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SPECIFIC LABELLING OF THE ACTIVE SITE OF THE PHOSPHATE TRANSLOCATOR IN SPINACH CHLOROPLASTS BY 2,4,6-TRINITROBENZENE SULFONATE[1)]

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SUMMARY: Phosphate transport across the chloroplast envelope is rapidly inactivated by the amino-group reagent 2,4,6-trinitrobenzene sulfonate. Subsequent exposure to $[^3H]NaBH_4$ leads to an incorporation of the trinitrophenyl moiety into envelope membrane preparations. From the membrane proteins only a polypeptide with 29000 dalton molecular weight is labelled. The inactivation of phosphate transport and the incorporation of radioactivity are both specifically reduced by the presence of substrates.

The results lead to the conclusion that a polypeptide with a molecular weight of 29000 dalton and containing a lysyl residue at the substrate binding site is involved in the phosphate translocator function.

INTRODUCTION: The inner membrane of the chloroplast envelope contains a specific translocator, which facilitates the transport of inorganic phosphate, 3-phosphoglycerate and dihydroxyacetone phosphate (1). The main function of this metabolite carrier is to enable the export of fixed carbon from the chloroplasts to the cytosol in the form of triose phosphates and 3-phosphoglycerate in exchange with inorganic phosphate.

In the course of our studies on the characterization of the

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ABBREVIATIONS: TNBS, 2,4,6-Trinitrobenzene sulfonate.

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membrane protein involved in this transport it was found that diazobenzene sulfonate, known to react with various amino acid side chains, preferentially with histidine, tyrosine, cysteine and lysine residues (2), inhibited the phosphate transport and was incorporated into a membrane protein with a molecular weight of 29000 dalton (3). It was shown recently that pyridoxal 5'-phosphate, a potent inhibitor of the phosphate transport in chloroplasts and which is known to react with lysine residues, is also specifically incorporated into the 29000 dalton polypeptide (4). These findings indicated that a polypeptide with a molecular weight of 29000 dalton participates in specific phosphate transport across the inner envelope membrane. 2,4,6-trinitrobenzene sulfonic acid, a reagent reacting selectively with primary amino groups (5) as present in lysine, has been described as an inhibitor of the phosphate translocator in chloroplasts (6). Due to the sulfonate residue this compound does not readily permeate membranes and is therefore specially suited to react with the protein exposed to the outer surface of the inner envelope membrane. In the present report this substance is used as a tool in order to characterize further the active site of the translocator and to label the transport protein involved.

METHODS: The chloroplasts were prepared from fully-grown spinach leaves according to the method of Cockburn et al. (7) modified by Heldt and Sauer (8). For the assay of chlorophyll see (9) and of protein see (10). Phosphate transport was measured by silicone-layer filtering centrifugation (1).

For the labelling procedure intact chloroplasts equivalent to 3 mg chlorophyll were suspended in 7 ml medium containing 0.33 M sorbitol, 50 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes), pH 7.6, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. This suspension was treated with 0.2 - 0.6 mM 2,4,6-trinitrobenzene sulfonate (Sigma) for 3-15 min. A three-fold molar excess of $[^3H]NaBH_4$ (30 mCi/mmol) was then added and

the incubation proceeded at 4^{o} for 10 min. The chloroplasts were washed three times with the medium. In order to remove the envelope membranes by osmotic shock, 4 ml 10 mM N-tris-hydroxylmethyl-methyl-glycine (Tricine) pH 7.8 and 4 mM $MgCl_2$ were added. After 1.5 min at $4^{o}C$, sucrose was added to a final concentration of 0.33 M. Purification of envelope membranes was achieved by discontinuous sucrose-gradient centrifugation as described earlier (3) according to the method of Douce et al. (11). The purified envelope membranes were analyzed by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) (12). The separation gel contained 11.5% acrylamide and 0.1% N,N'-methylene bis-acrylamide. Tandem gels were run and were either stained with Coomassie Blue (13) and scanned at 546 nm or analyzed for radioactivity by slicing and digestion with NCS (Amersham and Searle) (14) followed by liquid scintillation counting.

All steps following the incubation with 2,4,6-trinitrobenzene sulfonate were done with minimal exposure to light.

RESULTS AND DISCUSSION: 2,4,6-trinitrobenzene sulfonate is a very effective inhibitor of the phosphate transport. Fig. 1 shows the concentration dependence of this inhibition. The full extent of the inhibition is reached after an incubation time with the inhibitor of 3-5 min. The question arises whether the inactivation of the transport is due to a reaction of the inhibitor with a lysyl group at the binding site of the carrier protein or whether the reaction with a lysyl group located elsewhere in the protein has caused conformation changes leading to the inactivation of the transport. In an attempt to answer this question it was investigated whether substrates bound to the carrier have a protective effect towards the inhibition of the transport. The experiment of Table 1 shows that this is indeed the case. The inhibition by 2,4,6-trinitrobenzene sulfonate is largely decreased when phosphate and 3-phosphoglycerate, both substrates of the phosphate translocator, are added to the chloroplasts prior to the addition of the inhibitor. In order to exclude the possibility that the observed decrease of the inhibition was due to an unspecific anion effect, the experiment was also

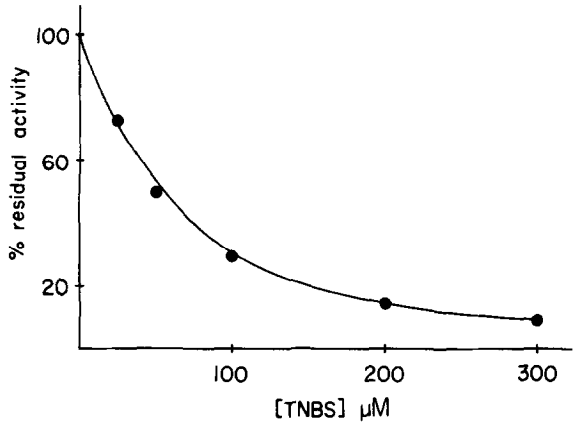

Fig. 1: Inhibition of phosphate transport by TNBS. Intact chloroplasts (0.1 mg/ml) were preincubated with the indicated concentrations of TNBS for 10 min. Phosphate transport was measured as described in Methods. The uninhibited rate was 43 µmol (mg chl.)$^{-1}$h^{-1}.

Tab. 1: Inhibition of phosphate transport by TNBS and the effect of substrates.
Spinach chloroplasts (0.15 mg chl/ml) were preincubated with TNBS (0.2 mM) for 5 min with or without substrates (10 mM for 10 min before the addition of TNBS). Incubations were stopped by centrifugation (SS 34 rotor, 1000xg for 1 min) and washing. Chloroplasts were resuspended and phosphate transport was measured as described earlier (1).

Additions	Phosphate transport* µmol (mg chl.)$^{-1}$h^{-1}	Inhibition (%)
control	43	-
TNBS	15	64
3-phosphoglycerate then TNBS	27	36
2-phosphoglycerate then TNBS	16	63
phosphate, then TNBS	29	33

*Mean values of four different experiments.

performed in the presence of 2-phosphoglycerate. This substance which is not transported by the phosphate translocator (1) has also no protective effect against the inhibition. These

data show that binding of phosphate and 3-phosphoglycerate to the carrier causes a specific protection against the reaction with 2,4,6-trinitrobenzene sulfonate. A lysyl group located at the active site of the phosphate carrier appears to be involved in substrate binding.

In the experiment of Fig. 2 the binding of 2,4,6-trinitrobenzene sulfonate to membrane proteins of the envelope was studied. The chloroplasts were first incubated with the inhibitor. Subsequently the bound inhibitor was radioactively labelled by incubation with $[^3H]NaBH_4$, leading to the irreversible formation of a 1:1 or a 1:2 adduct between the trinitrophenyl moiety and hydride ions (15). The envelope membranes were then isolated and analyzed for radioactivity by SDS-polyacrylamide gel electrophoresis as described in Methods. The resulting pattern shows that the radioactivity is appearing exclusively together with the major protein peak of a molecular weight of 29000 dalton.

A preincubation of the chloroplasts with 3-phosphoglycerate or inorganic phosphate prior to the addition of 2,4,6-trinitrobenzene sulfonate results in a large decrease of the incorporation of the tritium label into the 29000 dalton polypeptide, whereas 2-phosphoglycerate does not affect the incorporation of the label (Table 2). Apparently, the protective effect of substrates against the incorporation of the radioactivity has the same specificity as the protection against the inhibition of the transport by 2,4,6-trinitrobenzene sulfonate. These findings strongly indicate that the incorporation of the tritium label reflects the binding of the inhibitor.

Three different inhibitors of the phosphate translocator in chloroplasts, namely diazobenzene sulfonate (3), pyridoxal

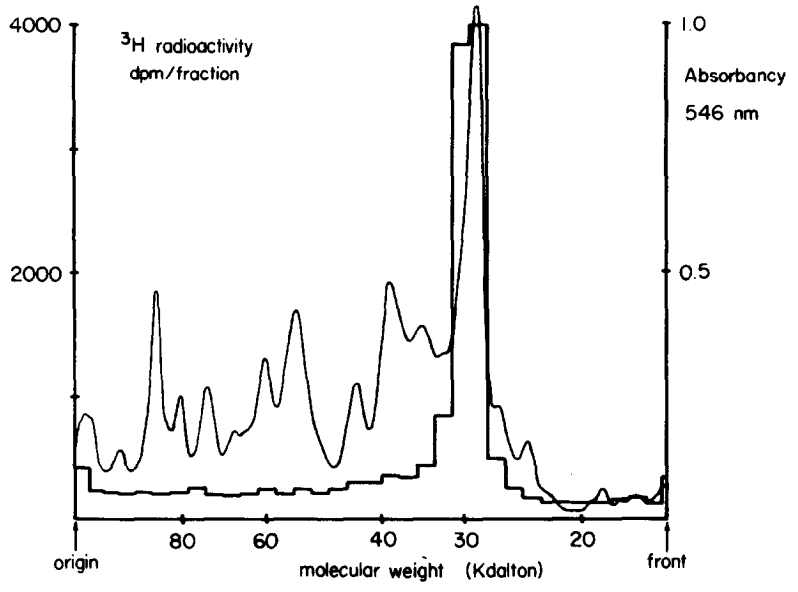

Fig. 2: SDS-polyacrylamide gel electrophoresis of envelope membrane proteins. The membrane proteins were allowed to react with TNBS. The incubation of intact chloroplasts with TNBS, the subsequent labelling by $[^3H]NaBH_4$ and the isolation of envelope membranes was carried out as described in Methods. Membranes equivalent to 100 µg protein were applied to SDS-polyacrylamide gel electrophoresis. Separate gels were used for the absorbance scan (continuous curve) and for radioactivity scan (discrete lines).

Tab. 2: Incorporation of TNBS into the 29000 dalton polypeptide and the effects of substrates.
For experimental conditions see Methods and legend of figure 2.

Additions before the incubation with TNBS/$[^3H]NaBH_4$	dpm*	% of control
none	4150	100
phosphate (10 mM)	1850	45
3-phosphoglycerate (10 mM)	2450	58
2-phosphoglycerate (10 mM)	4200	101

*Mean values of three different experiments.

5'-phosphate (4) and 2,4,6-trinitrobenzene sulfonate, as presented here, were shown to be specifically incorporated

into the same polypeptide band of envelope membrane preparations. Recent studies from our laboratory (unpublished data) showed that these three inhibitors are bound to the same substrate binding site of the membrane protein.

These concurring results from independent measurements may be regarded as convincing evidence that a membrane protein with a molecular weight of 29000 dalton, as evaluated from SDS-polyacrylamide gel electrophoresis, is involved in the function of the phosphate translocator in chloroplasts.

The binding of 2,4,6-trinitrobenzene sulfonate and pyridoxal 5'-phosphate to the substrate binding site of the membrane protein reveals the presence of a lysyl group at the active center. Further studies using phenylglyoxal gave evidence that also an arginine residue is located at the substrate binding site (Flügge and Heldt, unpublished data). It has been demonstrated that the phosphate translocator of chloroplasts transports the twice negatively charged anions of phosphate, dihydroxyacetone phosphate and 3-phosphoglycerate (6). It is feasable that the two positive charges required for the binding of these anions to the transport protein are supplied by one lysine and one argine present in the active center.

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