Is There an Alternative Pathway for Starch Biosynthesis in Cereal Seeds?

Per Villand and Leszek A. Kleczkowski

Plant Molecular Biology Laboratory, Agricultural Research Council of Norway, P.O. Box 51, 1432 Aas, Norway

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A hypothesis is presented concerning a putative extra-amyloplastic location of barley seed endosperm ADP-glucose pyrophosphorylase (AGPase), a key enzyme of starch biosynthesis. The hypothesis is based both on indirect and direct evidence obtained in our laboratory as well as on data of other investigators. It is proposed that ADP-glucose formed by the extra-amyloplastic enzyme is transported to the amyloplasts *via* an ADP-glucose carrier in the plastid membrane, and then is utilized by the starch biosynthesizing machinery of these organelles. In addition to the extra-amyloplastic form of AGPase, barley endosperm contains also a second isozyme of AGPase, located in the amyloplasts. Presence of isozymes of AGPase in cereal seed endosperm is consistent with biochemical, molecular and genetic data on starch biosynthesis in this tissue.

Introduction

ADP-glucose pyrophosphorylase (AGPase) carries out the first committed step of starch biosynthesis in all plants (Kleczkowski *et al.*, 1991; Stark *et al.*, 1992). The enzyme is composed of two subunit-types, encoded by different genes (Okita *et al.*, 1990; Kleczkowski *et al.*, 1991, 1993a,c; Villand *et al.*, 1992a). In plants, up to three isozymes of AGPase, based on the number of distinct mRNAs encoding the large subunit of AGPase, have been proposed (Villand *et al.*, 1993; Kilian *et al.*, 1994). Biochemical evidence indicates the presence of at least two distinct AGPase enzymes in barley (Kleczkowski *et al.*, 1993a,b,c) and wheat (Riffkin *et al.*, 1993) tissues. Isozymes of AGPase are usually located in different tissues of a given plant (Kleczkowski *et al.*, 1993; Riffkin *et al.*, 1993), but they may also be present in the same tissue, as suggested for rice seeds (Nakamura and Kawaguchi, 1992). Also, the *rb* mutation in pea embryos, resulting in structural and regulatory changes of AGPase (Hylton and Smith, 1992), may perhaps be related to the deficiency of one of the isozymes of AGPase in this tissue. In leaves, AGPase is believed to be located entirely in the chloroplast stroma, utilizing ATP produced during photophosphorylation of ADP, and being activated by 3-phosphoglycerate (PGA), the first stable product of photosynthesis in many plants (Kleczkowski *et al.*, 1991).

It has been assumed that the mode of starch synthesis is the same in chloroplasts and in starch-biosynthesizing plastids in non-photosynthetic tissues (amyloplasts), and that all reactions of starch biosynthesis, starting with ADP-glucose formation by AGPase, are confined to the plastid compartment (Echeverria *et al.*, 1988; Okita, 1992; Stark *et al.*, 1992; Emes and Tobin, 1993). This view was based mostly on organelle fractionation experiments and on metabolic transport studies with isolated amyloplasts. Although the recovery of total activity of AGPase in the amyloplasts was frequently low and the enzyme appeared unstable during fractionation [*e.g.* (Echeverria *et al.*, 1988)], AGPase has routinely been used as a marker for the plastid fraction. Studies with isolated amyloplasts from various tissues have indicated that an externally provided glucose-1-P, glucose-6-P and/or triose-P can be incorporated into starch (Heldt *et al.*, 1991; Emes and Tobin, 1993), supporting the plastid location of AGPase. While there is no doubt that amyloplasts do contain AGPase activity, the experiments described above can not rule out the

Abbreviations: AGPase, ADP-glucose pyrophosphorylase; PGA, 3-phosphoglycerate; Pi, inorganic phosphate.

Reprint requests to Dr. L. A. Kleczkowski (present permanent address: Department of Plant Physiology, University of Umeå, S-901 87 Umeå, Sweden).

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possibility that non-photosynthetic tissues may contain other isozymes of AGPase, located outside of amyloplasts. In the present paper, a possibility of an extra-amyloplastic AGPase involved in starch biosynthesis in seeds of barley and, perhaps, of other cereal plants is discussed.

Evidence for an extra-amyloplastic location of AGPase

In our recent studies on the biochemistry and molecular biology of AGPase from barley seed endosperm, we have uncovered several indirect and direct lines of evidence challenging the commonly held view of an exclusively amyloplast-based location of AGPase in non-photosynthetic tissues. These and other arguments in favour of an extra-amyloplastic isozyme of AGPase in barley seeds are listed (**A-F**) below:

(**A**) The cDNA-derived amino acid sequences of the small (P. Villand, unpublished) and large subunits (Villand *et al.*, 1992b) of barley endosperm AGPase do not contain any obvious motifs characteristic of a transit peptide sequence that is believed to be cleaved upon import of any nuclear-encoded protein to the plastid compartment (Keegstra *et al.*, 1989). The same is true for the derived amino acid sequence of the cDNA encoding the small subunit of rice seed AGPase (Anderson *et al.*, 1989), even though the authors did propose the presence of the transit peptide in this protein. Unfortunately, we do not know the N-terminal sequences of mature AGPase polypeptides from barley endosperm [the enzyme was only partially purified (Kleczkowski *et al.*, 1993a)] and, to our knowledge, no N-terminal sequence of the mature small subunit AGPase protein from rice seeds has been published. Comparison of the cDNA-derived amino acid sequences to those of mature AGPase proteins is required to finally prove or disprove the existence of the transit peptide for seed AGPase.

(**B**) Expression of full-length cDNAs encoding both subunits of endosperm AGPase in *Escherichia coli* (P. Villand, unpublished) resulted in polypeptides with apparent molecular masses (*ca.* 51 and 60 kDa for the small and large subunit, respectively) similar to those of the equivalent polypeptides in barley endosperm extracts (Kleczkowski *et al.*, 1993a), as found on immunoblots of crude proteins. This suggests that no transit peptide sequence is cleaved for the mature AGPase proteins. Were AGPase amyloplast located, one would expect lower molecular masses for each of the subunits of AGPase in barley endosperm when compared to those expressed in the heterologous system.

(**C**) Presentation of the *in vitro* generated polypeptides of barley endosperm AGPase to isolated pea chloroplasts gave no indication of import of the proteins into the plastidic compartment (J. E. Froehlich and K. Keegstra, personal communication). Although it is possible that barley endosperm amyloplasts contain a protein transport machinery distinct from the pea chloroplast system, it is believed that plastids of developmentally different tissues are fully capable of importing precursor proteins that are normally not found in these tissues. Various plastid types, though functionally and morphologically differentiated, have similar or identical protein import mechanisms when compared to the chloroplasts in green tissue (de Boer *et al.*, 1988; Klösgen *et al.*, 1989).

(**D**) The AGPase from barley endosperm, either crude or partially purified, was markedly insensitive to regulation by PGA (activator) and Pi (inhibitor) (Kleczkowski *et al.*, 1993a,b,c), in contrast to a pronounced effect of these metabolites on the leaf enzyme (Kleczkowski *et al.*, 1993c). When full length cDNAs encoding both subunit-types of barley endosperm AGPase were expressed in *E. coli* or Baculovirus systems, the heterologously formed enzyme had the same regulatory characteristics (insensitivity of effectors) as crude or partially purified AGPase from endosperm extracts (P. Villand and D. Doan, unpublished), indicating that the insensitivity does indeed represent an intrinsic property of the seed enzyme. Insensitivity to PGA/Pi regulation was also recently reported for AGPase from wheat seeds (Riffkin *et al.*, 1993). In contrast to leaf AGPase which is regulated by PGA and Pi, important metabolite indicators of the photosynthetic rate in chloroplasts, there is no apparent reason for regulation of seed AGPase by these compounds, especially if it is located outside of plastids.

(**E**) Preliminary immunogold-labelling analyzes (electron microscopy) of the barley endosperm tissue (A. M. Kram and G. T. Oostergetel, manuscript in preparation), using antibodies specific for AGPase (Kleczkowski *et al.*, 1993a, c), revealed

the label both in the cytosol and in amyloplasts. In the cytosol, the label appeared to be concentrated in cluster-like patterns rather than being uniformly distributed throughout the compartment. The amyloplast-labelling may correspond to a "leaf-type" of AGPase, which is expressed in the endosperm in addition to the seed-specific form of the enzyme, as found by polymerase chain reaction amplification of cDNA prepared from endosperm RNA (Villand *et al.*, 1992a) and by isolating the "leaf-type" mRNA encoding the large subunit of AGPase from a barley endosperm cDNA library (P. Villand, unpublished). The same antibodies labelled only the amyloplast compartment in potato tubers (A. M. Kram and G. T. Oostergetel, unpublished), which is consistent with previous data that the tuber AGPase is located exclusively in the amyloplasts (Kim *et al.*, 1990; Kram *et al.*, 1993). Potato tubers are derived from stem tissue and they do not represent a typical non-photosynthetic system, as cereal seeds do. Antibody-labelling of both the amyloplast and extra-amyloplastic compartments has also been observed for AGPase from maize seeds. In this tissue, the extra-amyloplastic label was found mostly associated with cell wall structures (P. S. Chourey and M. E. Miller, personal communication).

(**F**) The extra-amyloplastic location of AGPase in certain tissues is strongly supported, in our opinion, by the capability of various plastids to incorporate externally added ADP-glucose into starch (Pozueta-Romero *et al.*, 1991a, b; Liu *et al.*, 1991, 1992; Ardila *et al.*, 1993). In contrast to the well-known mitochondrial adenylate translocator (Klingenberg, 1989), an adenylate translocator in the amyloplasts can transport not only ATP and ADP but also AMP and ADP-glucose, the latter being effectively utilized for starch biosynthesis (Pozueta-Romero *et al.*, 1991a, b; Liu *et al.*, 1991). The adenylate translocator is present in all plastid types, even at the proplastid stage of plastid development (Ardila *et al.*, 1993).

A putative topology of ADP-glucose formation in barley seeds

The evidence described above strongly suggests that a portion of AGPase activity in barley endosperm might be located in the cytosol of this tissue. The biochemical implication of this spatial arrangement is that ADP-glucose can be formed outside the amyloplasts and then transported to the amyloplast where it enters the final steps of the starch synthetic pathway (Fig. 1). This may actually represent a specialization of seed tissue for starch biosynthesis, since: (**a**) the sucrose-derived carbon entering the amyloplast is a substrate for the starch synthesis pathway only; (**b**) the presence of AGPase in the cytosolic compartment may as-

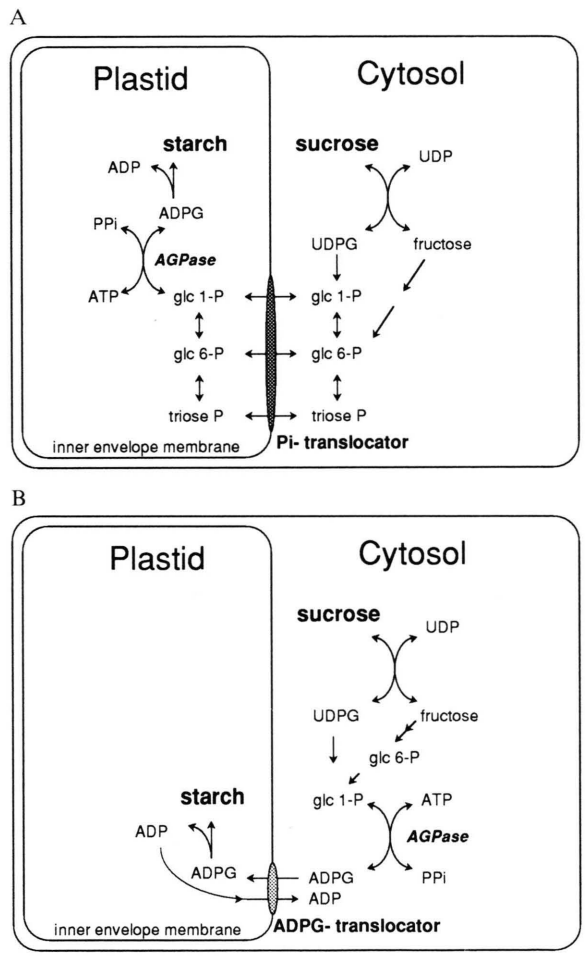

Fig. 1. Mechanisms of starch formation in plants. **A.** The mechanism based on an exclusive location of AGPase in plastids (*e.g.* in leaves, potato tubers). **B.** A putative additional mechanism of starch biosynthesis operating in barley seed endosperm, based on the presence of an extra-amyloplastic isozyme of AGPase. Please note that both mechanisms A and B are likely to operate in barley endosperm, and that, for sake of clarity, only details concerning operation of the extra-amyloplastic AGPase are depicted here. ADPG, ADP-glucose; UDPG, UDP-glucose.

sure a close integration between sucrose metabolism, occurring in the cytosol, and the first committed step of starch biosynthesis (AGPase) through glucose-1-P, the common intermediate of the two pathways; and (**c**) there should be no need for ATP generation in the amyloplast. In the latter case, import of ADP-glucose into the amyloplast may be countered by export of ADP, the product of starch synthase reaction (Fig. 1).

Assuming that AGPase activity is located outside of amyloplasts, a direct transport of ADP-glucose into amyloplasts does not represent an alternative pathway by-passing AGPase (Pozueta-Romero *et al.*, 1991 a, b, c; Ardila *et al.*, 1993), but rather could be regarded as a modified and specialized way of starch biosynthesis. The extra-amyloplastic AGPase is a better candidate to form ADP-glucose in the cytosol than sucrose synthase, which was previously proposed to produce ADP-glucose through a non-specific reaction (Pozueta-Romero *et al.*, 1991 c). The main argument against the involvement of sucrose synthase in ADP-glucose formation was that mutants deficient in AGPase activity/protein were starch-deficient [reviewed in (Okita, 1992)]. On the other hand, the concept of an extra-amyloplastic AGPase does not challenge the genetic evidence, and is consistent with the key role of AGPase in starch formation.

It should be emphasized that the extra-amyloplastic location of AGPase is proposed for cereal seeds, based on data on barley AGPase, but not for all non-photosynthetic tissues. It is well established that in potato tubers, for instance, the enzyme is localized exclusively in the amyloplasts, based on immunocytochemical evidence (Kim *et al.*, 1990; Kram *et al.*, 1993) and on studies with transgenic plants transformed with a heterologous cDNA for AGPase (Stark *et al.*, 1992). Also, the evidence discussed above does not preclude the presence of the amyloplast-located AGPase in barley endosperm. Based on immunocytochemical studies (see above), barley endosperm amyloplasts do contain AGPase. These data are supported by presence of both the "endosperm-type" and "leaf-type" mRNAs for AGPase in barley endosperm, while only the "leaf-type" mRNA was found in barley leaves [(Villand *et al.*, 1992 a), P. Villand, unpublished]. The presence of distinct isozymes of AGPase has already been proposed for rice seed endosperm (Nakamura and Kawaguchi, 1992).

The relative contribution of AGPase isozymes to an overall starch formation in the endosperm is unknown at present, requiring detailed studies both at the protein/enzyme and mRNA levels. The role of the extra-amyloplastic AGPase would certainly depend on the efficiency of import of ADP-glucose into the amyloplast stroma through ADP-glucose (adenylate) translocator. The translocator itself may be under some developmental control, as suggested for amyloplasts from maize kernels where starch biosynthesis from external ADP-glucose depended on seed development stage (Liu *et al.*, 1992). Concerning the role of the amyloplast-based AGPase, starch production through this enzyme would, in turn, depend on the efficiency of glucose-1-P, glucose-6-P and/or triose-P production from sucrose in the cytosol, and on their transport and metabolism within amyloplast stroma. While there is still much to be learned about starch biosynthesis in seeds of barley and other cereals, it seems likely that the concept of an extra-amyloplastically-located AGPase may contribute to better understanding of starch production in seeds, and should help in integrating our knowledge about mechanisms of sucrose-derived metabolite fluxes and glucose activation in this tissue.

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