RESEARCH COMMUNICATION

Identification of the putative hexose-phosphate translocator of amyloplasts from cauliflower buds

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Starch synthesis in amyloplasts isolated from cauliflower buds is strongly inhibited by the addition of micromolar concentrations of 4,4'-di-isothiocyano-2,2'-stilbenedisulphonic acid (DIDS). Using [³H]DIDS it was possible to label specifically a 31.6 kDa membrane protein of the envelope fraction of isolated amylo-

plasts. The intensity of the radioactive label was decreased in the presence of glucose 6-phosphate or dihydroxyacetone phosphate, indicating that this protein might be the amyloplastic hexose-phosphate translocator.

INTRODUCTION

Starch synthesis in heterotrophic amyloplasts is driven by the uptake of external carbon skeletons. Most of the amyloplasts analysed so far import hexose phosphates (for review see Okita, 1992). Amyloplasts purified from pea roots are able to import glucose 6-phosphate (Glc6P) (Borchert et al., 1989) and use this metabolite to support the oxidative pentose-phosphate pathway (Bowsher et al., 1992). Tyson and ap Rees (1988) demonstrated that starch synthesis in isolated amyloplasts from wheat endosperm is driven by uptake of glucose 1-phosphate, whereas pea endosperm and cauliflower bud amyloplasts most efficiently use Glc6P for starch biosynthesis (Hill and Smith, 1991; Neuhaus et al., 1993). Short-term uptake experiments by Borchert et al. (1989) have shown that Glc6P transport into plastids isolated from pea roots is mediated by a protein with transport properties that are similar to the chloroplastic triose-phosphate translocator. In addition, Glc6P-dependent starch synthesis in isolated cauliflower bud amyloplasts is strongly inhibited by addition of dihydroxyacetone phosphate (DHAP) or P_i to the incubation medium (Neuhaus et al., 1993). In contrast with the chloroplastic triose-phosphate translocator (Flügge et al., 1991), the nature of the amyloplastic hexose-phosphate translocator is unknown. The aim of this work was to identify the membrane protein involved in the transport of hexose phosphate across the envelope of cauliflower bud amyloplasts in order to enable future attempts to purify this protein.

MATERIALS AND METHODS

Isolation of cauliflower bud amyloplasts

Cauliflower buds (*Brassica oleracea* L., var. Prince de Bretagne) were purchased from the local market. Isolation of highly intact amyloplasts with low contamination by other cellular components was performed by the method of Journet and Douce (1985), modified as described by Neuhaus et al. (1993).

Starch synthesis in isolated cauliflower bud amyloplasts

This was performed essentially as described in Neuhaus et al.

(1993), except that BSA was omitted from the incubation medium. The effect of 4,4'-di-isothiocyano-2,2'-stilbenedisulphonic acid (DIDS) on the rate of starch synthesis was analysed by addition of various concentrations of this inhibitor to the incubation assay.

Isolation of envelopes of cauliflower bud amyloplasts and spinach chloroplasts

The envelope fraction was obtained from isolated plastids by the protocol of Alban et al. (1988). Inner membranes of spinach chloroplast envelopes were purified by the method of Block et al. (1983).

Labelling of isolated envelopes

Envelope membranes corresponding to 100 µg of protein were incubated at 10 °C for 45 min in a medium (0.1 ml) consisting of 10 mM Tricine/NaOH, pH 7.8, and 10 µM [³H]DIDS (7 µCi/nmol). The labelling was terminated by addition of 1 mM lysine to the incubation medium and a subsequent centrifugation step in order to obtain the membranes (100 000 g, 45 min; Beckman Airfuge). The membrane pellet was washed once with 10 mM Tricine/NaOH, pH 7.8. The effect of different metabolites on the intensity of the radioactive labelling was estimated after addition of various concentrations of Glc6P or DHAP to the incubation medium.

SDS/PAGE, protein and radiolabel detection

Isolated labelled envelope membranes were subjected to SDS/PAGE essentially as described by Laemmli (1970). After electrophoresis the gel was stained for protein with Coomassie Brilliant Blue. For fluorography, the gel was treated as described by Bonner and Laskey (1974) and subsequently dried between Cellophane. The dried gel was exposed to a Kodak X-OMAT AR-5 film at -78 °C for 5–7 days.

Protein determination

Total protein in isolated amyloplasts and envelopes was determined with a bicinchoninic acid CuSO₄ solution (Sigma,

Deisenhofen, Germany) in accordance with the supplier's instructions.

RESULTS AND DISCUSSION

To identify an unknown membrane protein, it is necessary to use a probe which allows specific labelling. The chloroplast triose-phosphate translocator has been labelled with two different substances. Flügge and Heldt (1978) used NaB^3H_4 in combination

Figure 1 Inhibition by DIDS of Glc6P-dependent starch synthesis in isolated cauliflower bud amyoplasts

Starch synthesis was performed as described by Neuhaus et al. (1993) with a complete incubation medium containing 0.5 mM [^{14}C]Glc6P and the effectors 3-phosphoglycerate and ATP. The rate of starch synthesis was estimated as incorporation of glucose moieties into starch. DIDS was added immediately before addition of the amyoplasts. Results are means $\pm$ S.E.M. of three independent samples.

Figure 2 Polypeptide pattern of isolated envelopes from cauliflower bud amyoplasts and radioactive labelling of the putative hexose-phosphate translocator with [^3H]DIDS

Isolation of amyoplast envelopes was performed as described by Alban et al. (1988); 50 μg of the envelope protein was applied to each lane. Lane 1, molecular-mass standards; lane 2, Coomassie-Blue-stained polypeptide pattern of the envelope fraction; lane 3, fluorogram of amyoplast membranes labelled with [^3H]DIDS; lane 4, fluorogram of chloroplast inner-envelope membranes labelled with [^3H]DIDS. The arrow indicates the position of the hexose-phosphate translocator.

Figure 3 Effect of Glc6P and DHAP on the incorporation of [^3H]DIDS

Isolated amyoplast envelopes were incubated with 10 μM [^3H]DIDS and in addition with DHAP or Glc6P. Lane 1, control; lane 2, 5 mM DHAP; lane 3, 0.5 mM Glc6P; lane 4, 5 mM Glc6P; lane 5, 50 mM Glc6P. Molecular-mass markers are indicated by arrows.

with pyridoxal phosphate, whereas Rumpho et al. (1988) applied [^3H]DIDS to chloroplasts purified from different species in order to identify the translocator protein. They demonstrated that [^3H]DIDS led to more specific labelling than did NaB^3H_4 .

Assuming that a functionally similar protein is involved in carbohydrate transport across the amyoplast envelope, we conducted experiments to reveal the effect of DIDS on Glc6P-dependent starch synthesis in amyoplasts isolated from cauliflower buds. As shown in Figure 1, addition of increasing concentrations of DIDS leads to a pronounced inhibition of amyoplast starch synthesis. This inhibition occurs already at micromolar concentrations, indicating the specific action of this inhibitor. Rumpho and Edwards (1985) demonstrated the high affinity of DIDS to the triose-phosphate translocator protein of the C_4 plant *Digitaria sanguinalis* L. They showed that DIDS also binds tightly to the triose-phosphate translocator of pea and spinach chloroplasts (Rumpho et al., 1988).

Envelope membranes were prepared from isolated cauliflower bud amyoplasts by the method of Alban et al. (1988). Figure 2 (lane 2) shows that the polypeptide pattern was similar to that obtained by these authors. In contrast with the polypeptide pattern of isolated spinach chloroplast envelopes (Block et al., 1983), the amyoplast envelopes did not contain a major band at 29 kDa that had been identified as the phosphate translocator in chloroplasts (Flügge and Heldt, 1978). This can be explained in two ways: (1) the hexose-phosphate translocator has a molecular mass of 29 kDa, but, in contrast with the situation in chloroplast envelopes (Flügge and Heldt, 1978), this protein does not represent 12–15% of the total envelope protein; (2) the hexose-phosphate translocator protein differs in its molecular mass from the triose-phosphate translocator.

Because of the strong inhibition by DIDS of Glc6P-dependent starch synthesis in isolated cauliflower bud amyoplasts, we attempted to label the protein involved in the transport of Glc6P across the envelope membrane with this reagent. As in chloroplasts, [^3H]DIDS leads to a specific labelling of only one single protein in the amyoplast envelope. Figure 2 reveals that the labelled protein of the amyoplast envelope (lane 3) has a significantly higher apparent molecular mass than the chloroplast phosphate translocator (lane 4) as judged by SDS/PAGE. We calculated a molecular mass of 31.6 kDa for the amyoplastic hexose-phosphate carrier in three independent preparations. Rumpho and Edwards (1985) demonstrated that the inhibitory effect of DIDS on photosynthesis of isolated chloroplasts can be overcome by applying DIDS in the presence of high concen-

trations of transport metabolites. This is probably due to the fact that DIDS interacts with a lysine residue that is involved in substrate binding (Gross et al., 1990). In order to provide further evidence for the specificity of the radioactive DIDS label, we incubated isolated envelope membranes with [3 H]DIDS in the presence of Glc6P or DHAP. These metabolites are known to interact with the hexose-phosphate translocator of amyloplasts (Borchert et al., 1989; Neuhaus et al., 1993). As shown in Figure 3, the presence of both metabolites, Glc6P and DHAP, leads to a substantial decrease in the intensity of the radioactive label.

In conclusion, the present results appear to indicate that a single protein has been labelled specifically, thus allowing one to purify and further characterize this translocator. The identification of the putative hexose-phosphate translocator will also allow an N-terminal amino acid sequence to be obtained. These data will then provide a useful tool with which to isolate a corresponding clone from a cDNA library.

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