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Passive anion transport through the chloroplast inner envelope membrane measured by osmotic swelling of intact chloroplasts

Bruno Fuks, Fabrice Homblé *

Laboratoire de Physiologie Végétale, Campus Plaine, C.P. 206/2, Université Libre de Bruxelles, B-1050 Brussels, Belgium

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Abstract

It has been shown that chloride channels are located in the envelope membranes of chloroplasts [5,11]. In this report, we use the light-scattering technique to measure quantitatively the rate of anion transport through the inner envelope membrane of isolated intact chloroplasts. Our results permit to assign the anion transport to the inner envelope of chloroplasts. The anionic selectivity determined from the kinetics of light scattering indicates that the chloride pathway is also highly permeable for NO_2^- and NO_3^-. The sulfate and phosphate anions are impermeant. The chloride flux is not inhibited by DIDS or NEM and is temperature-dependent. The activation energy of the transport process suggests that the Cl^- flux occurs through a channel.

Keywords: Bioenergetics; Photosynthesis; Chloride channel

1. Introduction

The chloroplast is enclosed by two envelope membranes. The outer envelope does not play any part in selective transport of metabolites [1]. This membrane contains nonspecific large pores which permit the flux of ions and molecules smaller than 10 kDa and acts as a barrier to large solutes only [2,3]. The inner envelope membrane is permeable for small neutral molecules and controls the passage of solutes into and out of the stroma via several metabolite translocators [4]. The molecular mechanisms of transport of small ions across the inner envelope are largely unknown. It has been shown that light-induced H^+ flux outside the chloroplast mediated by a H^+-ATPase is balanced by K^+ counter-exchange through an inner envelope K^+ channel [5]. Early studies of the swelling rate of isolated chloroplasts upon addition of valinomycin and isotope flux experiments indicated that the inner envelope is also permeable to chloride and other halides [6–10]. It has been suggested that H^+ efflux across the envelopes could be balanced in part by a passive chloride efflux [6]. These results did not provide any information about the translocation processes for anion across the membrane. More recently, chloride channels have been identified in chloroplast envelopes [5,11]. One of them has been shown to be involved in chloroplast protein import. However, their exact location and biophysical properties have not yet been characterized. A third anionic channel, located in the inner envelope, has been studied at the molecular level in our laboratory [12]. This channel has a large

* Corresponding author. Fax: +32-2-650-5382;
E-mail: fhomble@ulb.ac.be

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conductance and a poor selectivity. It is closed at negative membrane potentials suggesting that it might not be involved in Cl^- flux regulation during photosynthesis.

The study of ion transport across intracellular membranes such as chloroplast inner envelopes cannot be easily investigated at the molecular resolution provided by the patch clamp technique mainly because the small size of the organelles and the difficulty to purify large amount of membrane. Measurements of the swelling rate of the organelle by light scattering is an alternative strategy to study ion transport processes through membranes. It has been successfully used to calculate ion flux through ion channels from inner membrane of plant and animal mitochondria [13–16].

In this paper, the light scattering of intact chloroplast is used in order to characterize and quantify anion transport across the inner envelope membrane of this organelle. We measure the effect of temperature on the chloride transport and its anionic selectivity. Our results suggest that this passive anion transport through the inner envelope membrane is mediated by a specific pathway, a chloride channel.

2. Materials and methods

2.1. Isolation of intact chloroplasts

Spinach leaves were purchased from a local market. About 50 g of deribbed fresh leaves were homogenized with a Waring blender in 200 ml of grinding medium GM (0.33 M Sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, 50 mM Tricine–KOH (pH 7.8), and 0.1% bovine serum albumin). All operations were carried out at 4°C in a cold room. The mixture was filtered through eight layers of cheesecloth and one layer of cotton wool, and then were centrifuged at $2000\times g$ for 30 s (Sigma centrifuge). The pellet was collected, resuspended in 2×10 ml of GM without BSA, and layered over 2×15 ml of 40% (v/v) Percoll (Pharmacia Biotech) in GM. The two tubes were centrifuged at $2000\times g$ for 2.5 min and then brought to rest without braking (Heraeus, swinging buckets). The broken chloroplasts and contaminant organelles that formed a band above the pellet were discarded. Purified intact chloroplasts were recovered at the bottom of the tube and gently resuspended with GM. The isolation procedure was completed in 25 min. After isolation, intact chloroplasts were stored at 1 mg chl/ml on ice in the dark prior to use. Intacteness was routinely determined by the ferricyanide method [17] and varied between 95% and 99%. The intact chloroplasts demonstrated high photosynthetic rates over 100 μmol O_2(mg chl)$^{-1}$ h^{-1}. The K^+ content of intact chloroplasts determined by flame photometry (Perkin–Elmer 300) was not altered during the isolation procedure and was found to be 200 mM, a value similar to that measured in other laboratories assuming a stromal volume of 24 μl/mg chl in isotonic conditions [18].

2.2. Absorbance measurements

The absorbance measurements of intact chloroplast suspensions started 15–30 min after the isolation was complete. Absorbance changes were measured in the dark after the addition of 20 μl of intact chloroplasts (containing 1 mg chl^{-1} ml^{-1}) to 2 ml solution containing 10 mM Hepes buffer (pH 7.2) and K^+ salts to make the osmolality as indicated. Absorbance was measured at 550 nm with a Hach spectrophotometer connected to a computer with an A/D interface (Candibus, Grenoble, France). The wavelength of 550 nm was chosen because it is not absorbed by chloroplast pigments. During the experiment the content of the cuvette was continuously stirred using a magnetic stirrer. Valinomycin, from a stock solution in ethanol (9×10^{-6} M), was added prior to the injection of the chloroplast suspension. The final concentration of ethanol in the suspension (1.5%) did not affect the osmotic response of the chloroplasts. Unless otherwise indicated, the temperature in the cuvette ranged from 21°C to 23°C. The lamp for the illuminating beam did not increase the cuvette temperature more than 2°C at the end of the 25-s measurement.

2.3. Other methods

Chl content was measured in 80% acetone according to Arnon's method [17]. Protein concentration was determined using serum albumin as standard (BCA kit, Bradford). Osmolality of salt and sorbitol solutions was measured with a cryoscopic osmometer

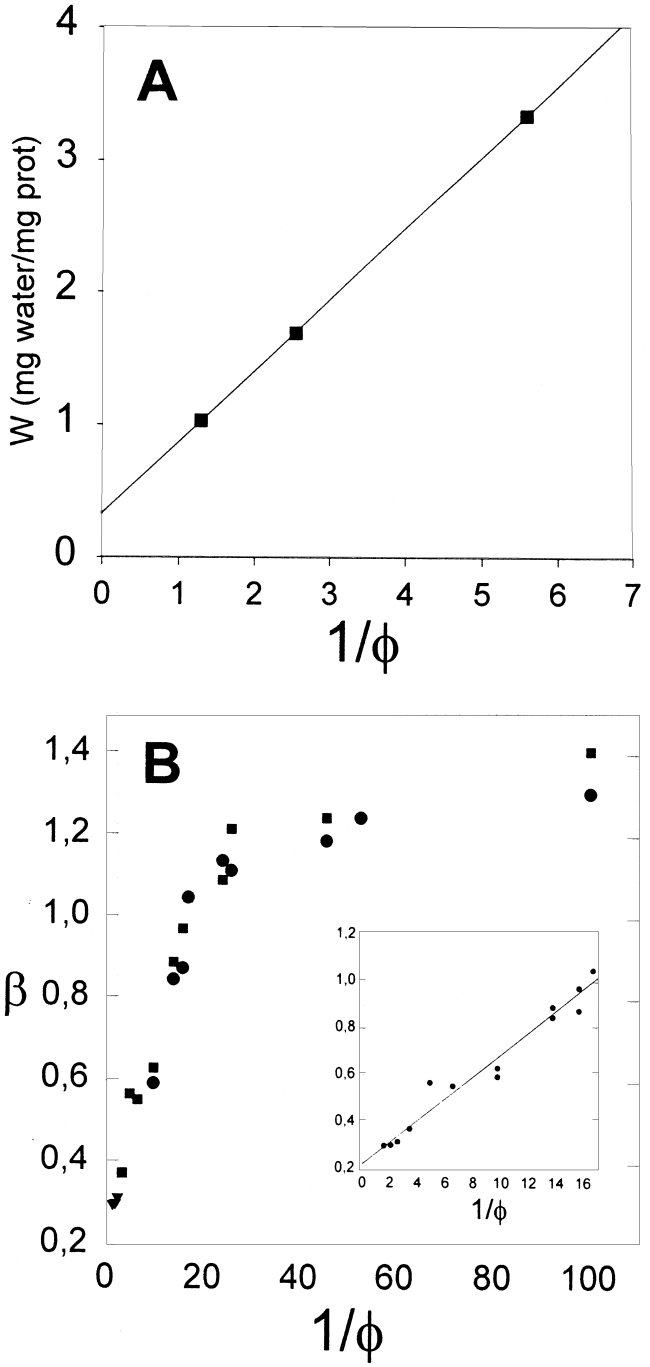

Fig. 1. (A) Relationship between the stromal water content, W (mg water per mg protein) and the reciprocal osmolality of the medium $1/\phi$ (osmol^{-1}). W values are derived and averaged from previously published stromal volumes [1,18,19]. Volumes (μl (mg chl)$^{-1}$) and concentrations (M) are converted to W and ϕ which are the proper variables for osmotic experiments. (B) The scattered light intensity β versus the reciprocal osmolality of the resuspension medium. One aliquot of an intact chloroplast suspension was added to 2 ml salt solution of various osmolalities in the measuring cuvette to make 0.14 mg protein/ml. Inset: the data below 17 osmol^{-1} are fitted by a straight light (slope = 47 mosmol; $r^2 = 0.982$).

(Roebling). Data are expressed as means ± S.E. (n = number of replicates).

3. Results

Anion transport was measured by following osmotic swelling, which arises from salt uptake, using the light-scattering technique described in detail by Beavis, Garlid and coworkers [13–16]. Their method permits quantitative estimates of solute flux through organelles. It requires the knowledge of the relationship between the light scattered by an organelle suspension and the water content of the organelle at different osmolalities. Early studies of the osmotic properties of isolated intact chloroplasts have indicated that they undergo rapid changes in volume when suspended in sorbitol, KCl or NaCl of various osmolalities because they behave as an osmometer [6,8]. Determination of the sorbitol-inaccessible space has shown that the inner envelope membrane is semipermeable [1] and confers the osmotic properties to the chloroplast. The osmotic response of the chloroplast is predicted by the Boyle van't Hoff law:

$$W = W_b + S_o \cdot (1/\Phi), \quad (1)$$

where W is the stromal water content (mg water per mg protein), W_b is the osmotically inactive water (mg water per mg protein), Φ is the medium osmolality and S_o is the stromal solute content (nosmol per mg protein).

Stromal volumes of spinach chloroplasts at various sorbitol concentrations have been previously measured by the silicone-layer centrifugation: 47.8 μl (mg chl)$^{-1}$ in 0.16 M sorbitol, 24.2 and 25 μl (mg chl)$^{-1}$ in 0.33 M sorbitol, 12.2 and 18.9 μl (mg chl)$^{-1}$ in 0.67 M sorbitol [1,18,19]. These data were converted to stromal water content (mg water per mg protein) [13] which is more relevant for our purpose because it is not dependent upon temperature. In order to plot the stromal water content as a function of the reciprocal osmolality (Fig. 1a), we have also measured the osmolality of each sorbitol solution and converted chlorophyll into protein content (protein/chl = 14 ± 1; n = 7).

In Fig. 1A, the intercept of the line with the ordinate (W_b = 0.329 mg (mg protein)$^{-1}$) yields the non-osmotic water which consists mainly of membrane and protein associated water. About 20% of the water content measured in isotonic solution is non-osmotic. The stromal solute content S_o was found to be 538 nosmol (mg protein)$^{-1}$.

Rapid changes in volume are readily monitored by the absorbance of a chloroplast suspension. The

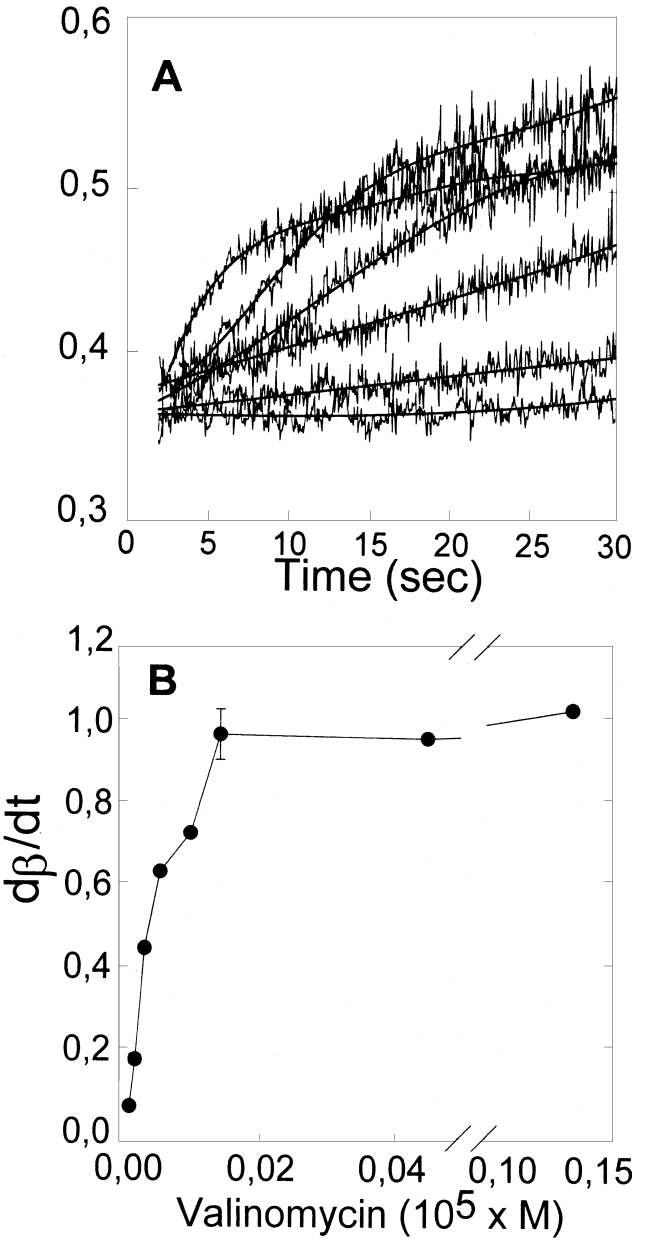

Fig. 2. (A) Light-scattering kinetics of intact chloroplasts (0.14 mg protein ml^{-1}) suspended in 292 mosmol KCl at valinomycin concentrations ranging from 9 nM to 0.45 μM. (B) Dependence of the light-scattering rate, dβ/dt (min^{-1}), on the valinomycin concentration in the KCl suspension medium.

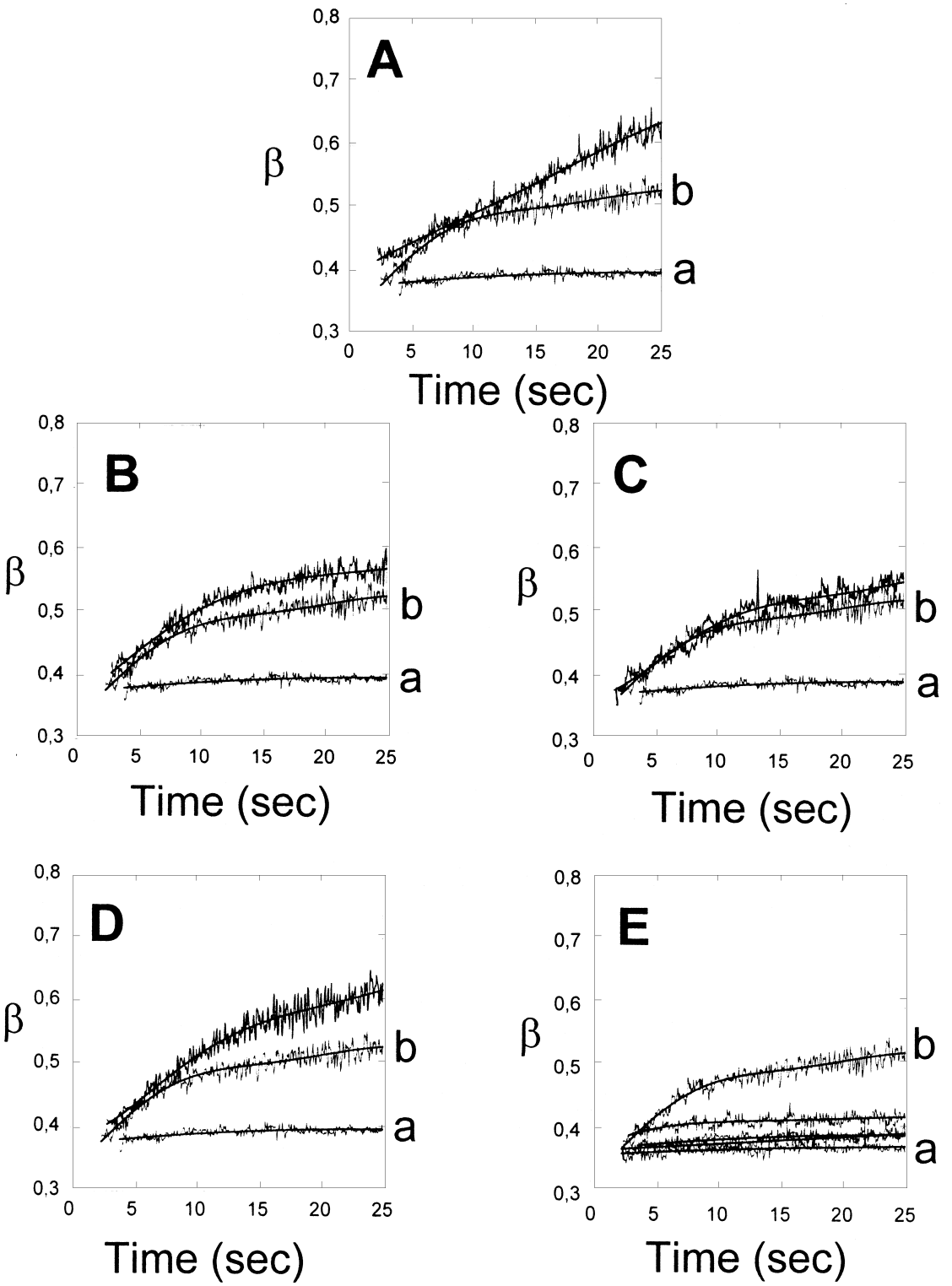

Fig. 3. (A–E) Swelling kinetics obtained with chloroplasts suspended in potassium salt of various anions. For each experiment, rates were measured in Cl^- (trace a), Cl^- and 1.4×10^{-7} M valinomycin (trace b) as a control. The anion tested was: A, I^-; B, Br^-; C, NO_2^-; D, NO_3^-; E, SO_4^{2-}, PO_4^{3-} and CH_3COO^- in the presence of 1.4×10^{-7} M valinomycin. Concentrations were chosen to make all media have the same osmolality (293 ± 5 mosmol).

swelling of intact chloroplasts in hypotonic medium is followed by a decrease in the light scattered by the suspension which yields to a decrease of absorbance (A). The osmotic adjustment of the chloroplast volume was extremely rapid (<1 s) and the absorbance of the chloroplast suspension remained constant after each osmolality change. The solute content, S_o, and $1/A$, measured at various osmolalities, can be used to quantify solute transport if $1/A$ is normalized to the protein concentration P (mg ml^{-1}) [13–16]). We found a linear relationship between $1/A$ and $1/P$ (results not shown):

$$1/A = \alpha + \beta \cdot 1/P \quad (2)$$

The constant α, an instrument parameter, equals 0.2 and does not depend of the type of suspension used. The variable β gives the light-scattering intensity of the intact chloroplast suspension and normalizes reciprocal absorbance for protein concentration as follows:

$$\beta = P/P_s(1/A - \alpha) \quad (3)$$

where $P_s = 1$ mg/ml to make β dimensionless [13–16]. Our values of $P = 0.14$ mg/ml and $\alpha = 0.2$ have been used to calculate β during all the study. The mean value of β in 292 mosmol KCl ($1/\Phi = 3.42$ osmol^{-1}) was found to be 0.371 ± 0.011 ($n = 14$) with seven different chloroplast purifications. Fig. 1B shows the osmotic curve obtained when intact chloroplasts were resuspended in KCl solutions of various osmolalities. At low osmolality, the deviation from linearity corresponds to excessive osmotic swelling and bursting of chloroplasts. The slope of the linear part of the curve before the osmotic breakage (see inset of Fig. 1B) can be fitted with the equation:

$$\beta = \beta_o + b \cdot 1/\Phi \quad (4)$$

The slope, b, equals 47 mosmol and is proportional to the solute content S_o of the chloroplast. Since β and W increase linearly as a function of $1/\Phi$ then we can relate these two parameters by combining Eq. 1 with Eq. 4:

$$\beta = \beta_o + (b \cdot (W - W_b))/S_o \quad (5)$$

Using Eq. 5 we can directly relate the light-scattering intensity (given by β) to the water content of the organelle (W), the other parameters being constant.

In order to quantify the ion flux through the inner envelope membrane, the light-scattering kinetic must be recorded. When intact chloroplasts are resuspended in 292 mosmol KCl, there was no change in chloroplast osmotic volume with time (Fig. 2A, lower trace). However, addition of the K^+-ionophore valinomycin induces time-dependent swelling of the chloroplasts. Because of electrical neutrality, K^+ uptake mediated by valinomycin will be accompanied by a Cl^- influx if a pathway for chloride transport exists in the membrane. The increase in internal concentration of KCl will cause an osmotic equivalent of water to enter the chloroplast, resulting in chloroplast swelling. In Fig. 2A, the increase of the light scattering due to salt uptake is shown at different concentrations of valinomycin ranging from 9 nM to 0.45 μM. Since chloroplasts are in extremely rapid osmotic equilibrium then the rate of water movement and swelling $d\beta/dt$ (min^{-1}) are limited by the rate of ion transport. The rate of salt uptake, J_i (mol min^{-1} (mg protein)$^{-1}$) is proportional to the initial increase of light scattering $d\beta/dt$ according to the equation [13–16]:

$$J_i = (\Phi \cdot S_o/n \cdot b) \cdot d\beta/dt \quad (6)$$

where n is the number of osmotically active ions.

With sufficient K^+ permeability induced by valinomycin, the rate of swelling would be limited only by the influx of Cl^-. The dose–response curve (Fig. 2B) obtained from the initial slope of Fig. 2A indicates that above 1.4×10^{-7} M the flux of K^+ is no longer a limiting factor for the swelling. Thus above this concentration of valinomycin, the Cl^- influx is the only rate limiting factor controlling the rate of chloroplast swelling. The maximal swelling rate, $d\beta/dt$, measured

Table 1
Selectivity of the anion transport

	J_i (μmol min^{-1} (mg protein)$^{-1}$)	J_i/J_{Cl^-} (%)
Cl^-	1.64 ± 0.10	100
NO_3^-	1.48 ± 0.04	90
NO_2^-	1.45 ± 0.23	88
Br^-	1.31 ± 0.03	80
I^-	1.01 ± 0.07	62
SO_4^{2-}	0.13 ± 0.02	8
PO_4^{3-}	0.08 ± 0.02	5
CH_3COO^-	0.06 ± 0.01	4

The flux of each anion is calculated from the initial rate of light scattering, $d\beta/dt$, in the presence of 1.4×10^{-7} M valinomycin ($n = 3$).

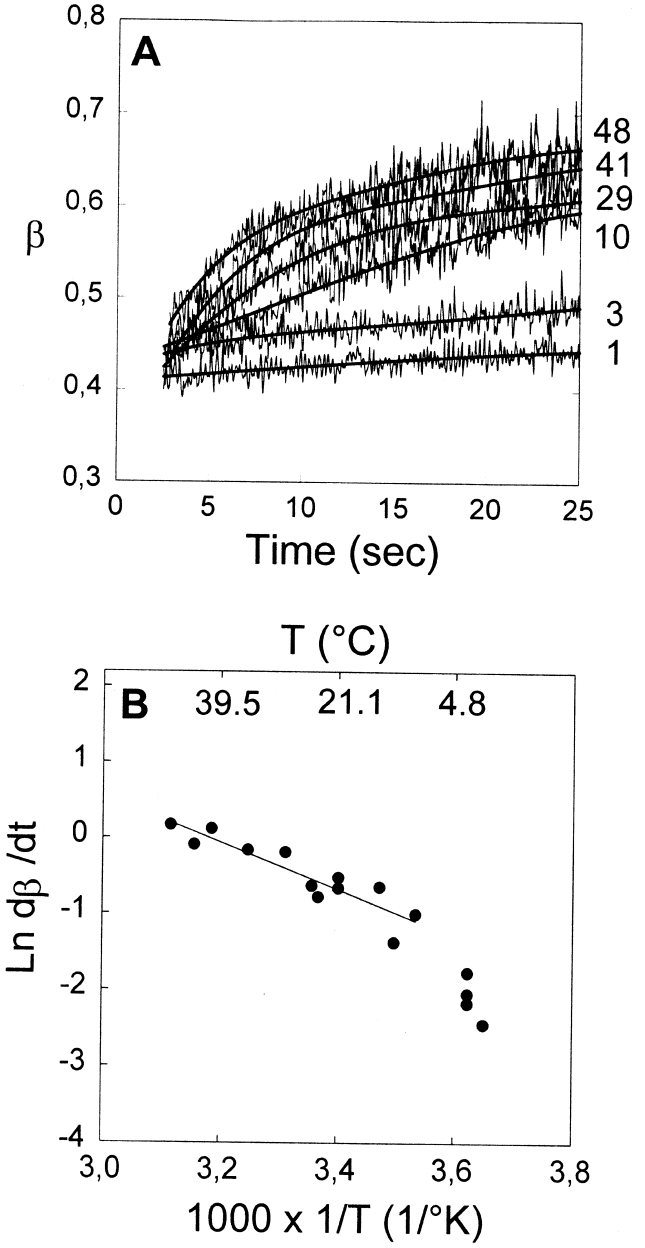

Fig. 4. (A) Light-scattering kinetics of intact chloroplasts in 292 mosmol KCl containing valinomycin (1.4 μM) at temperatures ranging from 1°C to 48°C. (B) Arrhenius plot for light-scattering rates $d\beta/dt$ (min^{-1}) derived from the traces shown in A.

at 1.4×10^{-7} M valinomycin, was 0.98 ± 0.06 min^{-1} ($n=9$) in 292 mosmol KCl. This will correspond to a chloride flux J_{Cl} of 1.64 μmol min^{-1} (mg protein)$^{-1}$ across the inner envelope membrane.

To investigate the selectivity of this chloride transport, intact chloroplasts were resuspended in media of the same osmolality (293 ± 5 mosmol; $n=9$) in which Cl^- had been substituted with I^-, $Br-$, NO_2^-, NO_3^-, SO_4^{2-}, PO_4^{3-} and CH_3COO^-. Then, we measured the rate of increase in light scattering, $d\beta/dt$, in the presence and in the absence of valinomycin (Fig. 3A–E). The fluxes of each anion normalized with the chloride flux are reported in Table 1.

Treatment of the chloroplast suspension at 4°C for duration from 10 to 120 min. with 1 mM DIDS, a classical anion transport inhibitor, or 1 mM NEM which can inhibit transport proteins by reacting with thiol groups did not inhibit the valinomycin-induced swelling of chloroplasts (not shown).

The rate of swelling measured with the addition of a high valinomycin concentration (1.4 μM) showed a marked temperature dependence and was inhibited by low temperatures (Fig. 4A). The Arrhenius plot is linear between 10°C and 48°C (Fig. 4B) and permits calculation of an activation energy required for the transport of Cl^- of about 26 kJ/mol.

4. Discussion

The light-scattering technique has been used to investigate the properties of anion transport through the inner envelope membrane.

The coincident increase of water content in intact chloroplasts (Fig. 1A) and the light-scattering parameter β (Fig. 1B) with decreasing osmolalities of the medium was used to convert the rate of swelling $d\beta/dt$ into anion fluxes. Our results show that in the presence of valinomycin ($\geq 1.4\times10^{-7}$ M), the rate of swelling is limited by the influx of anion through the inner envelope (Fig. 2).

The rates of swelling measured in the presence of various anions (Fig. 3) have permitted calculation of the selectivity of the anion transport (Table 1). The selectivity sequence is consistent with the idea that the inner envelope contains a transport protein selective to Cl^-. In the 1970s, Heber and coworkers also demonstrated that the anion transport is selective [6–9]. They reported a selectivity sequence which differs with that found in our work, since their data followed a Hofmeister power series where the diameter of the hydrated species determines the diffusion rate. However, these authors did not use initial swelling rates for selectivity estimates which preclude any quantitative analysis of the data. More-

over, their chloroplast samples contained a large amount of broken chloroplasts, making the comparison with our work difficult. Indeed, thylakoid membranes released in solution may have produced significant light scattering changes due to their swelling in the presence of valinomycin.

As expected, the selectivity sequence reported in Table 1 confirms that unidirectional transport of PO_4^{3-} and SO_4^{2-} do not occur through the Cl^- pathway. Indeed, phosphate is carried by the phosphate translocator which catalyzes counter-exchange with phosphate esters and SO_4^{2-} probably also uses the same transporter [4,20]. At high anion concentration ($\geq$10 mM), the phosphate translocator behaves like an anion channel which is inhibited by DIDS [21]. The poor selectivity of the Cl^- transport for PO_4^{3-} and SO_4^{2-} and the absence of effect of DIDS on the Cl^- influx (not shown) suggest that the chloride influx occurs through a distinct membrane protein and that the uniport property of the phosphate translocator is inhibited in our experimental conditions.

It has been suggested that NO_2^- could be transported across the inner envelope by means of a NO_2^- carrier [22]. The uptake of NO_2^- is inhibited by NEM whereas the chloride transport reported here is not. However, this does not mean that two different NO_2^- pathways exist in the envelope. Indeed, most of the evidence for a NO_2^- carrier rests on the effect of NEM on the NO_2^- uptake during photosynthesis [22]. As NEM could affect more than one physiological process in these experiments, any comparison must be made with great caution.

The chloride flux calculated in 292 mosmol KCl is equivalent to $J_{Cl} = 8 \times 10^{-10}$ mol/cm^2 per second. Considering an inner envelope membrane area of 50 μm^2 per chloroplast [9], this value corresponds to a permeability coefficient of about 10^{-6} cm s^{-1} in a large salt gradient. Thus, the chloride flux through the inner envelope reported here does not occur via an unspecific lipid leak since the permeability coefficient of glycolipids for chloride is 2.3×10^{-11} cm s^{-1} [23]. Furthermore, the activation energy of this chloride passive transport (Fig. 4) suggests that a channel is involved in this process. Indeed, its value of 26 kJ mol^{-1} is lower than that measured for anion carriers [21] and is similar to that reported for ion channels [21,24]. The effect of temperature can be ascribed to the chloride flux and not to the diffusion of valinomycin or lipid matrix phase transition, because the valinomycin is in large excess and the temperature considered here is well above the critical temperature for the envelope lipid phase transition [25]. In view of the evolutionary and functional relationship between mitochondria and chloroplasts, it is interesting to point out that mitochondria also contain an inner membrane anion channel [26]. This channel is believed to be involved in mitochondrial volume homeostasis. It has an activation energy of 21 kJ mol^{-1} around 30°C which is similar to the value reported in this paper.

In conclusion, the light-scattering technique is a useful tool to measure quantitatively the rates of an anion transport across the chloroplast inner envelope membrane. Our results suggest that an anion pathway highly selective for Cl^-, NO_3^- and NO_2^- over PO_4^{3-} and SO_4^{2-} is located in this membrane and provides information about the maximum anion flux across the chloroplast envelope.

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