

Since its discovery, many studies have been performed on this IDP. The aim of the present mini-review is to provide an update on the important role CP12 plays in regulating the Calvin cycle and the many other putative functions performed by this fascinating protein.

Distribution and sequence analysis of CP12
CP12 appears to be universally distributed in oxygenic photosynthetic organisms [14] and has been found in higher plants, green and red algae, diatoms and cyanobacteria [12,13,15–18]. Intriguingly, it has been reported recently in viruses: cyanophages which infect the marine cyanobacteria Prochlorococcus and Synechococcus carry and express CP12 [19].

CP12 is enriched in charged amino acids and has a low number of hydrophobic residues [14,20]. The mean net charge/hydrophobicity ratio of this protein is thus distinctly higher than that of structured proteins. CP12...
has characteristics of IDPs [2,4] because it is enriched in disorder-promoting residues (in particular alanine, glutamine and lysine) and depleted in order-promoting residues (phenylalanine, tryptophan, tyrosine, asparagine and leucine).

CP12 proteins invariably include a pair of conserved cysteine residues at their C-terminus [11]. A second pair of N-terminal cysteine residues is conserved in most CP12 proteins, but may be absent from some photosynthetic organisms, e.g. in rhodophytes, Cyanophora and Synechococcus [14,21]. Because of its order-promoting cysteine residues, CP12 stands just at the boundary between folded and unfolded protein spaces. Lastly, the hallmark of all CP12 proteins is the core consensus sequence A\textsuperscript{34}WDTVEEL\textsuperscript{41} (numbered from the mature Chlamydomonas reinhardtii CP12 sequence; Figure 1). However, in CP12 from the cyanophage [19], this sequence is absent, but the C-terminal part, including the disulfide bridge, is present.

Lower photosynthetic organisms generally contain a single copy gene for CP12, whereas CP12 in seed plants is encoded by small gene families. In the case of Arabidopsis thaliana, three CP12 proteins have been identified. CP12-1 and CP12-2 are 86% identical in amino acid sequence, and the expression of CP12-1 and CP12-2 genes is generally co-ordinated to the expression of GAPDH and PRK in different organs and growth stages [22]. On the other hand, CP12-3 is less than 50% identical with CP12-1 and CP12-2, its expression is extremely low and follows a different pattern, suggesting an unrelated and still undefined physiological role. Also, even if CP12 was primarily regarded as a chloroplast protein, it has also been found in roots, siliques and flowers of higher plants [22].

### Structural characterization of CP12

Consistent with their amino acid composition, CP12 proteins of the green alga C. reinhardtii and the higher plant A. thaliana were shown by NMR to be disordered in solution. Under reducing conditions, studies using CD showed that CP12 (Figure 2A) is completely disordered [10,20,23]. Under oxidizing conditions, owing to the formation of the two disulfide bridges, one at the N-terminus (Cys\textsuperscript{23} and Cys\textsuperscript{31}, numbered from C. reinhardtii CP12) and one at the C-terminus (Cys\textsuperscript{66} and Cys\textsuperscript{75}, Figure 2B), the overall disorder of the protein decreased and some α-helices were present, but oxidized CP12 still remained very flexible. Mutants of the CP12 have been produced using site-directed mutagenesis to replace one of the cysteine residues by a serine residue, thus disrupting the bridges. The lack of structure in IDPs makes them sensitive to proteolysis in vitro [24]. Wild-type CP12 is sensitive to protease attack, but the more unstructured cysteine mutants are even more easily degraded (B. Gontero, unpublished work). The cysteine mutants, like the wild-type, have an abnormal migration on SDS/PAGE with an apparent molecular mass that is much higher than the theoretical one. This electrophoretic behaviour is also characteristic of an IDP.

A bioinformatic approach, using the C. reinhardtii CP12, was used to produce a structural model of oxidized CP12 that proposed the existence of two α-helices located in the N-terminal and central parts, whereas the C-terminal part remains flexible. In this model, CP12 exhibits two faces with distinct electrostatic properties [25]. This charge distribution, with positively and negatively charged residues clustered, might be involved in the dimerization of CP12 in a ‘head-to-tail’ or antiparallel way [13] to facilitate the binding of target enzymes [25].

### CP12: master of the Calvin cycle

The flexibility and the net negative charge of CP12 may increase its reactive area and ‘stickiness’ compared with rigid proteins, thus enhancing the ability of this protein to act as a ‘scaffold protein’. The involvement of CP12 in supramolecular complexes containing GAPDH and PRK has been demonstrated in several photosynthetic organisms [10,12,13,16–18,22,26–29]. By interacting with PRK and GAPDH, CP12 indirectly regulates the Calvin cycle responsible for CO\textsubscript{2} assimilation. This cycle, driven by ATP and NADPH produced by the primary phase of photosynthesis, needs to be finely tuned in order to prevent a futile cycle either with the oxidative pentose-phosphate pathway or with glycolysis. The Calvin cycle is inactive in the dark and active in the light. Many mechanisms are involved in this control such as pH, magnesium and metabolite concentrations, and protein–protein interactions such as the ferredoxin-thioredoxin system [30,31]. An additional level of regulation involves the association–dissociation of supramolecular complexes involving CP12 [23]. The benefit of having CP12 for co-ordinated regulation of photosynthetic activity via modulation of PRK and GAPDH activities was
Figure 2 | CD studies and model of C. reinhardtii CP12
(A) CD spectra of oxidized (ox, black line) and reduced (red, grey line) CP12 [10]. Because of the small amount of structural information and poor template similarities, the derivation of the model of oxidized CP12 (B) consisted of an iterative trial-and-error procedure using the comparative modelling program MODELLER, the three structure validation programs PROCHECK, PROSA and WHATIF, and molecular mechanics energy refinement of the model using the program CHARMM. The two disulfide bridges are between Cys23 and Cys31 and between Cys66 and Cys75. C-ter, C-terminus; N-ter, N-terminus.

CP12: jack of all trades
The absence of rigid globular structure under physiological conditions represents a considerable functional advantage by allowing ‘one-to-many partners’ or protein promiscuity. It had been thought until recently that upon binding, disordered proteins become ordered, but closer inspection of the complexes of IDPs reveals that they can preserve functionally significant disorder in the complex with their binding partner(s), a phenomenon termed ‘fuzziness’ [39]. IDPs that remain flexible, even in their bound state, often bind to multiple partners as a consequence of their plasticity. The crystal structure of a binary GAPDH–CP12 complex demonstrated in cyanobacteria where cells with a deletion of the CP12 gene grew more slowly than the wild-type cells under light/dark conditions, but not under continuous light [18].

Supramolecular complexes are found in cyanobacteria (Synechocystis PCC6803 [12], Synechococcus PC7942 [18], C. reinhardtii [10,12] and higher plants [13,16,29]). In higher plants, oligomerization of PRK in a supramolecular complex with GAPDH corresponded to the rapid modulation of these enzyme activities in response to changes in light intensity [32]. In most cases, the complex formation was triggered or promoted by NAD(H) and by oxidizing conditions in the dark leading to a decrease in activity. In contrast, NADP(H), reducing agent in vitro or light in vivo resulted in dissociation of the PRK–GAPDH–CP12 complex, leading to an increase in activity [10,12,13,16,18,29,32,33]. Moreover, CP12 has been shown to be redox-regulated by thioredoxins [32,33]. As oxidized CP12 is involved in the formation of the PRK–GAPDH–CP12 complex and reduced CP12 in the dissociation of this complex, CP12 allows modulation of the activities of PRK and GAPDH and down-regulates the Calvin cycle in the dark. The CP12 produced by cyanophages is also used to down-regulate the Calvin cycle of the host. Under phage infection, the ATP and NADPH produced by the cyanobacterium is re-routed towards the biosynthesis of dNTP for phage replication [19].

Analyses of the properties of PRK and GAPDH showed that the regulatory mechanisms differed in the higher plant A. thaliana and the green alga C. reinhardtii [10,16]. The strength of binding between the PRK–GAPDH–CP12 complex appears to differ between these two species and probably among other species [34]. For example, the dissociation constant for GAPDH–CP12 is in the micromolar range in A. thaliana, but in the nanomolar range in C. reinhardtii [10]. Therefore CP12 in the green alga was responsible for a down-regulation of GAPDH within the GAPDH–CP12 subcomplex that was only observed in the higher plant when PRK was present, i.e. within the PRK–GAPDH–CP12 complex. Phylogenetic variation in the amino acid sequence of CP12 [14] could partly explain the different patterns of regulation of PRK and GAPDH [15,35,36].

Higher plants contain two tetrameric isoforms of GAPDH based on GapA and GapB subunits [21]. The formation of a complex between GapA and CP12 provides a mechanism to regulate GAPDH because GapA lacks redox-sensitive cysteine residues. However, the GapB subunit contains a C-terminal extension that is homologous with the C-terminal part of CP12 and contains regulatory cysteine residues and therefore has the potential to be independently redox-regulated [21,37]. This suggests that in species such as potato where the A2B2 form is found inside the GAPDH–CP12–PRK [34], CP12 may not just regulate GAPDH, and indeed it has been shown that CP12 in C. reinhardtii could bind other enzymes of the Calvin cycle, such as fructose-1,6-bisphosphate aldolase [38].
from Synechococcus elongatus has been solved [40,41]. These data indicated that the only region of oxidized CP12 that is structured upon binding is the C-terminal region lying within the groove of the active site of GAPDH. These structural data are in good agreement with the kinetic data obtained with C. reinhardtii showing that the last residues of CP12 interact with the S-loop of GAPDH hindering NADPH entry, thus down-regulating chloroplast GAPDH [42]. The crystal structure data also corroborate results obtained by EPR showing that the C. reinhardtii CP12 remains very mobile when bound to GAPDH [43]. These data indicate that CP12-GAPDH is a fuzzy complex and the role of CP12 in chloroplast metabolism is probably not restricted to regulating the activity of enzymes belonging to the Calvin cycle.

Expression analysis in A. thaliana showed that the CP12 gene family is also expressed in non-photosynthetic tissues [44]. In an attempt to decipher the role of CP12 in tobacco metabolism, CP12 antisense lines were constructed [45,46]. Despite the gross morphological and metabolic changes observed, the number of transcriptional changes was small. For instance, in the antisense CP12 plants, the up-regulated transcripts were the genes for thylakoid polyphenol oxidase and polyamine metabolism. Analysis of CP12 antisense tobacco plants, possessing a severe leaf phenotype, also revealed modifications of the activity of two other enzymes, glucose-6-phosphate dehydrogenase and the chloroplast malate dehydrogenase. Moreover, in a previous study, other proteins such as malate dehydrogenase, elongation factor 1α2 and 38 kDa ribosome-associated protein were able to interact with CP12, but to a lesser extent than PRK, GAPDH and the aldolase [38]. So, clearly, the role of CP12 in vivo is more widespread than initially envisaged, but probably other approaches such as use of specific knockouts of CP12 genes or induced gene silencing will be required to elucidate it.

In C. reinhardtii, CP12 was also able to protect GAPDH against heat-induced inactivation and aggregation. CP12 thus appears to act as a chaperone, but, unlike other plant chaperones such as ERD10 and ERD14, two IDPs involved in protection against abiotic stresses that have a broad spectrum of substrates [7], the protective function of CP12 is specific to GAPDH [47].

Lastly, it has been shown that CP12 specifically binds Cu^{2+} and Ni^{2+} ions with a low affinity (dissociation constants of 26 and 11 μM respectively). The Cu^{2+} ion was able to oxidize the reduced CP12 and triggered the formation of the two disulfide bridges, but was also able to bind to oxidized CP12. Using hydrophobic cluster analysis, CP12 was shown to be similar to A. thaliana metallochaperones [48,49].

Conclusions
IDPs are important in human health and are involved in diseases such as cancer, Parkinson’s disease and Alzheimer’s disease. IDPs are also important in the plant kingdom, where proteins such as CP12 play key roles as a hub protein that can affect crop productivity and hence food security. In order to increase our mechanistic understanding of the roles that CP12 and other IDPs play in plants and algae, we need to exploit new techniques, bring together expertise from different disciplines such as biophysics, molecular biology, biochemistry and bioinformatics, and explore the large phylogenetic diversity of CP12 and other IDPs.

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