Fluorescence microscopy and radiolabeling of C\textsubscript{3} and C\textsubscript{4} chloroplasts using diisothiocyanatostilbene disulfonic acid as a marker for the phosphate translocator

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Abstract. The usefulness of 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) for in-situ studies of the chloroplast phosphate translocator was evaluated by fluorescence microscopy and radiolabeling of spinach (\textit{Spinacia oleracea} L.) (C\textsubscript{3} plant) and maize (\textit{Zea mays} L.) (C\textsubscript{4} plant) chloroplasts. In maize mesophyll and bundle-sheath chloroplasts and in spinach chloroplasts that were either intact, broken or swollen, DIDS fluorescence was only associated with the chloroplast envelope. Intact chloroplasts often had fluorescent patches corresponding to concave regions of the chloroplast which we assume to be regions enriched in DIDS-binding sites.

Incubation of intact or broken spinach chloroplasts or maize mesophyll chloroplasts with \textsuperscript{3}H\textsubscript{2}DIDS resulted in the labeling of a single polypeptide (relative molecular mass, \(M_r\), ~30 kDa) in the envelope fraction, in each case. Label in the stromal fraction was not detected when intact chloroplasts were incubated with \textsuperscript{3}H\textsubscript{2}DIDS. However, when broken chloroplasts were incubated with \textsuperscript{3}H\textsubscript{2}DIDS, several polypeptides of various molecular masses were labeled, but not the 30 x 31-kDa polypeptide. In thylakoid fractions from both broken and intact chloroplasts, a single 30 x 31-kDa polypeptide was labeled inconsistently. When a mixture of intact maize mesophyll and bundle-sheath chloroplasts was labeled with \textsuperscript{3}H\textsubscript{2}DIDS, extracts of whole chloroplasts displayed radioactivity only in the 30 x 31-kDa band.

We conclude that DIDS is a valuable probe for the in-situ identification and characterization of the ~30-kDa protein – the presumptive phosphate translocator – in C\textsubscript{3} and C\textsubscript{4} chloroplasts since DIDS (1) does not penetrate the inner membrane of the envelope of intact chloroplasts and, therefore, (2) does not bind internal sites in intact chloroplasts, and (3) only binds the ~30-kDa protein in the inner membrane of the envelope.

Key words: Anion transport – Chloroplast membrane (phosphate translocator) – 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) – Fluorescence microscopy – \textit{Spinacia} (chloroplast membrane) – \textit{Zea} (chloroplast membrane)

Introduction

The exchange of photosynthetic intermediates across the inner membrane of the chloroplast envelope by the phosphate translocator (Pi-translocator) has been well documented in C\textsubscript{3} chloroplasts (Heldt and Rapley 1970; for review see Heber and Heldt 1981), to a lesser extent in C\textsubscript{4} mesophyll chloroplasts (see Hatch and Osmond 1976; Day and Hatch 1981), and only indirectly in C\textsubscript{4} bundle-sheath chloroplasts (Hatch et al. 1975; Jenkins and Boag 1985). All three chloroplast types possess a Pi-translocator protein that is similar, yet distinguishable based on specificity of metabolite transport. The Pi-translocator of C\textsubscript{3} chloroplasts catalyzes the preferential export of dihydroxyacetone phosphate in exchange for inorganic phosphate and 3-phosphoglycerate (PGA), all as divalent an-
ions in the light and at physiological pH (Heldt and Rapley 1970; Hatch and Osmond 1976; Day and Hatch 1981). The C₄ mesophyll chloroplast Pi-translocator is characterized by its unique ability to rapidly exchange phosphoenolpyruvate for inorganic phosphate, in addition to transporting dihydroxyacetone phosphate and PGA (Huber and Edwards 1977; Day and Hatch 1981; Rumpho and Edwards 1984). Furthermore, it is believed that the Pi-translocator of bundle-sheath chloroplasts (particularly NADP-malic enzyme-type C₄ plants such as maize) preferentially exports PGA as a trivalent anion for reduction to dihydroxyacetone phosphate in the mesophyll (Hatch et al. 1975).

Despite the importance of the Pi-translocator in regulating carbon flow and the implied differences in the structure and/or function of the protein(s), only the C₃ Pi-translocator of spinach chloroplasts has been extensively studied (Flügge and Heldt 1981, 1986). Recently, Flügge et al. (1989) sequenced the cDNA encoding the 29-kilodalton (kDa) Pi-translocator polypeptide of spinach chloroplasts and deduced its amino-acid sequence. They were also able to demonstrate that the precursor protein, synthesized in vitro, could be inserted into the chloroplast envelope. Inferences from the primary sequence should prove useful in characterizing the structure-function relationship(s) of the Pi-translocator. However, the existence of a suitable inhibitor and in-situ marker is necessary to provide direct evidence for these deductions, e.g., by specific and irreversible labeling of the protein, followed by in-situ proteolysis.

Rumpho and Edwards (1985) have previously demonstrated that the stilbene disulfonate derivative, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), inhibits PGA-dependent O₂ evolution in both C₃ and C₄ mesophyll chloroplasts. This inhibition was alleviated by preincubation with the substrates for the C₃ and C₄ Pi-translocators, respectively. Further work demonstrated that radiolabeled DIDS bound irreversibly to a ~30-kDa polypeptide of the envelope fraction of both C₃ and C₄ mesophyll chloroplasts (Rumpho et al. 1988). The permeability of the chloroplast envelope to DIDS and the labeling of internal chloroplast fractions, were not examined.

The anion-exchange inhibitor, DIDS, has been used in studies of anion transport in plant tissues (Lin 1981; Kiefer et al. 1982; Churchill and Sze 1984; Deane-Drummond and Gates 1987), but the specificity and irreversibility of DIDS binding were not demonstrated. In contrast, the stilbene disulfonates, and in particular DIDS, have been invaluable in the identification, isolation, and in-situ characterization of the topographical arrangement of the erythrocyte anion-exchange transport protein (band 3) (Rothstein et al. 1980; Jennings et al. 1986).

One objective of our study was to determine the distribution of the ~30-kDa polypeptide within the inner membrane of the envelope of intact C₃ and C₄ chloroplasts by observing the pattern of DIDS fluorescence. Attempts at localizing the translocator in isolated, intact spinach chloroplasts by immunofluorescence have proved unsuccessful (Joyard et al. 1983; Van Berkel et al. 1986). A second objective was, using fluorescence microscopy and radiolabeling, to determine whether C₄ bundle-sheath chloroplasts also bind DIDS given the potential differences in structure and function of the bundle-sheath chloroplast Pi-translocator as discussed above. Thirdly, we wanted to determine the usefulness of DIDS for in-situ structure-function studies of the proposed ~30-kDa Pi-translocator. A truly useful marker for the translocator would: (a) bind specifically, irreversibly, and inhibit transport activity, (b) not bind at any other site on or within the chloroplast, and (c) not cross the inner membrane of the envelope. Previous studies established that DIDS binds one protein in the envelope and inhibits PGA-dependent oxygen evolution (Rumpho et al. 1988). Internal binding and permeability of the envelope are examined here.

**Materials and methods**

**Fluorescence studies**

*Isolation of chloroplasts and incubation with DIDS. Spinacia oleracea L. (spinach) was purchased from a local supermarket. Intact chloroplasts were isolated from spinach leaves according to Leegood and Walker (1983). Zea mays L. cv. Aztec (maize; Asgrow Seed Co., Kalamazoo, Mich., USA) was grown in a growth chamber (Rumpho et al. 1988). For the fluorescence studies, a mixture of maize mesophyll and bundle-sheath chloroplasts was used. This preparation was obtained as described previously for the mechanical isolation of mesophyll chloroplasts (Rumpho et al. 1988) except that homogenization with a Polytron PT-36 probe (Brinkmann Instruments, Westbury, N.Y., USA) was increased to four 5-s bursts at setting 8 to break open the bundle-sheath cells. All chloroplast preparations were centrifuged through 25% (v/v) Percoll, washed and resuspended in 330 mM sorbitol, 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid-KOH (Hepes) buffer (pH 7.8) (isotonic wash medium), to a concentration of 1 mg chlorophyll/ml. Chlorophyll was measured according to Wintermans and De-Mots (1965).

Intact chloroplasts (5 mg chlorophyll) were incubated isotonically with 2-5 mM DIDS (Sigma Chemical Co., St. Louis, Mo., USA) for 15 min at 28°C and in room light. A second preparation of maize and spinach chloroplasts was incubated with DIDS in a hypotonic medium consisting of 50 mM sorbitol, 50 mM Hepes (pH 7.8). In addition, a third spinach chloroplast preparation was subjected to two freeze-thaw treat-