The novel cytochrome $c_6$ of chloroplasts: a case of evolutionary bricolage?

Christopher J. Howe1*, Beatrix G. Schlarb-Ridley1, Juergen Wastl1,†, Saul Purton2 and Derek S. Bendall1

1 Department of Biochemistry, University of Cambridge, Downing Site, Tennis Court Road, Cambridge CB2 1QW, UK
2 Department of Biology, University College London, Gower Street, London WC1E 6BT, UK

Received 14 June 2005; Accepted 21 October 2005

Abstract

Cytochrome $c_6$ has long been known as a redox carrier of the thylakoid lumen of cyanobacteria and some eukaryotic algae that can substitute for plastocyanin in electron transfer. Until recently, it was widely accepted that land plants lack a cytochrome $c_6$. However, a homologue of the protein has now been identified in several plant species together with an additional isoform in the green alga *Chlamydomonas reinhardtii*. This form of the protein, designated cytochrome $c_6A$, differs from the ‘conventional’ cytochrome $c_6$ in possessing a conserved insertion of 12 amino acids that includes two absolutely conserved cysteine residues. There are conflicting reports of whether cytochrome $c_6A$ can substitute for plastocyanin in photosynthetic electron transfer. The evidence for and against this is reviewed and the likely evolutionary history of cytochrome $c_6A$ is discussed. It is suggested that it has been converted from a primary role in electron transfer to one in regulation within the chloroplast, and is an example of evolutionary ‘bricolage’.

Key words: Arabidopsis, bricolage, *Chlamydomonas*, cytochrome, endosymbiosis, immunophilin, plastocyanin, photosystem, thioredoxin, thylakoid.

Introduction

Cytochrome $c_6$ is a low molecular weight soluble redox carrier found in oxygenic photosynthetic bacteria and in eukaryotic algae, where it is widely distributed among green, red, and ‘brown’ (i.e. those that contain chlorophyll $c$) algal lineages (Sandmann *et al.*, 1983; Kerfeld and Krogmann, 1998). It is located in the thylakoid lumen, and functions in photosynthesis in the transfer of reducing equivalents from cytochrome $f$ of the cytochrome $b_6f$ complex to Photosystem I (Merchant and Dreyfuss, 1998). In oxygenic photosynthetic prokaryotes, where the photosynthetic membranes also contain a cytochrome oxidase, cytochrome $c_6$ also serves as an electron donor to this complex (Lockau, 1981; Obinger *et al.*, 1990; Moser *et al.*, 1991; Nicholls *et al.*, 1992). The mature cytochrome $c_6$ polypeptide typically comprises 83–90 residues, with a single haem, and has a midpoint redox potential of 335–390 mV. Under copper-replete conditions, many cyanobacteria and green algae such as *Chlamydomonas* replace cytochrome $c_6$ with the copper-containing redox carrier plastocyanin, synthesis of which is repressed under copper starvation (Wood, 1978; Ho and Krogmann, 1984). Although the structures of the two proteins are different, they are similar in size and midpoint redox potential, reflecting their similar functions. There is considerable variation across species in the pI of both cytochrome $c_6$ and plastocyanin. However, in any one species, the pIs of both proteins are similar, again reflecting their similar functional interactions with other proteins (Ho and Krogmann, 1984; De la Rosa *et al.*, 2002). It was widely accepted that land plants lacked cytochrome $c_6$, having replaced it with plastocyanin (Kerfeld and Krogmann, 1998). Nevertheless, recent studies have shown that plants do have a form of cytochrome $c_6$. There are differing views in the literature of its function. In this review, the question of whether the ‘plant’ form of the protein functions as a substitute for plastocyanin and, if it does not, what its role might be, is discussed.

Discovery of cytochrome $c_{6A}$ in plants

The model whereby land plants had abandoned cytochrome $c_6$ in favour of plastocyanin became significantly more
complicated in 2002 when two groups reported nearly simultaneously the existence of a modified form of cytochrome $c_6$ in a range of land plants. This modified form of the cytochrome has subsequently been named cytochrome $c_{6A}$ for reasons that are discussed below, and this nomenclature will be used in the rest of this review. The report of Wastl et al. (2002) was based on searching of plant genomic and EST databases with a cyanobacterial cytochrome $c_6$ sequence. This identified homologues of cytochrome $c_6$ coding sequences in a number of land plants, including Arabidopsis, rice, wheat, barley, potato, and soybean. Although this report did not demonstrate the existence of the cytochrome at the protein level, the fact that the gene was transcribed (and therefore represented in EST databases) and conserved indicated that it encoded a functional protein. Remarkably, all the predicted amino acid sequences for the plant protein contained a conserved insertion of 12 amino acids compared with the bacterial and algal proteins. This insertion occurs in a region corresponding to a loop in the known three-dimensional structure of the bacterial and algal proteins. For brevity, the insertion will be referred to as the LIP (Loop Insertion Peptide). It contains two cysteine residues that are conserved in all the sequences studied. These sequences came from a wide range of plant species, and it was suggested that a disulphide bridge (or bridges) involving the cysteine residues was an important part of the function of the protein. Homology modelling, based on the structure of cytochrome $c_6$ from the eukaryotic green alga Monoraphidium, allowed a structure for the cytochrome $c_{6A}$ to be proposed (Fig. 1). This structure suggested that the surface properties of cytochrome $c_{6A}$ from Arabidopsis are quite different from those of Arabidopsis plastocyanin, implying that cytochrome $c_{6A}$ does not function as a simple substitute for plastocyanin in the light reactions of photosynthesis. In addition, it was noted that several of the EST libraries from which cytochrome $c_{6A}$ sequences had been identified were derived from non-photosynthetic tissue.

About the same time as the report of Wastl et al. (2002), a separate study independently reported the existence of the same plant homologue of cytochrome $c_6$. In this case, the initial discovery of cytochrome $c_{6A}$ had been as a protein interacting with the chloroplast immunophilin FKBP13 in a yeast two-hybrid assay (Gupta et al., 2002a; Buchanan and Luan, 2005). Gupta et al. (2002a) showed that the mRNA for cytochrome $c_{6A}$ was present in leaf tissue of Arabidopsis, with protein (as determined by western analysis using antibodies to an over-expressed glutathione S-transferase fusion protein) present in leaf and (at lower abundance) root tissue. Gupta et al. (2002a) also expressed the protein in the cyanobacterium Synechocystis sp. PCC 6803 and provided evidence that it could simulate photosynthetic electron transport in inside-out thylakoid membrane vesicles from Arabidopsis. They identified an Arabidopsis line with a T-DNA insertion mutation for cytochrome $c_{6A}$, but this strain had no visible phenotype even if homozygous plants were grown under conditions of copper deprivation. (Interestingly, no increase in levels of cytochrome $c_{6A}$ was observed in wild-type plants under copper deprivation.) When plastocyanin levels were depleted using RNAi in an otherwise wild-type background, the resulting plants were significantly smaller. Depletion of plastocyanin levels by RNAi in the cytochrome $c_{6A}$ mutant plants was lethal. Gupta et al. (2002a) interpreted all these data as indicating that cytochrome $c_{6A}$ was a functional substitute for plastocyanin in photosynthetic electron transfer in Arabidopsis.

However, Weigel et al. (2003b) subsequently showed that if both of the plastocyanin genes of Arabidopsis were inactivated by stable frameshift mutations resulting from transposon insertion, the resulting plants were unable to grow photoautotrophically, even if cytochrome $c_{6A}$ was

![Fig. 1. Homology model of Arabidopsis thaliana cytochrome $c_{6A}$ (left panel) based on that of cytochrome $c_6$ from Monoraphidium braunii (right panel). The haem is indicated in purple and the cysteines of the LIP are indicated in yellow. The models were prepared with Modeller4 and displayed with Molscript (Kraulis, 1991).](https://academic.oup.com/jxb/article-abstract/57/1/13/442096/2?612.0x791.0)
artificially overexpressed at the same time using the Cauliflower Mosaic Virus 35S promoter. Fluorescence data indicated a block in electron transfer downstream of the cytochrome b₆f complex, consistent with the loss of plastocyanin function. This study was carried out using a different ecotype from that used by Gupta et al. (2002a), but Weigel et al. (2003b) argued that the role of cytochrome c₆A was unlikely to be fundamentally different between the two ecotypes, and that their own results showed the protein could not substitute for plastocyanin in vivo. This disagreement was further addressed by Molina-Heredia et al. (2003), who showed that cytochrome c₆A could not substitute for plastocyanin in vitro. Their study used Arabidopsis cytochrome c₆A expressed in Escherichia coli, thus avoiding any possibility of contamination with cytochrome c₆ when using a cyanobacterium as the expression host. The protein expressed in E. coli showed spectroscopic characteristics expected of a functionally-folded protein. However, it was found to have a redox midpoint potential for the haem over 200 mV lower than that for plastocyanin from Arabidopsis (365 mV) or cytochrome c₆ from the eukaryotic green alga Monoraphidium (358 mV). The low midpoint potential of the Arabidopsis cytochrome c₆A makes it extremely unlikely that it could oxidize cytochrome f (its substrate if it substituted for plastocyanin) efficiently in vivo, because cytochrome f has a midpoint potential similar to that of plastocyanin or cytochrome c₆. Although the redox potential of cytochrome c₆A would, in principle, allow it to reduce photosystem I, Molina-Heredia et al. (2003) also showed that cytochrome c₆A was at least 100 times less efficient at doing so in vitro than plastocyanin. This was not unexpected, given the very different surface electrostatic potentials predicted for cytochrome c₆A and plastocyanin. However, increasing the ionic strength of the reaction conditions to screen the likely charge repulsion between cytochrome c₆A and Photosystem I failed to increase the rate of reaction significantly, suggesting that charge repulsion was not the only factor prohibiting the reaction. It is not clear why the midpoint potential of cytochrome c₆A is so different from cytochrome c₆. The difference is not due to the LIP, since its removal, or mutation of the cysteine residues in it to serine, did not greatly affect either the midpoint potential or the reactivity of the protein towards Photosystem I in vitro (Wastl et al., 2004a).

More doubt was cast on the ability of cytochrome c₆A to substitute for plastocyanin in higher plants by the observation that the mRNA expression profile of a set of over 3000 nuclear genes in a homozygous Arabidopsis cytochrome c₆A mutant was significantly different from the profiles in mutants lacking either or both functional plastocyanin genes (Weigel et al., 2003a). However, this study also noted that transcript levels of some genes for the photosynthetic machinery were similarly altered in the cytochrome c₆A mutant and the plastocyanin single mutants, and interpreted this as consistent with the notion that cytochrome c₆A plays a role in some aspect of the regulation of photosynthesis.

Cytochrome c₆A in algae

It is also now clear that cytochrome c₆A is not restricted to higher plants, as searching of the complete genome sequence of the green alga Chlamydomonas reinhardtii showed that this organism contains a gene (designated CYC4) for cytochrome c₆A (Wastl et al., 2004b) in addition to its much-studied cytochrome c₆ (Gorman and Levine, 1966; Merchant and Bogorad, 1987). The cytochrome c₆A gene has also been identified in sequence data (http://genome.jgi-psf.org/chlre2/chlre2.download.ftp.html) from the multicellular green alga Volvox carteri (S Purton, unpublished observation). However, not all eukaryotic algae contain cytochrome c₆A, as the red algal and diatom genomes do not contain a recognizable gene for it (Wastl et al., 2004b). The presence of both cytochrome c₆A and cytochrome c₆ genes in Chlamydomonas also adds weight to the proposal that cytochrome c₆A is not a simple substitute for plastocyanin, as it would presumably be redundant in the presence of a conventional cytochrome c₆.

Questions raised

This survey of the experimental information available about cytochrome c₆A raises several interlinked questions. For clarity and convenience, it is important to decide what the most appropriate name should be. A second question is how the existence and taxonomic distribution of cytochrome c₆ and cytochrome c₆A evolved. The remaining questions relate to the function of the protein. Where is it located, what is its structure, and what is its function?

Nomenclature

Initially, the protein was referred to simply as cytochrome c₆ or ‘the cytochrome c₆-like protein’. Weigel et al. (2003a) subsequently proposed the term cytochrome cₓ, as the protein had a different function from conventional cytochrome c₆. However, this nomenclature runs the risk of confusion with the c-type ‘haem x’ discovered in the cytochrome b₆f complex (Kurisu et al., 2003; Stroebel et al., 2003) and the cytochrome cₓ of purple photosynthetic bacteria (Jones et al., 1990). The name cytochrome c₆A seems the most appropriate because it distinguishes the protein from cytochrome c₆, while reflecting its origin from the cytochrome c₆ family (Wastl et al., 2004b).

Evolution

A model for the evolution of cytochrome c₆A is shown in Fig. 2. The earliest electron donors both to Type I and to Type II reaction centres (as seen today in, for example,
Chlorobium and Rhodobacter, respectively) were probably c-type cytochromes (Mathis, 1994; Itoh et al., 2002; Meyer and Cusanovich, 2003). It therefore seems likely that the original electron donor to Photosystem I in oxygenic photosynthetic bacteria was also a c-type cytochrome, which gave rise to present-day cytochrome c₆.

As atmospheric oxygen levels rose after the development of oxygenic photosynthesis, free iron would have become less readily available at the earth’s surface, copper would have become more available, and this would have provided a selective advantage for the development of the copper-containing redox protein plastocyanin (Osterberg, 1974; Wood, 1978; De la Rosa et al., 2002). The effect that the amount of iron required for synthesis of cytochrome c₆ has on the cell’s physiology presumably depends on its stoichiometry compared with the other components of the photosynthetic electron transfer chain, and may not be large (Raven et al., 1999). It is also likely that there would have been geographic heterogeneity in iron and copper levels, as well as variation with depth. However, iron-limitation is known to have an important effect on the metabolism of present-day cyanobacteria (Ferreira and Straus, 1994; Laudenbach et al., 1988; Laudenbach and Straus, 1988; Bibby et al., 2001; Boekema et al., 2001), so this explanation for the selective advantage of developing plastocyanin remains possible.

Based on ¹³C isotope signatures of carbonate in rocks, indicative of Form I Rubisco (found in oxygenic photosynthetic organisms), oxygenic photosynthesis developed some 2.7–3.0 billion years ago (reviewed by Nisbet and Fowler, 2004). Data on iron deposition and sulphur isotope fractionation indicate a major increase in oxygen levels around 2.3 billion years ago (Bekker et al., 2004; reviewed by Nisbet and Fowler, 2004). Phylogenetic analyses of present-day oxygenic photosynthetic bacteria (CJ Howe, unpublished results; Battistuzzi et al., 2004) indicate that the radiation of this group occurred more recently than 2.3 billion years ago (although the group itself has existed for longer). Thus if the development of plastocyanin was associated with the increase in oxygen levels at 2.3 billion years ago, both electron transfer systems would have been present at the time of the radiation of present-day oxygenic photosynthetic bacteria (Mathis, 1994; Itoh et al., 2002; Meyer and Cusanovich, 2003).

![Proposed evolutionary pathway for cytochrome c₆A](https://academic.oup.com/jxb/article-abstract/57/1/13/442096/16/Howe_et_al)
photosynthetic bacteria. This proposal is consistent with the observation that *Gloeobacter*, widely regarded on ultrastructural grounds as well as sequence-based studies as diverging early among the extant cyanobacteria (Nelissen *et al.*, 1995; Honda *et al.*, 1999), has both cytochrome *c*<sub>6</sub> and plastocyanin (Nakamura *et al.*, 2003). Presumably the maintenance of either or both of the two redox carriers in different extant species reflects the environments they have occupied during their evolution, and the consequences of lateral gene transfer.

Plastids are thought to have arisen about 1.5 billion years ago (reviewed by Falkowski *et al.*, 2004), by which time both cytochrome *c*<sub>6</sub> and plastocyanin would have been in existence according to the arguments above on oxygen levels. The chlorophyte lineage contains both cytochrome *c*<sub>6</sub> and plastocyanin, so is most likely to have derived from an endosymbiont containing both of these proteins. Duplication and divergence of cytochrome *c*<sub>6</sub> gave rise to cytochrome *c*<sub>6A</sub>. This probably took place in a chloroplast-containing lineage, rather than prior to endosymbiosis, as cyanobacteria do not appear to have cytochrome *c*<sub>6A</sub>. (However, it is possible that the duplication occurred earlier, in the prokaryotic ancestor to chloroplasts, and a prokaryotic cytochrome *c*<sub>6A</sub> remains to be discovered.) The cytochrome *c*<sub>6</sub> form was then lost from the lineage that gave rise to land plants after the divergence from the *Chlamydomonas* lineage. (This may indicate that the environments initially colonized by land plants contained adequate levels of copper. Furthermore, genuine copper starvation is relatively unusual in the wild among present-day land plants.) Cytochrome *c*<sub>6A</sub> and plastocyanin were retained in the land plant lineage.

Plastocyanin appears to be absent from red and brown algae (Sandmann *et al.*, 1983), and the complete genome sequences of the red alga *Cyanidioschyzon merolae* and the diatom *Thalassiosira pseudonana* do not contain a gene for cytochrome *c*<sub>6A</sub> or for plastocyanin. The significance of these observations for the origin of cytochrome *c*<sub>6A</sub> depends on whether a polyphyletic or a monophyletic origin of plastids is assumed (Lockhart *et al.*, 1999; Stiller *et al.*, 2003; McFadden and van Dooren, 2004). If polyphyletic origins of plastids are assumed, these observations can be most simply explained if the red algal lineage (and others derived subsequently by serial endosymbiosis) was derived from a symbiont possessing only cytochrome *c*<sub>6</sub>, and not plastocyanin. This lineage never developed cytochrome *c*<sub>6A</sub>. If a monophyletic origin is assumed, the simplest explanation is that loss of plastocyanin in the red/brown lineage and duplication of cytochrome *c*<sub>6</sub> in the green lineage happened after the separation of the two lineages. Other possibilities cannot be excluded, such as a monophyletic origin with an early duplication of cytochrome *c*<sub>6</sub> prior to divergence of the red and green lineages, together with subsequent loss in (at least some of) the non-green lineages, but these are more complex.

**Location**

Knowing where cytochrome *c*<sub>6A</sub> occurs, both in the whole plant and at the subcellular level, will help greatly in understanding its function. Using northern blots, Gupta *et al.* (2002a) showed that mRNA for cytochrome *c*<sub>6A</sub> was present in leaves, but barely detectable, if at all, in roots of plants grown in a greenhouse under long-day conditions. Using western blots with antibodies raised against the protein expressed in *E. coli*, they similarly showed that the protein was present in leaves, and at lower levels in roots. When the gene was placed under the control of a constitutive promoter, transcripts were found both in roots and leaves, but the protein appeared to be predominantly located in leaves, with an electrophoretic mobility that suggested it was post-translationally processed. Gupta *et al.* (2002a) also showed that the protein could be recovered in a thylakoid luminal fraction isolated from protoplasts of over-expressing plants. It was not found in the stromal fraction, although the efficiency of recovery of stromal proteins was not indicated. Although these data favour a location in the thylakoid lumen, they do not exclude other less likely possibilities, for example, that the protein is distributed between the thylakoid lumen and stroma (or even that it is mitochondrial, with a fraction mis-targeted to the chloroplast under over-expression conditions). A location in the lumen is consistent with the interaction between cytochrome *c*<sub>6A</sub> and the luminal immunophilin FKBP13.

Apart from these observations, there is little direct information on the expression or localization of the protein. A number of the EST databases from which cytochrome *c*<sub>6A</sub> sequences were identified by Wastl *et al.* (2002) were derived from non-photosynthetic tissue, such as anthers, suggesting that the transcript is present at significant levels in such tissue. Searching of microarray databases (Zimmermann *et al.*, 2004) also indicates the presence of transcripts for cytochrome *c*<sub>6A</sub> (as well as plastocyanin) in anthers of *Arabidopsis*. In general, however, cytochrome *c*<sub>6A</sub> transcripts are low in non-photosynthetic tissue (roots, petal, stamen, senescing leaf) and higher in photosynthetic tissue. This relative pattern is similar to that seen for plastocyanin, but with the cytochrome *c*<sub>6A</sub> transcripts less abundant.

Predictions as to the subcellular location of cytochrome *c*<sub>6A</sub> from the putative targetting sequence are inconclusive, with different algorithms variously predicting the protein to be in the chloroplast or the mitochondrial (Wastl *et al.*, 2004a). Proteomic analyses of the thylakoid lumen have failed to detect cytochrome *c*<sub>6A</sub> (Peltier *et al.*, 2002; Schubert *et al.*, 2003). It is unlikely that this failure was due to the size or isoelectric point of the protein, since plastocyanin was detected in these studies, yet has a lower predicted molecular mass and a more extreme isoelectric point than cytochrome *c*<sub>6A</sub> (10.5 kDa and 4.4 for plastocyanin compared with 11.8 kDa and 5.1 for cytochrome *c*<sub>6</sub>).
$c_{6A}$. This suggests that cytochrome $c_{6A}$ is of low abundance under the conditions used for those studies (but perhaps present at higher levels under stress conditions). More general proteomic studies of plastids, including differentiated chloroplasts and undifferentiated heterotrophic plastids from a tobacco BY-2 cell culture have likewise failed to detect cytochrome $c_{6A}$ (Baginsky et al., 2004; Friso et al., 2004; Kleffmann et al., 2004). It has also not been reported from other subcellular compartments, such as mitochondria (Millar et al., 2001).

Overall, therefore, it seems likely that, under ‘normal’ conditions cytochrome $c_{6A}$ is present at low levels in chloroplasts from green tissue, and may well be located in the thylakoid lumen. It is generally present at even lower levels (presumably in the plastid) in non-photosynthetic tissue. However, this model is based on a limited amount of data, and more detailed analyses, such as a more direct demonstration of the subcellular location and a study of the expression under different developmental and stress conditions, are needed.

Structure

No experimentally-determined three-dimensional structure of cytochrome $c_{6A}$ is available yet, although a structure has been modelled based on homology to cytochrome $c_6$ proteins whose structure is known (Fig. 1). Aspects of this have already been described. The 12 amino-acid insertion of cytochrome $c_{6A}$ occurs as an extension to a loop region of cytochrome $c_6$, but is not very distant from the haem group. The pI of Arabidopsis cytochrome $c_{6A}$ is 5.1, compared with 4.4 for Arabidopsis plastocyanin, and the electrostatic potential of cytochrome $c_{6A}$ indicates a much more positively charged surface than for plastocyanin (Molina-Heredia et al., 2003). It will be important to confirm this structure experimentally and to determine any structural differences between the oxidized and reduced forms of the protein.

Function

A role as a simple substitute for plastocyanin seems unlikely, given the redox characteristics of the protein (albeit expressed in a heterologous system), its inability to reduce Photosystem I in vitro, the lethality of plastocyanin mutants, the RNA microarray data described above, the failure to observe induction of cytochrome $c_{6A}$ under copper-deprivation, and the presence of a cytochrome $c_{6A}$ gene in Chlamydomonas, which also has a conventional cytochrome $c_6$. The contrary evidence of Gupta et al. (2002a, b) could be explained if gene inactivation in RNAi lines were incomplete (as is usually the case), and the cytochrome $c_{6A}$ used for reconstitution experiments contained cyanobacterial protein as contamination or was modified in some way. In any case, reactions can occur in vitro that do not necessarily occur in vivo.

So what does cytochrome $c_{6A}$ actually do? The following is known about the ‘physiology’ of the protein. It is expressed in chloroplast-containing tissue, at low levels under ‘normal’ growth conditions, and is probably located in the thylakoid lumen. It may also be present, at lower levels, in non-photosynthetic tissue. Loss of the protein leads to changes in transcript levels for, among others, proteins involved in photosynthesis. Loss in plants that already have reduced levels of plastocyanin is lethal. These observations suggest a regulatory role, as originally suggested by Wastl et al. (2002), in response to stress such as a disturbance of the light reactions of photosynthesis. Under ‘unstressed’ conditions, there is no need for the protein, so cytochrome $c_{6A}$ mutants have little phenotype. However, under serious stress conditions, including a major depletion of plastocyanin, the absence of cytochrome $c_{6A}$ is lethal, presumably because the organism is unable to respond appropriately.

How might the molecule perceive and transmit a signal? There are three obvious features; the haem, the LIP (which is absent from the conventional cytochrome $c_6$), and the apparent ability of the protein to interact with the chloroplast immunophilin FKBP13 in a two-hybrid screen (Gupta et al., 2002a; Buchanan and Luan, 2005). The binding of haem requires specific residues which are conserved in all cytochrome $c_{6A}$ proteins, suggesting that haem remains an important part of its function. The LIP is also highly conserved, with -C-P-G-CTFG identical from Chlamydomonas to higher plants. Interestingly, this sequence is similar in the spacing of the cysteine residues to an insertion EICDINGKC (in spinach) in the gamma subunit of land plant chloroplast ATP synthases that mediates the regulation of ATP synthase by thioredoxin. This insertion is absent from the cyanobacterial gamma subunit (reviewed by van Walraven and Bakels, 1996). Disulphide bridges are also known to be a mechanism for the perception and response to oxidative stress in other systems (Paget and Buttner, 2003). The reported interaction with an immunophilin is particularly interesting (although it should be noted that two-hybrid systems can often give false positive results). Immunophilins typically have peptidyl-prolyl cis-trans isomerase (PPI) activity, and there are a number of them in the thylakoid lumen (Romano et al., 2005). The PPI activity of the immunophilin AtrFKBP13 from the Arabidopsis thylakoid lumen can be modulated in vitro by a thioredoxin (Gopalan et al., 2004), and the precursor of AtrFKBP13 also interacts with the Rieske Fe-S protein of the photosynthetic electron transfer chain (Gupta et al., 2002b). At least one luminal immunophilin, TLP40, has been shown to participate in intraorganellar signalling as it is involved in modulating the phosphorylation state of a number of Photosystem II polypeptides, in addition to having a conventional PPI activity (Fulgos et al., 1998).

There are a number of different ways that cytochrome $c_{6A}$ might participate in regulation through the haem, the
LIP, or immunophilin-binding (Fig. 3A). One of the three elements might simply perceive a signal and then transmit it to another molecule without the involvement of the other two. However, these possibilities would not satisfactorily explain the existence of all three elements. Another possibility is that one element receives a signal and transmits it through one of the other two to a signalling pathway. For example, oxidation or reduction of one component might lead to oxidation or reduction of the other. Assuming it is similar to the midpoint potential of the disulphides in thioredoxin and thioredoxin-regulated enzymes, the midpoint redox potential of the putative disulphide bridge in the LIP is likely to be of the order of \(-300\) mV (Knaff, 2000). If electron transfer took place between the disulphide bridge and the haem, it would therefore be expected to require reduction of the former by another redox carrier (such as a thiol) followed by reduction of the haem by the cysteines and regeneration of the disulphide bridge. The haem could then be reoxidized by another redox carrier (perhaps plastocyanin, as discussed below). Electron flow from cysteines to haem would probably be consistent with the distance between them predicted from the homology model. However, haem reduction or oxidation is a one-electron process, whereas the reduction or formation of the disulphide bridge is a two-electron process, with a reactive intermediate generated after single electron transfer. Such an intermediate would need to be suitably safeguarded. Regulatory pathways involving only two of the three elements again leave the role of the third unexplained.

The most attractive possibility is that all three features are involved, perhaps with the protein functioning in the integration of different signals. For example, the haem might serve to transmit a signal based on the redox state of some component of the photosynthetic machinery, and its ability to do this might be influenced by a conformational change in the protein that depends on the LIP or on immunophilin-binding. Alternatively, the LIP may function in signalling, with its role likewise modified in response to the redox state of the haem or immunophilin-binding. It is suggested that the protein is used to sense both the redox state of the inter-photosystem electron transfer chain via the haem, and the state of the thioredoxin pool (which might reflect the overall flux through the chain) via the LIP, in order to transmit a signal through an immunophilin (Fig. 3B). For example, a change in oxidation state of the haem or the LIP might lead to a conformational change that modifies the ability of cytochrome \(c_{6A}\) to bind to an immunophilin, either by directly altering the immunophilin binding site or causing the release of cytochrome \(c_{6A}\) from some other complex and freeing it to bind with an immunophilin. Many processes in chloroplasts are regulated in response to the redox state of the plastoquinone pool, and thus the poise of the chain can influence the activity of many enzymes. For example, cytochrome \(c_{6A}\) is reduced by plastocyanin in the light, and this reduction is accompanied by a conformational change that allows the protein to bind to the immunophilin (Fig. 3B). This binding can then activate the enzyme, leading to an increase in the rate of photosynthesis. However, in the dark, the protein is not reduced, and its conformational change is not observed. This suggests that the protein is used to sense both the redox state of the inter-photosystem electron transfer chain and the state of the thioredoxin pool, and its ability to do this might be influenced by a conformational change in the protein that depends on the LIP or on immunophilin-binding.
of the electron transfer chain in general. These processes include the phosphorylation of light-harvesting and other proteins of the thylakoid membrane (Allen et al., 1981) and chloroplast gene expression (Pfannschmidt et al., 1999). The midpoint potential of the haem of cytochrome c6A would allow it to be reduced by the plastoquinone pool, or something closely linked to it, and would be consistent with the protein having a role in these regulatory processes.

Furthermore, many chloroplast functions are influenced by thioredoxins, including thylakoid protein phosphorylation (Carlberg et al., 1999). Although they are generally located in the stroma, at least one thiorexin-like protein, Hcf164, is associated with the thylakoid membrane and lumen (and, interestingly, is required for the biogenesis of the cytochrome b6f complex and is similar to bacterial proteins that are required for maturation of c-type cytochromes (Lennartz et al., 2001)). There is also at least one putative thiol disulfide transporter in the thylakoid membrane, CCDA, which may serve to link the state of the luminal thiorexin pool to that in the stroma (Page et al., 2004). It has also been suggested that cytochrome c6A may interact with stromal redox proteins on its way into the thylakoid lumen (Weigel et al., 2003b). Given the implication of immunophilins in regulatory processes as well as folding (Fulgosi et al., 1998), cytochrome c6A provides an attractive link to redox processes in the chloroplast. It is, of course, possible that a signal might move in a different direction through the molecule. For example, it might serve to detect the activity of the protein folding machinery, and use this to modulate photosynthetic electron transfer.

A possible regulatory role of a different kind is prompted by the observation that FKBP13 is activated by oxidation of disulfide bridges within the molecule (Gopalan et al., 2004). Cytochrome c6A might therefore oxidize FKBP13 (or other disulfide containing proteins), reducing the disulfide bridge in the cytochrome. This, in turn, could reduce the cytochrome’s haem group, and the electrons could be passed from that onto another redox carrier, such as plastocyanin, with a higher midpoint potential (i.e. a stronger oxidant) as shown in Fig. 3B. In other words, cytochrome c6A would form part of a short electron transfer chain that served to oxidize and regulate other proteins, with the electrons being ‘dispersed’ of into the photosynthetic electron transfer chain. (A close and stable complex between cytochrome c6A and its substrate might help avoid damage caused by the single-electron transfer intermediate.) Given the thiol disulfide transporter referred to above, cytochrome c6A might also help to regulate the thiol balance of the stroma.

More distant roles for the protein have also been suggested, including a speculation that it functions in apoptosis (Weigel et al., 2003a). It was not detected as an up-regulated protein in heat- or senescence-induced cell death in Arabidopsis cell culture (Swidzinski et al., 2004), but this may reflect the small size of the protein. The preferred model for the role of the protein is therefore in modulating the function of the immunophilin FKBP13 and perhaps other proteins. Cytochrome c6A either directs a response to the redox poise of luminal thiols and the electron transfer chain, or it provides the actual electron transfer pathway for oxidative regulation of disulfide proteins. If the role of cytochrome c6A has indeed been switched from an ancestral one in electron transfer to a regulatory one, it is an excellent example of evolutionary ‘tinkering’ or bricolage (Jacob, 1977). The protein was no longer needed for dealing with copper starvation (either because there was an existing functional cytochrome c6, as in Chlamydomonas, or because copper starvation was rare in plants) and was then used for a different function. No cytochrome c6A has yet been identified in a cyanobacterium. This may simply be because the protein failed to evolve until after chloroplasts were formed. It is possible that cytochrome c6A controls a process that does not occur in cyanobacteria, or that the process is controlled differently, or not at all.

Although the functional models are speculative, they help us to identify important areas for further study of this protein. More information is clearly needed on the subcellular location, and its location within whole plants, as well as the range of conditions under which it is expressed, both in land plants and in Chlamydomonas. Structural data are needed to help us to understand the possible interactions between the haem and the LIP as well as possible conformational changes on oxidation or reduction. Data are needed on the nature of the interaction with immunophilin(s) and data are also needed on how chloroplast control systems, especially those linked to the plastoquinone pool and to thioredoxins, are disturbed in plants deficient in cytochrome c6A, given the exciting possibility that this protein plays a key role in regulatory pathways in the thylakoid.

Acknowledgements

Work on cytochrome c6A in the authors’ laboratories was supported by the EU ‘Transient’ network, and by the Biotechnology and Biological Sciences Research Council. We thank Euan Nisbet for helpful discussions. CJH is grateful to Trish McLenachan, Peter and Toby Lockhart, and the Allan Wilson Centre, Massey University, NZ, for their hospitality during the writing of this review.

References


Baginsky S, Siddique A, Gruissem W. 2004. Proteome analysis of tobacco bright yellow-2 (BY-2) cell culture plastids as a model