RESEARCH COMMUNICATION

Evidence for two types of phosphate translocators in sweet-pepper (*Capsicum annuum* L.) fruit chromoplasts

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We have investigated whether there is evidence for the presence of different types of phosphate translocators in envelopes purified from pepper-fruit chromoplasts. A method was developed that allowed the purification of envelope membranes from isolated pepper-fruit chromoplasts. Proteoliposomes containing envelope-membrane proteins are able to import inorganic phosphate (P_i) or glucose 6-phosphate (Glc6P). In both cases, the rate of import is strongly dependent upon preloading of proteoliposomes with either P_i , dihydroxyacetone phosphate (DHAP) or Glc6P. This demonstrates the presence of a phosphate translocator

activity catalysing a counter exchange of phosphorylated intermediates. Interestingly, a high external concentration of Glc6P does not strongly inhibit P_i uptake into proteoliposomes preloaded with DHAP, whereas external Glc6P strongly inhibits P_i uptake into proteoliposomes preloaded with Glc6P. This observation strongly indicates that two types of phosphate translocator are present in chromoplast envelopes from red-pepper fruits. These data are discussed with respect to the possible physiological function of two types of phosphate translocator in one type of plastid.

INTRODUCTION

The co-ordinated transport of a wide range of compounds across cellular membranes is a prerequisite for control of metabolism. In plant cells, plastids are surrounded by two envelope membranes. The outer membrane is freely permeable to molecules with a molecular mass of less than 10 kDa, owing to the presence of porins [1]. The co-ordinated transport across the plastid envelope is achieved by the presence of several transporter proteins located in the inner envelope membrane [2].

One of the best-characterized transporters of this membrane is the triose phosphate translocator (TPT), which has been well characterized at both the protein and molecular levels [3]. This protein mediates the counter exchange of inorganic phosphate (P_i) and several phosphorylated three-carbon metabolites and is responsible for the export of triose phosphates during photosynthesis [4]. Interestingly, heterotrophic plastids possess an additional transport property that allows the uptake of hexose phosphates in counter exchange to P_i or phosphorylated three-carbon units [5,6]. The transporter responsible is generally referred to as the hexose-phosphate translocator (HPT).

However, until now it has not been analysed in detail whether heterotrophic plastids possess two different phosphate translocators, one with similar transport properties to the TPT and a second transporting in addition hexose phosphates. This question is particularly relevant, as we have recently shown that spinach chloroplasts, which normally contain only a TPT, can be induced by glucose feeding to develop glucose 6-phosphate (Glc6P) uptake capacity in counter exchange with P_i [7]. Since the capacity for Glc6P uptake into isolated chloroplasts appears within 24 h of glucose feeding and continues to increase for several days, we concluded that, at least under our artificial experimental conditions, the expression of an HPT had been induced. The aim of this work was to analyse whether the HPT

is present in addition to the TPT or instead of the TPT. Rather than glucose-induced HPT activity in chloroplasts, we have utilized the more physiologically relevant heterotrophic chromoplasts isolated from red-pepper fruits.

MATERIALS AND METHODS

Isolation of pepper-fruit chromoplasts and corresponding envelope membranes

Sweet-pepper plants (*Capsicum annuum* L., var. Yolo Wonder) were grown in soil under field conditions in the botanical garden of the University of Osnabrück. Fully ripened fruits were harvested, and the chromoplasts were purified according to a previously published method [8]. This method leads to pure chromoplast preparations, as revealed both by electron microscopy [8] and determination of the activity of compartment-specific enzymes (results not shown).

Chromoplast-envelope membranes were purified according to a protocol that was developed for the enrichment of envelopes from various heterotrophic plastids [9,10]. The protein content of the enriched envelopes was determined using a detergent-insensitive assay containing a bicinchoninic acid/ Cu_2SO_4 solution (Sigma, Deisenhofen, Germany) according to the supplier's instructions.

Uptake of [^{32}P] P_i or [^{14}C] Glc6P into proteoliposomes prepared from red-pepper fruit chromoplast envelope membrane protein

Proteoliposomes with incorporated proteins from red-pepper fruit chromoplast envelope membranes were prepared similarly as described previously [6] but with some minor modifications. Vesicles were prepared using 100 mg/ml acetone-washed phosphatidylcholine (Type IV-S from soybean; Sigma) in

Abbreviations used: DHAP, dihydroxyacetone phosphate; Glc6P, glucose 6-phosphate; HPT, hexose phosphate translocator; OPP, oxidative pentose-phosphate pathway; P_i , inorganic phosphate; TPT, triose phosphate translocator.

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100 mM Tricine/NaOH (pH 7.5)/30 mM potassium gluconate. For counter-exchange experiments the given metabolites were added at the indicated concentrations. The suspension was sonicated 2×5 min for homogenization. Envelope membrane protein (1 mg) was added per 4 ml of the liposome suspension. Membrane proteins were solubilized by adding Triton X-100 to a final concentration of 0.2 % (v/v); the sample was then rapidly (less than 10 s) combined with the liposome suspension (at a resulting lipid/detergent ratio of 50:1) and was immediately transferred into liquid nitrogen. Incorporation of the membrane proteins was achieved by freeze-thawing. After thawing at room temperature, the proteoliposomes were sonicated for 20 s (40 % line voltage, 20 % duty cycle; Vibra-Cell; Bioblock-Scientific Instruments, Freiburg, Germany) under a permanent stream of nitrogen gas. Unincorporated solution was removed from the liposomes by passing the preparation over an NAP-5 gel-filtration column (Pharmacia, Freiburg, Germany) equilibrated with 10 mM Tricine/NaOH (pH 7.5)/100 mM potassium gluconate/50 mM sodium gluconate. The eluted proteoliposomes (1 ml) were stored on ice and used for transport measurements within the next 60 min.

Uptake experiments were performed in 1.5 ml reaction vessels. The incubation was initiated by adding 100 μ l of proteoliposome suspension to 100 μ l of extraction medium containing the given radioactively labelled substrates (specific activities used were 0.5–1 MBq/mmol; NEN DuPont, Dreieich, Germany). The latter medium was kept at 30 °C by incubation in an Eppendorf incubator. The uptake was carried out for 2 min and was stopped by transfer to a 1 ml Dowex AG-1X8 column (chloride form, 100–200 mesh) equilibrated with 200 mM Tricine/NaOH, pH 7.5. Proteoliposomes were eluted from the column by washing with 3×500 μ l of the equilibration medium, and the radioactivity in the eluate was quantified in a Tricarb 2500 scintillation counter (Canberra-Packard, Frankfurt, Germany).

Linearity of uptake with time and increasing amounts of proteoliposomes was demonstrated before experiments (results not shown). All data presented were corrected for transport occurring on ice, which was always less than 2 % of the observed rates.

RESULTS AND DISCUSSION

Transport of P_i or Glc6P into proteoliposomes

It is well known that several types of heterotrophic plastids possess the ability to transport Glc6P in counter exchange for phosphorylated intermediates. Recently, we have demonstrated that isolated chloroplasts from green-pepper fruits were able to import Glc6P and to use this precursor for starch biosynthesis or as a substrate for the plastidic oxidative pentose-phosphate pathway [11,12]. To reveal whether chromoplasts isolated from red-pepper fruits also possess the ability to take up Glc6P, we isolated chromoplasts from the fruit and purified the corresponding envelope membranes. These membranes were then used to generate proteoliposomes containing the proteins residing in the chromoplast envelope.

The purification protocol leads to the enrichment of a relatively pure plastid preparation with similar size and shape of organelles [8]. Therefore, we suppose that the transport properties described here represent the characteristics of typical pepper-fruit chromoplasts. However, we cannot completely rule out the possibility that more than one type of chromoplast exist in pepper fruits with different transport properties, although we feel this is unlikely, as the purification procedure is very sensitive to minor differences in the density of both the plastids and the envelope membranes.

Table 1 Uptake of [14 C]Glc6P or [32 P] P_i into proteoliposomes preloaded with various metabolites

Proteoliposomes were prepared as described in the Materials and methods section. Preloading was achieved by addition of the indicated metabolites (10 mM). Data are the means $\pm$ S.E.M. of three independent measurements.

Counter-exchange substrate	Rate of Glc6P uptake (nmol/h per mg of protein)	Rate of P_i uptake (nmol/h per mg of protein)
None	10.1 $\pm$ 0.3	5.7 $\pm$ 1.2
P_i	80.8 $\pm$ 1.2	272.3 $\pm$ 6.4
Glc6P	96.2 $\pm$ 0.1	129.8 $\pm$ 3.0
DHAP	112.4 $\pm$ 2.8	289.0 $\pm$ 3.4

As demonstrated in Table 1, the proteoliposomes are able to import both Glc6P and P_i at low rates. The rate of import was strongly stimulated by preloading the vesicles with 10 mM of either P_i , Glc6P or dihydroxyacetone phosphate (DHAP). It is noticeable that the P_i import driven in counter exchange with P_i or DHAP was about twice as much as that driven by counter exchange to Glc6P (Table 1).

In contrast to P_i uptake, Glc6P import into proteoliposomes preloaded with P_i was lower than into proteoliposomes preloaded with Glc6P (Table 1). From the data given in Table 1 it is clear that the Glc6P-transporting phosphate translocator (or HPT) is able to transport Glc6P, P_i and DHAP. However, the rates of Glc6P uptake in counter exchange are always lower than the rates of P_i import (Table 1).

The observation that P_i import into proteoliposomes preloaded with high concentrations (10 mM) of P_i or DHAP is about twice as much as into proteoliposomes preloaded with Glc6P can be explained if we assume that pepper-fruit chromoplasts possess two types of phosphate translocators. According to such a model, P_i import driven in counter exchange to endogenous P_i or DHAP is most likely catalysed by both translocators, the TPT and the HPT. This simultaneous use of both transporters would explain why P_i import into proteoliposomes preloaded with DHAP or P_i is significantly higher than P_i import into vesicles preloaded with Glc6P, as the latter restricts P_i import to the activity of the HPT.

Effect of exogenous Glc6P on P_i uptake into proteoliposomes preloaded with DHAP or Glc6P

If two types of phosphate translocator are present in envelopes from pepper-fruit chromoplasts, then we would expect that exogenous Glc6P would act differently on P_i import into vesicles preloaded with either DHAP or Glc6P. Figure 1 shows the substrate saturation of P_i import into proteoliposomes preloaded with either DHAP (Figure 1A) or Glc6P (Figure 1B). Increasing concentrations of external P_i induce a rapid increase in the uptake rate, which approaches saturation at higher substrate concentrations (Figure 1A), giving a $V_{\max}$ of about 1250 nmol/h per mg of protein (Figure 1A, inset). Interestingly, even a high external concentration of Glc6P (10 mM) additionally present in the medium did not strongly inhibit P_i uptake (Figure 1A).

This effect of Glc6P on P_i uptake is in strong contrast to the effect of Glc6P on P_i transport into proteoliposomes preloaded with Glc6P. As described above, rising concentrations of P_i induced a rapid increase of P_i import, reaching saturation at about 0.6 mM (Figure 1B) and giving a $V_{\max}$ of 300 nmol/h per mg of protein (Figure 1B, inset). However, the additional presence of Glc6P strongly inhibits P_i transport (Figure 1B); about 85 %

Figure 1 Substrate saturation of P_i uptake and Glc6P effect upon P_i uptake into proteoliposomes preloaded with either DHAP or Glc6P

Proteoliposomes were prepared as described in the Materials and methods section. Preloading with 10 mM of either DHAP (A) or Glc6P (B) was achieved by addition of these intermediates during reconstitution. Exogenous Glc6P was supplied (if indicated) at a 10 mM concentration. Data are the means $\pm$ S.E.M. of three independent measurements. The insets depict double reciprocal plots of the data given, indicating K_m values of phosphate uptake of 0.6 mM (A) and 0.46 mM (B).

of the P_i uptake is inhibited even under saturating P_i concentrations.

This observation is further evidence for the presence of two types of phosphate transporter in red-pepper fruit chromoplasts. It is well known that Glc6P is not transported by the TPT from spinach chloroplasts [4]. Obviously, the TPT present in pepper-fruit chromoplasts does not transport this intermediate, as Glc6P

Figure 2 Effect of rising DHAP concentrations on P_i uptake into proteoliposomes preloaded with P_i

Proteoliposomes were prepared as described in the Materials and methods section. Preloading with P_i (10 mM) was achieved by addition of this compound during reconstitution. Radioactively labelled Glc6P was supplied at a concentration of 2 mM. Data are the means $\pm$ S.E.M. of three independent measurements.

does not competitively inhibit the rate of P_i uptake into proteoliposomes preloaded with DHAP. Moreover, there are further indications that the residual P_i uptake into vesicles preloaded with DHAP is due to a TPT activity. (i) The inhibitory effect of Glc6P on P_i uptake into vesicles preloaded with Glc6P (Figure 1B) is substantially higher than into vesicles preloaded with DHAP (Figure 1A). If only one membrane protein was responsible for the transport of all phosphorylated intermediates tested, the degree of inhibition at a given external P_i /Glc6P ratio should always be in the same range. (ii) We can calculate from the data given in Figure 1(B) that Glc6P reduces the rate of P_i uptake (at saturating P_i concentrations) by about 180 nmol/h per mg of protein. A similar decrease of P_i uptake into vesicles preloaded with DHAP can be observed when Glc6P is present also (Figure 1A).

Interaction between triose and hexose phosphates

These data show that chromoplasts isolated from red-pepper fruits possess two types of phosphate transporters, one responsible for the transport of triose phosphates and one responsible for the transport of hexose phosphates. To analyse a possible interaction of both intermediates in more detail, we have determined how rising concentrations of DHAP influence the rate of Glc6P uptake. The data shown in Figure 2 clearly demonstrate that submillimolar concentrations of DHAP strongly inhibit the rate of Glc6P import into vesicles preloaded with P_i . This competitive inhibition of Glc6P uptake is in agreement with the data given in Table 1 (Glc6P–DHAP exchange) and with data published on isolated pea-root plastids [5].

Strong evidence is presented for the presence of two phosphate transporters in the envelopes of heterotrophic plastids. The

physiological reason for this phenomenon is still far from clear, but we propose that the presence of two transporters allows for hexose phosphate and triose phosphate transport to occur independently and that hexose phosphate uptake into plastids is strongly controlled by the concentration of triose phosphates. In heterotrophic plastids the plastidic oxidative pentose-phosphate pathway (OPP) is the sole source for reducing equivalents needed for nitrite reduction and the Gogat reaction [13–15]. The carbohydrate required to drive this metabolic pathway appears to be Glc6P, which is imported into the intact organelle [13–16]. Using germinating *Ricinus communis* seedlings, it was clearly shown that the addition of ammonia or glutamine (the products of nitrite reduction and Gogat reaction) induce a rapid stimulation of glycolysis, leading to an almost doubled triose phosphate/hexose phosphate ratio [17]. Such a metabolic change in the cytosol would prevent the entry of Glc6P into the plastidic OPP (Figure 2) and thus would act as a feedback signal to allow high cytosolic synthesis of carbon skeletons that would be required for transamination of glutamine and for incorporation of ammonia. As under these conditions the reducing equivalents synthesized in the plastidic OPP are sufficient for the synthesis of ammonia or glutamine, there would be no further need for the uptake of hexose phosphates into the plastidic compartment. This hypothesis concerning the interaction between triose phosphates and hexose phosphates is further strengthened by the observation that rising levels of exogenous DHAP strongly inhibit the rate of Glc6P-dependent starch synthesis in isolated amyloplasts purified from cauliflower buds [18]. Furthermore, studies with intact plastids isolated from pea embryos also demonstrated that the measured inhibition of Glc6P uptake by DHAP was not as large as predicted theoretically (61 % instead of 91.4 %) if the two substrates were solely transported by a

single HPT, again suggesting the presence of a second transporter [19].

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