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The unique features of starch metabolism in red algae

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Red algae (Rhodophyceae) are photosynthetic eukaryotes that accumulate starch granules outside of their plastids. The starch granules from red algae (floridean starch) show structural similarities with higher plant starch granules but lack amylose. Recent studies have indicated that the extra-plastidic starch synthesis in red algae proceeds via a UDP glucose-selective α-glucan synthase, in analogy with the cytosolic pathway of glycogen synthesis in other eukaryotes. On the other hand, plastidic starch synthesis in green cells occurs selectively via ADP glucose in analogy with the pathway of glycogen synthesis in prokaryotes from which plastids have evolved. Given the emerging consensus of a monophyletic origin of plastids, it would appear that the capacity for starch synthesis selectively evolved from the α-glucan synthesizing machinery of the host ancestor and its endosymbiont in red algae and green algae, respectively. This implies the evolution of fundamentally different functional relationships between the different subcellular compartments with regard to photosynthetic carbon metabolism in these organisms. It is suggested that the biochemical and molecular elucidation of floridean starch synthesis may offer new insights into the metabolic strategies of photosynthetic eukaryotes.

Keywords: starch synthesis; red algae; starch synthase; α-glucan synthase; plastid evolution; UDP glucose

1. INTRODUCTION

Most living cells contain intracellular storage α-glucans that are deposited as soluble or insoluble polymers. The most common storage α-glucans are glycogen and starch. Glycogen, which is a polymer formed by α-1,4-linked D-glucose residues with numerous α-1,6-glucosidic branch points, is found in the form of amorphous granules in the cell cytoplasm of most bacteria, yeast, fungi and animal cells. Starch is found as semi-crystalline granules in the chloroplasts of green algae and higher plants. These starch granules are anhydrous structures that are formed by a mixture of an essentially unbranched α-1,4-linked D-glucose polymer (amylose) and a larger polymer (amylopectin) with the same basic structure and frequent α-1,6 branch points.

The growth of α-glucan molecules occurs by apposition of glucose residues to the non-reducing end of existing chains and is catalysed by α-glucan synthases. These enzymes use the sugar nucleotides ADP glucose (ADPGlc) or UDP glucose (UDPGlc) as glucosyl donors for chain elongation. At present, the European Commission's classification for different α-glucan synthases is based on their selectivity for a glucosyl donor *in vitro* and is the source of some confusion. For example, bacterial glycogen synthases are specific for ADPGlc (ADPGlc: α-1,4-glucan 4-α-D-glucosyltransferase) (EC 2.4.1.21) and are distinct from eukaryote glycogen synthases, which use UDPGlc (UDPGlc: glycogen 4-α-D-glucosyltransferase) (EC 2.4.1.11). Plants contain ADPGlc-specific soluble starch synthases and these are classified together with bacterial glycogen synthases. However, plants also contain forms of starch synthase associated with the starch granule (granule-bound starch synthase) that are responsible for the elongation of amylose chains. These forms can use both ADPGlc and, with lower efficiency, UDPGlc as glucosyl donors *in vitro* and, consequently, are sometimes grouped with the eukaryote glycogen synthases. However, the use of UDPGlc by these enzymes *in vivo* is thought to be unlikely for various reasons (for a discussion see Kleczkowski 1994), including the absence of UDPGlc pools in the plastids. The concept of ADPGlc being the only glucosyl donor for all starch synthases *in vivo* is now well-established (Preiss 1988) and, according to this criterion, all forms of plant starch synthase should be best classified together with bacterial ADPGlc glycogen synthases.

There are also substantial structural and kinetic differences between ADPGlc- and UDPGlc-selective α-glucan synthases. There is no homology in the amino-acid sequences of mammalian UDPGlc:glycogen synthase and the ADPGlc:glycogen synthase of *Escherichia coli* (Leung & Preiss 1987). Conversely, the ADPGlc:glycogen synthase from *E. coli* shares some homology (*ca.* 30%) with plant starch synthases (Dry *et al.* 1992; Ainsworth *et al.* 1993; Baba *et al.* 1993). Eukaryotic UDPGlc:glycogen synthases are oligomeric enzymes (Nimmo *et al.* 1976; Carabaza *et al.* 1992) that are often found complexed with other proteins (e.g. glycogenin and glycogenin synthase) (Nimmo *et al.* 1976; Gosh *et al.* 1989) and are widely regarded as the rate-limiting enzyme in glycogen biosynthesis through modulation of their activity by allosteric mechanisms and through protein phosphorylation (reviewed in Cohen 1982, 1986; Roach 1986). In contrast, plant starch synthases are monomeric enzymes (Preiss 1988; Ainsworth *et al.* 1993; Marshall *et al.* 1996) and their activity is not regulated. The control of storage α-glucan synthesis in plants and bacteria generally resides with substrate (ADPGlc) production (Preiss & Romero 1994; Martin & Smith 1995).

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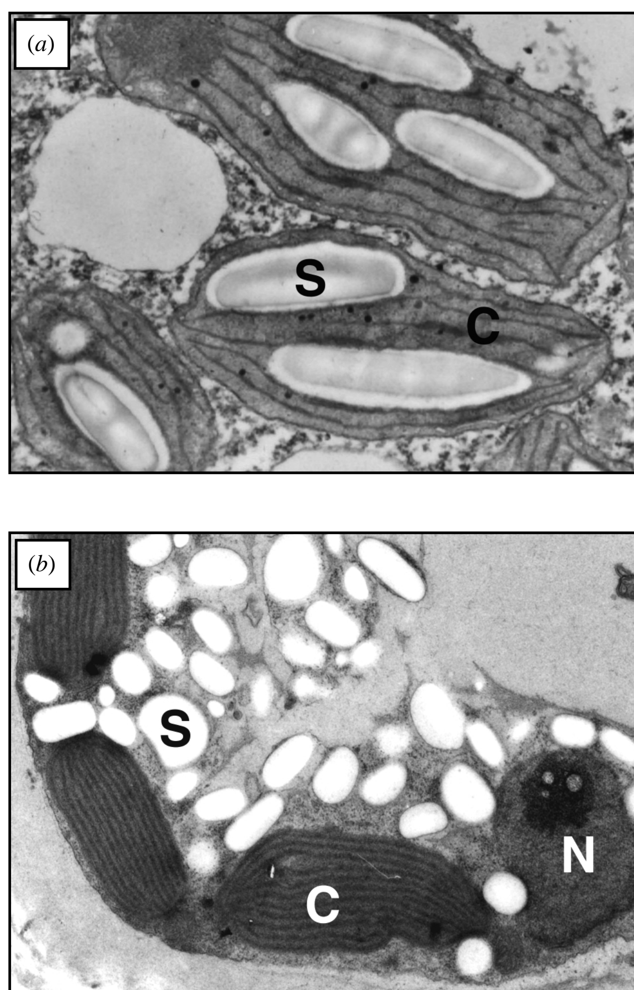

Figure 1. Appearance of starch granules within (*a*) the chloroplasts of a needle of *Pinus sylvestris* and (*b*) the cytosol of the red alga *G. tenuistipitata*. S, starch granule; C, chloroplast; N, nucleus. Micrograph of *Pinus sylvestris* courtesy of B. Walles.

2. FLORIDEAN STARCH

It is generally accepted that the many similar features between starch synthesis in Chlorophyta and glycogen synthesis in bacteria are attributable to the origin of chloroplasts from endosymbiotic cyanobacteria (Bhattacharya & Medlin 1995; Delwiche *et al*. 1995; Reith 1995). Red algae (Rhodophyta) constitute an exception to this rule as they, unlike chlorophytes, synthesize and store starch as granules outside their plastids in the cytosol (figure 1) (Pueschel 1990). The starch from red algae also differs from higher plant starches in its apparent lack of amylose (except in some unicellular species) (McCracken & Cain 1981) and is known as 'floridean starch' (Meeuse *et al*. 1960). Compositionally, floridean starch granules are constructed from a polymer more similar to amylopectin than glycogen (Peat *et al*. 1959; Manners & Wright 1962; Craigie 1974) and have similar structural features to higher plant starches, e.g. a radially arranged fibrillar-like pattern and concentric layers (Meeuse *et al*. 1960; Sheath *et al*. 1981*a*). In most Rhodophyta floridean starch represents the major sink for photosynthetically fixed carbon and, under certain growth conditions, floridean starch granules can amount to as much as 80% of the total cell volume (Ekman *et al*. 1991). The unusual compartmentalization of starch granules in red algae raises the question as to whether its biosynthetic pathway is more similar to that of starch synthesis in plants' plastids/glycogen biosynthesis in bacteria or to that of glycogen synthesis in eukaryotes (figure 2).

3. FLORIDEAN STARCH SYNTHESIS

Most of the research on floridean starch synthesis has focused on the nature of the α-glucan donor for chain elongation. Early studies showed that both ADPGlc and UDPGlc served as substrates *in vitro* for α-glucan synthases from red algal extracts (Fredrick 1967; Nagashima *et al*. 1971), thus resembling the specificities of the granule-bound starch synthases of higher plants. Nagashima *et al*. (1971) found that partially purified starch synthase incorporated ADPGlc, UDPGlc and GDPGlc into polymeric glucan in the coralline red alga *Serraticardia maxima*, although ADPGlc was the most efficient glucosyl donor. Sheath *et al*. (1979, 1981*b*) detected incorporation of ADP[^{14}C-Glc] into floridean starch in crude extracts of *Porphyridium purpureum*. However, incorporation of UDPGlc was not examined. More recently, Sesma & Iglesias (1998) reported lower incorporation of UDP[^{14}C-Glc] into glycogen compared with ADP[^{14}C-Glc] by crude extracts of *Gracilaria gracilis*. These authors also studied the ADPGlc:α-glucan synthase activity in the alga and found that it showed broadly similar kinetic properties to plant starch synthases. These results led to the hypothesis that the cytosolic pathway of floridean starch synthesis in red algae may be similar to plastidic synthesis in Chlorophyta (Nagashima *et al*. 1971; Sesma & Iglesias 1998). However, recent evidence has been obtained which disputes this claim. Nyvall *et al*. (1999) investigated starch synthases in *Gracilaria tenuistipitata* and found that incorporation of UDP[^{14}C-Glc] (but not ADP[^{14}C-Glc]) into glycogen by crude algal extracts was highly sensitive to the presence of antioxidants and protease inhibitors during extraction. By optimizing the extraction conditions, these authors substantially increased the total extractable α-glucan synthase activity from the alga and found that incorporation into glycogen was between 8.6- and 16.2-fold higher with UDP[^{14}C-Glc] as compared with ADP[^{14}C-Glc]. Based on their results, Nyvall *et al*. (1999) suggested that the apparent preference for ADPGlc as a substrate for α-glucan synthesis in red algae extracts reported previously by Nagashima *et al*. (1971) was due to selective losses of UDPGlc:α-glucan synthase activity during the experimental procedures employed. In addition, the UDPGlc:α-glucan synthase activity from *G. tenuistipitata* was strongly stimulated by citrate, in analogy with the majority of α-glucan synthases described (Preiss 1988 and references therein). On the other hand, Sesma & Iglesias (1998) assayed starch synthase activity in crude extracts of *G. gracilis* in the absence of citrate and with a relatively low concentration of glycogen (660 μg ml^{-1}). When the UDPGlc:α-glucan synthase activity from *G. tenuistipitata* was assayed in the absence of citrate the V_{max} was only 30% of that in the presence of citrate and, crucially, the K_m for glycogen increased from 21 to 4700 μg ml^{-1}. Indeed, from the data

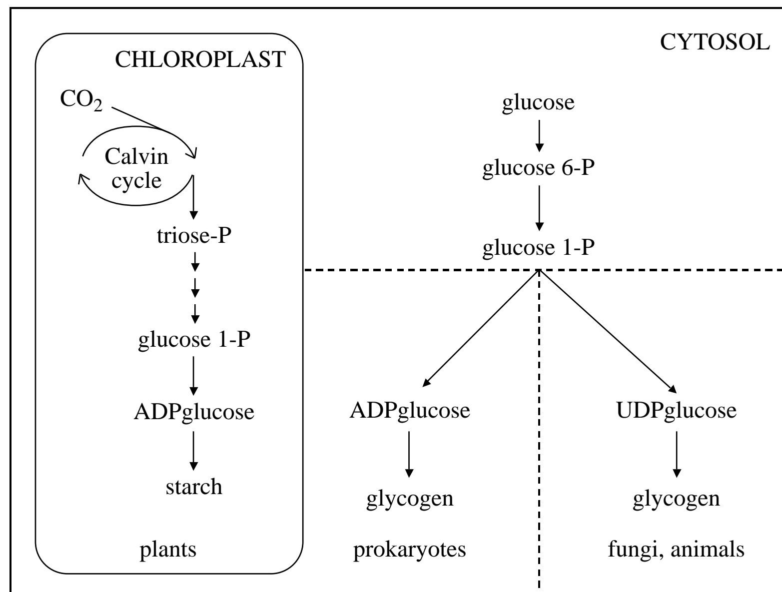

Figure 2. Pathways and compartmentalization of glycogen or starch synthesis in prokaryotes and eukaryotes.

published by Nyvall *et al.* (1999) one can extrapolate that the *G. tenuistipitata* UDPGlc:α-glucan synthase activity assayed with the method of Sesma & Iglesias (1998) would amount to less than 1% of the activity assayed under optimal conditions. Furthermore, when *G. tenuistipitata*, *Gracilaria sordida* and *Gracilariopsis lemaneiformis* (a closely related species of *G. gracilis*) were extracted with the protocol of Sesma & Iglesias, (1998) but assayed in the presence of citrate and excess glycogen (15 mg ml^{-1}), substantially higher α-glucan synthase activity was detected with UDPGlc as compared with ADPGlc (Nyvall 2000). UDPGlc:α-glucan synthase appears to be the only glucosyltransferase with activity sufficient for sustaining the observed rate of floridean starch synthesis in *G. tenuistipitata* (P. Nyvall *et al.*, unpublished data). Taken together, these findings strongly suggest that the UDPGlc:α-glucan synthase purified from *G. tenuistipitata* represents the 'floridean' starch synthase. This would make this enzyme the first known starch synthase specific for UDPGlc. Interestingly, only a minor proportion of UDPGlc:α-glucan synthase activity was found associated with the floridean starch granule in algal extracts. This may help in explaining the absence of amylose in the floridean starch granule in view of the requirement for starch synthase activity embedded within the starch granule for the synthesis of amylose in higher plants (Smith *et al.* 1997).

The use of UDPGlc as a substrate for floridean starch synthesis raises several questions in relation to the carbon storage strategies in red algae. UDPGlc is synthesized from carbon molecules exported from the rhodoplast and is used as a building block for the biosynthesis of various compounds including walls (galactans) and floridoside (α-D-galactopyranosyl 1→2′-glycerol) following its epimerization to UDP galactose. Floridoside is an important osmolyte but also represents the main soluble pool of storage carbon in most red algae and is used as a transportable form of carbon between cells in analogy with sucrose in higher plants (Ekman *et al.* 1991). A further analogy with sucrose biosynthesis is the floridoside biosynthetic pathway, which proceeds via the formation of a phosphorylated intermediate (floridoside-P) that is catalysed by the enzyme floridoside phosphate synthase (FPS) (Bean & Hassid 1955). Floridoside is thought to be synthesized in the cytosol, (Bisson & Kirst 1979; Kirst & Bisson 1979), i.e. in the same compartment as floridean starch granules. Thus, unlike in green plants, the distribution of assimilated carbon to insoluble and soluble products in red algae does not involve the exchange of metabolites across a plastidic membrane. At present, there is very little information on the regulation of photosynthetic carbon allocation in red algae. Both FPS and UDPGlc:α-glucan synthase activities appear inhibited by UDP (a reaction product) but are not affected by allosteric products (Meng & Srivastava 1991; Nyvall *et al.* 1999) unlike their counterparts (sucrose phosphate synthase and glycogen synthases) in other eukaryotes. As for the citrate activation of UDPGlc:α-glucan synthase or indeed the generality of α-glucan synthases, it is still not established whether this is due to binding of the acid to an allosteric site or to induction of conformational changes in the enzyme (Sevall & Kim 1971; Imparl-Radosevich *et al.* 1998). Allosteric regulation has been reported for α-glucan phosphorylase activity from *G. sordida* (Yu & Pedersén 1991). The enzyme is equally inhibited by low concentrations of UDPGlc and ADPGlc, unlike the enzyme from higher plants, which requires non-physiological levels of the sugar nucleotides (Steup 1988) or the enzyme from *Chlorella vulgaris*, which is more sensitive to inhibition by ADPGlc than UDPGlc (Nakamura & Imamura 1983). Thus, sugar nucleotide levels and relative concentrations in the cytosol may be important determinants of carbon allocation to end-products. Significantly, carbon metabolism in red algae appears not to be modulated by fructose-2,6-bisphosphate (Dancer & ap Rees 1989), a compound that serves as a regulatory link between chloroplasts and cytosol in plants (Stitt 1990) and in glycolysis in other eukaryotic cells

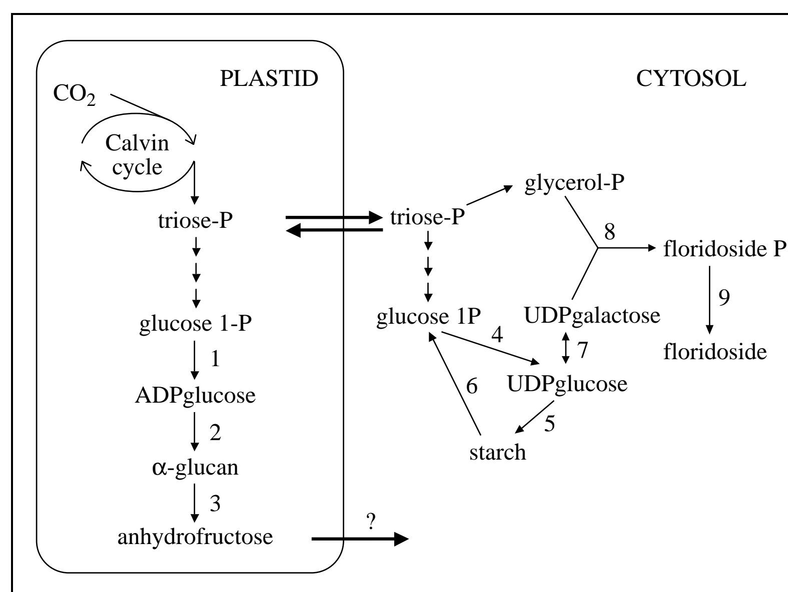

Figure 3. Pathways of carbon metabolism in red algae. Numbers indicate enzymes: [1] ADPGlc: pyrophosphorylase, [2] ADPGlc:α-glucan synthase, [3] α-glucan lyase, [4] UDPGlc: pyrophosphorylase, [5] UDPGlc:α-glucan synthase (floridean starch synthase), [6] α-glucan phosphorylase, [7] UDPGlc: epimerase, [8] floridoside phosphate synthase and [9] floridoside phosphate phosphatase.

(Hers 1985). Thus, it is plausible that the regulation of photosynthetic carbon flow in red algae may involve additional and as yet unidentified mechanisms.

4. THE COEXISTENCE OF TWO PATHWAYS OF α-GLUCAN BIOSYNTHESIS IN RED ALGAE?

The hypothesis that floridean starch synthesis operates via a UDPGlc-selective pathway conflicts with the findings of Sesma & Iglesias (1998) who reported on the purification and characterization of ADPGlc pyrophosphorylase (AGPase) in *G. gracilis*. The unique function of this enzyme in plants and bacteria appears to be the synthesis of ADPGlc substrate for α-glucan biosynthesis (Preiss 1988). In addition, there are several reports of the presence of ADPGlc:α-glucan synthase activity in cell-free extracts of various species of red algae (Nagashima *et al*. 1971; Sheath *et al*. 1981*b*; Nyvall *et al*. 1999). Nyvall *et al*. (1999) found that extraction conditions strongly affect the incorporation of UDPGlc into glycogen but not that of ADPGlc in extracts of *G. tenuistipitata*. Furthermore, the incorporation of ADPGlc into α-glucan primers is progressively lost during purification of the UDPGlc:α-glucan synthase. It would seem plausible that incorporation of UDPGlc and ADPGlc into α-glucan primers in protein extracts of red algae is attributable to separate enzymes. Sheath *et al*. (1979, 1981*b*) found that, in a study on *P. purpureum* cultures, ADPGlc:α-glucan synthase activity changed with the age of the culture, decreased during dark incubation and was inhibited by the addition of chloramphenicol but not cycloheximide in a dark to light transition. In contrast, α-glucan phosphorylase activity was inhibited by cycloheximide and not chloramphenicol. Immunocytochemical studies have shown that α-glucan phosphorylase is located exclusively in the cytosol, which is associated with the floridean starch granule (Yu *et al*. 1993*a*). The opposite effect of metabolic inhibitors on ADPGlc:α-glucan synthase may be interpreted as evidence for a plastidic functional assembly of the enzyme. Notably, the rhodoplasts of *G. lemaneiformis* were found to contain α-glucan lyase, a novel enzyme with starch hydrolysing activity (Yu *et al*. 1993*b*). This enzyme degrades α-1,4 glucans ranging from maltose to amylose or amylopectin *in vitro* by removing glucosyl units from the non-reducing end of the chain and generating 1,5 anhydrofructose (Yu *et al*. 1995). This product is ubiquitous in bacteria and eukaryotes and glucan lyase activity has been detected in rats (Kametani *et al*. 1996), fungi (Baute *et al*. 1988) and bacteria (Nakamura *et al*. 1986). The precise function of α-glucan lyase in algal metabolism is not known, particularly as it appears to be spatially segregated from the starch granules (Yu & Pedersén 1993). However, the possibility exists that this enzyme may be involved in the turnover of soluble α-glucans in the plastids, perhaps generated via an ADPGlc-selective pathway. This hypothesis implies the presence of two pathways of α-glucan biosynthesis in different compartments of red algal cells (figure 3). Further studies on unicellular red algae are required in order to establish whether these pathways coexist in the same cell or are spatially separated in different cell types.

5. FLORIDEAN STARCH SYNTHESIS AND THE EVOLUTION OF RHODOPHYTA

The origin of red algae and their enigmatic relationships with other eukaryotes have been the subject of many investigations. They have alternately been considered as plants (Bold & Wynne 1985) and as the earliest eukaryotes (Margulis & Schwartz 1982). Where the red algae emerge ties in closely with the question of plastid evolution. The hypothesis that all plastids originated from a single successful endosymbiotic event between a

cyanobacterium-like ancestor and a eukaryotic phagotrope has received support from mitochondrial and plastidic genome analyses (Delwiche & Palmer 1997; Douglas 1998). However, analyses of nuclear genomes have often proved less conclusive. For example, Stiller & Hall (1997) analysed sequence data from the nuclear-encoded largest subunit of RNA polymerase II (RPB1) and concluded that the evolutionary emergence of Rhodophyta preceded that of plants, animal or fungi. On the other hand, Moreira *et al.* (2000) recently carried out phylogenic analyses of the nuclear-encoded elongation factor 2 (EF2) and of a fusion of 13 nuclear markers and provided substantive evidence in support of the hypothesis that green and red algae are sister groups derived from a primary endosymbiotic event. These authors also suggested that previous inconsistencies resulting from RPB1 sequences might be attributable to phylogenetic reconstruction artefacts. On balance, the evidence in support of a monophyletic origin of all plastids now appears overwhelming. Thus, the divergence of red algae and green plants with regard to the compartmentalization of starch synthesis might have been the result of selective pressure leading to the evolution of starch synthesis from the α-glucan-synthesizing machineries of the original host or endosymbiont, respectively. A high evolutionary adaptability of glycogen synthases to different functional roles has been suggested on the basis of the lower than expected nucleotide sequence identity between closely related bacterial strains (Browner *et al.* 1989). Moreover, a gene encoding a starch branching enzyme was recently cloned from *G. gracilis* (Lluisma & Ragan 1998). The gene sequence lacked transit peptide-coding regions and phylogenetic analyses showed that it grouped with eukaryotic and not prokaryotic genes. This indicated that the gene might be derived from the eukaryotic ancestor rather than from an endosymbiotic cyanobacterium. A full biochemical and molecular characterization of the pathway of floridean starch biosynthesis is likely to identify a novel strategy for the storage of assimilated carbon in photosynthetic eukaryotes.

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