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Research article

Multimeric states of starch phosphorylase determine protein–protein interactions with starch biosynthetic enzymes in amyloplasts

Renuka M. Subasinghe [a], Fushan Liu [a], Ursula C. Polack [a], Elizabeth A. Lee [b], Michael J. Emes [a], Ian J. Tetlow [a, *]

[a] Department of Molecular and Cellular Biology, Science Complex, College of Biological Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada
[b] Department of Plant Agriculture, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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ABSTRACT

Protein–protein interactions between starch phosphorylase (SP) and other starch biosynthetic enzymes were investigated using isolated maize endosperm amyloplasts and a recombinant maize enzyme. Plastidial SP is a stromal enzyme existing as a multimeric protein in amyloplasts. Biochemical analysis of the recombinant maize SP indicated that the tetrameric form was catalytically active in both glucan-synthetic and phosphorolytic directions. Protein–protein interaction experiments employing the recombinant SP as an affinity ligand with amyloplast extracts showed that the multimeric state of SP determined interactions with other enzymes of the starch biosynthetic pathway. The monomeric form of SP interacts with starch branching enzyme I (SBEI) and SBEIIb, whereas only SBEI interacts with the tetrameric form of SP. In all cases, protein–protein interactions were broken when amyloplast lysates were dephosphorylated *in vitro*, and enhanced following pre-treatment with ATP, suggesting a mechanism of protein complex formation regulated by protein phosphorylation. *In vitro* protein phosphorylation experiments with [γ-^{32}P]-ATP show that SP is phosphorylated by a plastidial protein kinase. Evidence is presented which suggests SBEIIb modulates the catalytic activity of SP through the formation of a heteromeric protein complex.

1. Introduction

Starch is an important carbon reserve synthesized within the plastids of higher plants and green algal cells, allowing them to meet variable metabolic demands over a day/night cycle and in the case of plants also acts as a long-term carbon store in seeds and tuberous tissues. The major route of starch synthesis is *via* the nucleotide diphosphate sugar precursor ADP-glucose (ADP-Glc), synthesized by the highly regulated enzyme ADP-Glc pyrophosphorylase (AGPase, E.C. 2.7.7.27) (Greenberg and Preiss 1964). ADP-Glc is in turn the substrate for a class of glucan-elongating enzymes called starch synthases (SS, E.C. 2.4.1.21), which, together with starch branching enzymes (SBE, E.C. 2.4.1.18) and debranching enzymes (DBEs, including isoamylases [Iso], E.C. 3.2.1.41 and pullulanase [ZPU1], E.C. 3.2.1.68) are the three major groups of enzymes responsible for the synthesis of water-insoluble semi-crystalline starch granules (for recent reviews see Hennen-Bierwagen et al., 2012; Tetlow, 2011).

Amongst the many other enzymes involved in starch metabolism, starch phosphorylase (SP, E.C. 2.4.1.1) has long been implicated in the pathway of starch biosynthesis, although its precise role has yet to be defined (see review by Schupp and Ziegler, 2004). α-Glucan phosphorylases are found in many different organisms as dimers or tetramers (Nakano and Fukui 1986; Albrecht et al., 1998; Buchbinder et al., 2001; Higgins et al., 2013) and catalyze a

Abbreviations: ADP-Glc, ADP-glucose; *ae*$^{-}$, *amylose extender*; AGPase, adenosine 5′ diphosphate glucose pyrophosphorylase; APase, alkaline phosphatase; BSA, bovine serum albumen; Bt2, AGPase small subunit; DAP, days after pollination; DBE, debranching enzyme; D-enzyme, disproportionating enzyme; DP, degree of polymerization; DTT, dithiothreitol; GBSSI, granule-bound starch synthase I; Glc, glucose; Glc-1P, α-D-glucose 1-phosphate; Iso, isoamylase; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; MOS, malto-oligosaccharides; MS, mass spectrometry; NP-40, nonyl phenoxylpolyethoxyl ethanol; PBS, phosphate buffered saline; Pho1, plastidial starch phosphorylase; Pho2, cytosolic starch phosphorylase; Pi, inorganic orthophosphate; SBE, starch branching enzyme; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SP, starch phosphorylase (Pho1); SS, starch synthase; TRIS, tris-(hydroxymethyl)-aminomethane; Triton X-100, polyethylglycol *p*-*t*-octylphenol; U, units of enzyme activity (defined as 1 μmol per min); ZPU1, pullulanase.

* Corresponding author. Fax: +1 519 837 1802.
E-mail address: itetlow@uoguelph.ca (I.J. Tetlow).

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reversible (equilibrium) reaction; (α-1,4-linked Glc)$_n$ + Glc-1P ↔ (α-1,4-linked Glc)$_{n+1}$ + inorganic orthophosphate (Pi), meaning that subcellular Glc-1P and Pi levels will influence the direction of catalytic flux. Plants possess two forms of α-glucan phosphorylases, a plastidial form involved in starch synthesis/metabolism called Pho1 (or PhoL, because of its low affinity for hydro-soluble glycogen as a glucan substrate), and a cytosolic form termed Pho2, or PhoH, which has a high affinity for glycogen and high-molecular-weight heteroglycan (Shimomura et al., 1982; Yang and Steup, 1990). Pho2 is closely related to bacterial and mammalian α-glucan phosphorylases and is involved in cytosolic polyglucan metabolism, including metabolism of α-glucans derived from the products of starch degradation (exported from the plastid as maltose (Niittylä et al., 2004; Weise et al., 2004; Fettke et al., 2012)). Pho2 therefore plays no role in plastidial starch biosynthesis.

Expression studies of the plastidial SP gene (Pho1, hereafter referred to as SP) in leaf and storage tissues correlate closely with periods of active starch biosynthesis (Tsai et al., 1970; Duwenig et al., 1997; Van Berkel et al., 1991; Yu et al., 2001), and genetic evidence from *Chlamydomonas reinhardtii* and rice (*Oryza sativa* L.) indicates a role for SP in starch accumulation (Dauvillée et al., 2006; Satoh et al., 2008). In addition, there is no direct evidence for SP-mediated starch degradation; loss of SP in potato (*Solanum tuberosum* L.) and Arabidopsis (*Arabidopsis thaliana* L.) caused no measurable alteration to diurnal starch accumulation and turnover (Sonnewald et al., 1995; Zeeman et al., 2004), which is consistent with the prevailing view of a primarily amylolytic route of starch degradation in plants (Zeeman et al., 2010; Fettke et al., 2012). Further, amyloplasts possess high Pi/Glc-1P ratios which, it has been argued, would indicate that the SP reaction is favored in the direction of phosphorolysis (α-glucan degradation) (Wirtz et al., 1980; Tiessen et al., 2012). Studies of SP from maize amyloplasts showed that the phosphorolytic reaction was stimulated in the presence of malto-oligosaccharides (MOS), further supporting the notion of a predominantly phosphorolytic-acting SP in plastids. However, recent biochemical studies of rice endosperm SP clearly indicate that the SP reaction favors -glucan synthesis over degradation, even in the presence of high Pi levels (Hwang et al., 2010). Both SP reactions show high sensitivity to inhibition by ADP-Glc (Dauvillée et al., 2006; Burr and Nelson, 1975; Matheson and Richardson, 1978), and it has been suggested by Hwang et al. (2010) that low ADP-Glc levels are maintained early in endosperm development (3–5 DAP) by an active ADP-Glc pyrophosphatase (E.C. 3.1.4, Rodriguez-Lopez et al., 2000) thereby stimulating extension of short MOS (DP 4-19) by SP. SP is thus envisaged as having a role in starch granule initiation by producing MOS primers for subsequent action by SSs. Research on rice endosperm SP also supports the notion that SP is involved in granule initiation through provision of precursor branched maltodextrins *via* functional interactions with SBEs (Nakamura et al., 2012). Recent work with potato tuber discs and Arabidopsis leaves show that [U-^{14}C]-Glc from [U-^{14}C]-Glc-1P can be efficiently incorporated into starch granules following transport into plastids, potentially *via* a SP-mediated route (Fettke et al., 2010, 2011). In addition to the possible role of SP in granule initiation, an alternative route of starch synthesis is also borne out from genetic studies in rice which indicates a predominantly SP-mediated route of starch synthesis at low temperatures (Satoh et al., 2008).

There is increasing evidence that many of the enzymes of storage starch synthesis, including various SS and SBE isoforms, are found associated in multi-enzyme complexes which are assembled through the action of a phosphorylation-dependent regulatory pathway in amyloplasts (Tetlow et al., 2008; Hennen-Bierwagen et al., 2008, 2009; Emes and Tetlow 2012). In developing wheat endosperm amyloplasts, a proportion of SP is associated with SBEI and SBEIIb in a phosphorylation-dependent protein complex (Tetlow et al., 2004). SP has also been shown to associate in a multi-enzyme complex in amyloplasts of the maize *amylose extender* (*ae*$^-$) mutant lacking the SBEIIb protein (Liu et al., 2009). Interestingly, the phosphorylation-dependent association of SP in the *ae*$^-$ protein complex caused SP, normally a stromal enzyme, to become granule-bound, and this phenomenon has also been shown in high-amylose barley genotypes lacking SBEII (Ahmed et al., pers. comm.). The precise role of SP within the different protein complexes remains unclear. In sweet potato root, plastidial SP activity with different substrates is regulated by proteolytic cleavage mediated through the 20S proteasome, initiated by phosphorylation of serine residues on the protein (Chen et al., 2002; Young et al., 2006; Lin et al., 2012).

The following study aimed to examine the posttranslational regulation of SP in developing maize endosperm amyloplasts. A functional recombinant maize SP was purified and used as an affinity ligand in protein–protein interaction studies. Results show that SP is readily phosphorylated by a plastidial protein kinase, and that it interacts with different isoforms of SBE in a phosphorylation-dependent manner, depending upon its multimeric state.

2. Materials and methods

2.1. Plant material

Wild-type CG102 maize (*Zea mays*) was grown in a field in Guelph, Ontario during the summer of 2011. The *ae*$^-$ allele was examined in the common maize inbred line background, CG102. The mutant *ae*$^-$ allele was *ae1-ref* (stock#517B) obtained from the Maize Genetics Cooperation Stock Center and back-crossed into CG102 for three generations. Pollinated kernels were collected at 9–12 days after pollination (DAP), 20–25 DAP, and 29–35 DAP and used to prepare endosperm amyloplasts. Plastid preparations were flash frozen in liquid nitrogen and stored at −80 °C until future use.

2.2. Amyloplast isolation

Maize endosperm amyloplasts were isolated using a modification of the methods described by Tetlow et al. (2008). Fresh endosperm tissue was washed and chopped with a razor blade in ice-cold amyloplast extraction buffer (50 mM *N*-(2-hydroxyethyl) piperazine-*N*′-ethanesulfonic acid (HEPES)/KOH, pH 7.5, containing 0.8 M sorbitol, 1 mM KCl, 2 mM $MgCl_2$, and 1 mM Na_2-EDTA,). The resulting whole cell extract was then filtered through four layers of Miracloth (CalBiochem) wetted in the same buffer. Approximately 25 mL of the filtrate was then carefully layered onto 15 mL of 3% (w/v) Histodenz (Sigma) in amyloplast extraction buffer followed by centrifugation at 100*g* at 4 °C for 20 min and the supernatant was carefully decanted. Intact amyloplasts appeared as a yellow ring on top of the starch in the pellet and were lysed osmotically by the addition of ice-cold rupturing buffer containing 100 mM *N-tris* (hydroxymethyl) methyl glycine (Tricine)/KOH, pH 7.8, 1 mM Na_2-EDTA, 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, and a protease inhibitor cocktail (ProteCEASE™ [G-Biosciences, USA] used at 10 μL per cm^3). The plastid lysate was then centrifuged at 13,500*g* for 2 min at 4 °C to remove starch granules followed by ultracentrifugation at 120,000*g* for 15 min in a Beckman Optima Max-XP Ultracentrifuge to separate plastidial envelope membranes. The supernatant from the ultracentrifugation step, termed plastid stroma (0.5–1.2 mg protein per mL), was flash frozen in liquid nitrogen and stored at −80 °C until future use.