IDENTIFICATION OF A PROTEIN INVOLVED IN PHOSPHATE TRANSPORT OF CHLOROPLASTS

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1. Introduction

The inner membrane of the chloroplast envelope contains a specific translocator facilitating the transport of inorganic phosphate, triosephosphates and 3-phosphoglycerate [1]. The main function of this translocator is to enable the export of fixed carbon in the form of triosephosphates from the chloroplasts to the cell in exchange for inorganic phosphate. This transport can therefore be regarded as a partial step of the overall reaction of CO_2 fixation. The translocator is very specific. Thus the K_m for transport of 2-phosphoglycerate is 20 times higher than that of 3-phosphoglycerate [2]. Whereas in the mitochondria the inner membrane is the site of metabolite transport as well as of electron transport, in the chloroplasts the electron transport is restricted to the thylakoid membranes. The inner envelope membrane appears to have primarily transport functions. This membrane should be therefore a very good source for an isolation of a metabolite carrier.

A prerequisite for an isolation is the identification of the carrier protein. Despite extensive searches by our laboratory, we did not find any specific inhibitor with which the protein might be labelled. We therefore tested a number of reagents known to bind covalently to certain amino acids for their ability to inhibit the phosphate translocator in chloroplasts. We wanted to find a substance which binds to the carrier and the binding of which is specifically inhibited by substrates occupying the active site. In the following it will be shown that *p*-(diazonium)-benzene sulfonic acid, which is known to react with histidine and tyrosine [3], appears to be a suitable tool to label the protein involved in phosphate transport. This substance also offers the advantage, that due to its sulfonic acid group, it is not able to penetrate intact membranes and it therefore can be expected to react primarily with proteins exposed to the outside as might be the case with a carrier protein.

2. Methods

The chloroplasts were prepared from fully grown spinach leaves according to the method of Cockburn et al. [4] modified by Heldt and Sauer [5]. Chlorophyll was assayed according to the method of Whatley and Arnon [6]. Protein was measured by the method of Lowry et al. [7], phosphate transport was measured by silicone layer filtering centrifugation [1].

The purification of chloroplast envelope membranes was based on the methods described by Douce et al. [8] and Poincelot [9]. The membranes prepared in this way contain both the outer and the inner envelope membrane. Intact chloroplasts equivalent to 10 mg chlorophyll were suspended in 2 ml medium containing 50 mM sodium borate (pH 7.8), 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. To this 15 ml 10 mM *N*-trishydroxymethyl methylglycin (TRICINE) pH 7.8 and 4 mM $MgCl_2$ were added to remove the envelope membranes by osmotic shock. After 1 min at 4°C sucrose was added to a final concentration of 0.33 M 11 ml of the suspension were layered on top of a discontinuous gradient consisting of 9 ml 0.93 M sucrose in 50 mM *N*-trishydroxymethyl

methyl glycine, pH 7.8 + 4 mM $MgCl_2$ and 17 ml of the same buffer containing 0.63 M sucrose. The gradient was centrifuged for 120 min at 20 500 rpm (Beckman, rotor SW 27). A cloudy yellow band containing envelopes appears at the interface between the 0.93 M and 0.63 M sucrose layer. The envelope layer was carefully removed and diluted with 50 mM *N*-trishydroxymethyl methylglycine buffer, pH 7.8 to a sucrose concentration of 0.33 M. The envelope membranes were centrifuged for 30 min at 40 000 rpm (Beckman, Rotor Ti 50) and resuspended in 50 mM sodium borate (pH 7.8), 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. The washing was repeated twice.

Chloroplast membrane proteins were analyzed by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate [10]. The separation gel contained 11.5% acrylamide and 0.1% *N,N'*-methylene bisacrylamide. The gels were stained with Coomassie blue [11] and scanned at 546 nm. As marker enzymes triosephosphate isomerase, lactic dehydrogenase, serum albumin and ovalbumin were used. Gels were analyzed for radioactivity by slicing and digestion with NCS (Amersham, Searle) [12], followed by liquid scintillation counting.

Preparation of ^{35}S-*p*-(diazonium)-benzene sulfonic acid was carried out according to Gattermann-Wieland [13] using ^{35}S-labelled sulfanilic acid (Amersham-Buchler).

3. Results and discussion

Phosphate transport in intact chloroplasts can be inhibited by *p*-(diazonium)-benzene sulfonic acid, as shown in fig.1. The inhibition is a slow reaction and it requires a relatively high concentration of the inhibitor. The inhibition is largely decreased if phosphate or 3-phosphoglycerate are added during the preincubation with the inhibitor (table 1). 2-Phos-

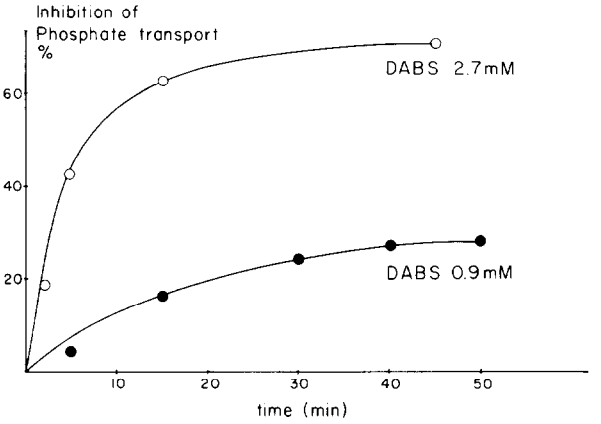

Fig.1. Inhibition of the transport of phosphate (0.47 mM, 0°C, pH 7.6) into intact isolated spinach chloroplasts by *p*-(diazonium)-benzene sulfonic acid (DABS). See methods. The uninhibited rate was 45 μmol/mg chlorophyll·h.

Table 1
Inhibition of phosphate transport into intact isolated chloroplasts by preincubation with *p*-(diazonium)-benzene sulfonic acid (DABS) and substrates

Preincubation	Phosphate transport μmol / mg chlorophyll·h	Inhibition (%)
–	45	–
DABS (1 mM)	30	33
DABS + phosphate (20 mM)	37	18
DABS + 3-phosphoglycerate (10 mM)	38	16
DABS + 2-phosphoglycerate (10 mM)	31	32

Spinach chloroplasts (4.7 mg chlorophyll/ml) in sorbitol-borate medium, pH 7.8 (see methods) were incubated for 40 min at 0°C in the presence of 1 mM *p*-(diazonium)-benzene sulfonic acid and substrates as indicated. Then 25 mM histidin were added, and the suspension was centrifuged for 1 min at 800 × *g* (Sorvall centrifuge, Rotor SS 34). The chloroplasts were washed twice, and the rate of phosphate transport was measured at 0°C with 0.47 mM phosphate. The data shown are mean values from three different experiments.

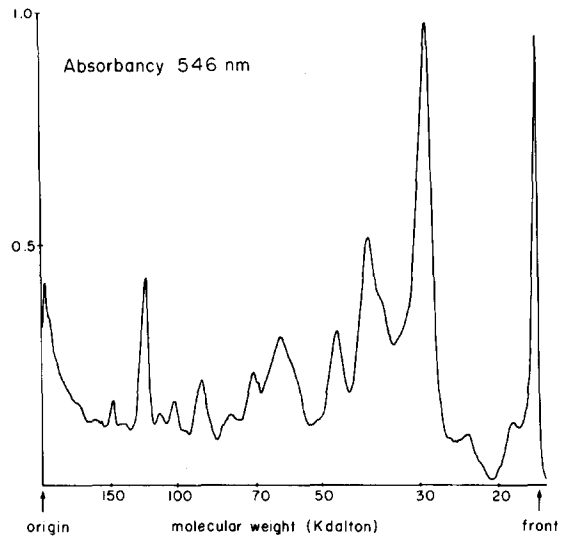

Fig.2. Densitometric trace of a sodium dodecyl sulfate polyacrylamide electrophoresis of protein in an envelope membrane preparation (150 μg total protein). For details see methods.

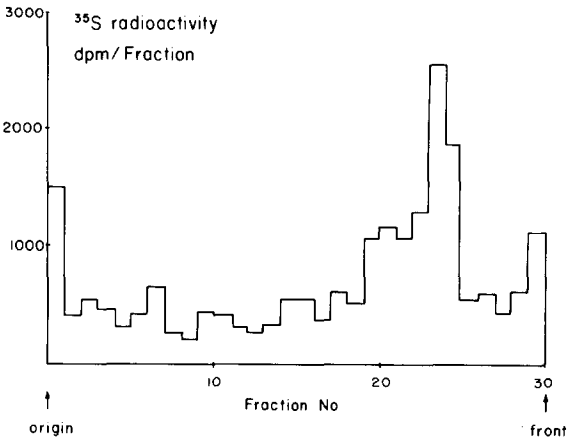

Fig.3. Incorporation of ^{35}S *p*-(diazonium)-benzene sulfonic acid into the protein of an envelope membrane preparation. The envelope membranes were incubated according to the legend of table 1 with 0.31 mM ^{35}S *p*-(diazonium)-benzene sulfonic acid (specific activity 11 Ci/mol). The membranes were then centrifuged and washed twice (see methods), and finally suspended in a medium containing 50 mM Tris (hydroxymethyl)aminomethane (Tris) pH 7.8, 0.07 M sodium dodecyl sulfate, 2 mM dithiothreitol and 0.15 M sucrose. Membranes equivalent to 150 μg protein were applied to gel electrophoresis (see legend fig.1). Subsequently, the gels were analyzed for radioactivity (see methods).

phoglycerate which is only a weak substrate of the phosphate translocator [2] also has little or no effect in decreasing the inhibition. Apparently, the carrier is protected by its substrates against the reaction with the inhibitor. In the experiment of fig.2 the binding of *p*-(diazonium)-benzene sulfonic acid to the proteins of the envelope membranes was investigated. For this the purified envelope preparations were incubated with radioactively labelled *p*-(diazonium)-benzene sulfonic acid and the protein was analyzed by sodium dodecyl sulfate gel electrophoresis. The resulting polypeptide pattern shows relatively few bands and a prominent peak with a molecular weight of about 29 000. Similar polypeptide patterns of the envelope membranes have been reported earlier [14,15].

As shown in fig.3, the radioactive label is found in various polypeptides, but the highest incorporation is observed in the polypeptide with a molecular weight

Table 2
Dependency of the incorporation of ^{35}S *p*-(diazonium)benzene sulfonic acid (DABS) into the 29 000 dalton polypeptide on substrates added during the incubation

Substrates added during the incubation with [^{35}S]DABS	[^{35}S]DABS incorporated nmol / mg protein	(% of control)
–	1.62	100
phosphate (20 mM)	1.25	77
3-phosphoglycerate (10 mM)	1.10	68
2-phosphoglycerate (10 mM)	1.67	103

For methods see legend fig.3. Mean values from three different experiments.

of 29 000. When 3-phosphoglycerate or phosphate were added during the incubation with the inhibitor, the labelling of the 29 000 dalton polypeptide was considerably decreased, whereas the labelling of the other protein peaks was not markedly changed (traces not shown). In table 2 the incorporation of ^{35}S *p*-(diazonium)-benzene sulfonic acid into the 29 000 dalton protein has been evaluated. It is clearly demonstrated that inorganic phosphate and 3-phosphoglycerate, which are both transported by the phosphate translocator, cause a specific protection against the binding of the inhibitor to the 29 000 dalton polypeptide. 2-phosphoglycerate, which is practically not transported also does not affect the binding of the inhibitor to the 29 000 dalton polypeptide. These data indicate that a polypeptide with a molecular weight of 29 000 contains a binding site essential for the transport of phosphate. It is not possible to assay from the densitometer trace the exact portion of the 29 000 dalton polypeptide in relation to the total protein of the envelope membranes. A rough estimation may yield about 25% (see also ref. [15]) of the total protein for this peak. If this peak has a uniform composition, which still has to be shown, it would indicate that the major protein of the envelope membranes plays a role in the functioning of the phosphate translocator.

Acknowledgement

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