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Regulation of the Anion Channel of the Chloroplast Envelope From Spinach

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Several anions such as Cl^-, NO_2^-, SO_4^{2-}, and PO_4^{3-} are known to modulate the photosynthetic activity. Moreover, the chloroplast metabolism requires the exchange of both inorganic and organic (e.g., triose phosphate, dicarboxylic acid, ATP) anions between the cytoplasm and the stroma. A chloride channel form the chloroplast envelope was reconstituted in planar lipid bilayers. We show that the channel is active in conditions prevailing in the plant. The open probability increases with the ionic strength of the experimental solutions and is maximal at 0 mV. This suggests that the channel could play a role in the osmotic regulation of the chloroplast. Amino group reagents affect the channel activity in a way that demonstrated that lysine residues are important for channel gating but not for ATP binding. Together, our results provide new information on the functioning of this channel in the chloroplast envelope membranes. They indicate that the open probability of the channel is low ($P_o \leq 0.2$) in vivo and that this channel can account for the chloride flux through the chloroplast envelope.

KEY WORDS: Chloride; ion channel; membrane; succinylation.

INTRODUCTION

Photosynthesis of green plants occurs in chloroplasts. A double membrane envelope that controls the exchange of solutes and water between the cytoplasm and the stroma surrounds each chloroplast. Several anions such as NO_2^-, SO_4^{2-}, Cl^-, and HPO_4^{2-} are known to modulate the photosynthetic activity. Moreover, the chloroplast metabolism requires the exchange of both inorganic and organic (e.g., triose phosphate, dicarboxylic acid, ATP) anions between the cytoplasm and the stroma.

Carriers or channels can mediate anion transport through the chloroplast envelope. Several carriers have been characterized in intact chloroplasts or purified envelope vesicles. The triose phosphate translocator and the 2-oxoglutarate/malate translocator have been characterized at the molecular level (Flügge, 1999; Flügge and Heldt, 1991). Previous results indicate that different anion channels can be found in the chloroplast envelope. A putative channel selective to Cl^- and NO_2^- was inferred from the light-scattering induced by the osmotic swelling of intact chloroplasts in response to a salt uptake (Fuks and Homblé, 1999). Patch clamping intact chloroplasts from *Nitellopsis obtusa* has allowed to identify a 160 pS (in 100 mM KCl) anion selective channel (Pottosin, 1992) and a high conductance channel with electric properties similar to the mitochondrial VDAC (Pottosin, 1993). A 60 pS (in 100 mM Tris/HCl) channel was observed in giant proteoliposomes formed from the whole chloroplast envelope (Heiber *et al.*, 1995). The characterization of voltage-dependent porin-like channels was achieved upon reconstitution of either inner or outer envelope membrane into planar lipid bilayers (Flügge and Benz, 1984; Fuks and Homblé, 1995; Heiber *et al.*, 1995).

Most of the chloroplast proteins are encoded in the nuclear genome. They are synthesized as precursors in the

Key to abbreviations: AS, succinic anhydride; DMSO, dimethylsulfoxide; OEP, outer envelope protein; TNBS, trinitrobenzene sulfonate.

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cytoplasm and then imported into the chloroplast. Each membrane of the chloroplast envelope has its own protein import machinery. An anion channel involved in the protein import machinery has been identified in both inner and outer membranes of the chloroplast envelope (Hinnah *et al.*, 1997; van den Wijngaard *et al.*, 1999; van den Wijngaard and Vredenberg, 1997, 1999). The channel is formed by Toc75 (145 pS in 150 mM KCl) and Tic110 (50 pS in 250 mM KCl) in the outer and inner membranes, respectively. Both proteins are thought to form the translocation pathway for precursor proteins.

Chloroplast envelope membranes cannot be purified on a large scale. This restricts the usefulness of the classical biochemical approach to purify ion channels from chloroplast envelopes. Recently, a proteomic approach has been preferred to identify new proteins with a putative solute transport feature in the envelope membrane (Seigneurin-Berny *et al.*, 1999).

Three channels have been characterized at the molecular level in the outer envelope membrane of pea chloroplasts. The outer envelope protein OEP16 is a channel selective to amino acids and amines (Pohlmeyer *et al.*, 1997). The OEP24 is a channel with properties similar to porins (Pohlmeyer *et al.*, 1998). Finally, Bolter *et al.* (1999) have cloned a cDNA encoding a 21-kDa protein (OEP21) which forms an anion-selective channel when reconstituted in planar lipid bilayers. The channel properties of the recombinant OEP21 were studied at salt concentrations (1 M NaCl) that are much higher than the in vivo concentrations. As it has been claimed previously that carriers, like the phosphate translocator of the inner membrane, can switch to channels at high salt concentrations (Schwartz *et al.*, 1994), further experiments are required to highlight the function of OEP21 in physiological conditions.

In the present work, an anion-selective channel from the spinach chloroplast envelope has been reconstituted in planar lipid bilayers. Our results indicate that the channel can be activated at salt concentration similar to that found in vivo. Moreover, we show that both ionic strength and positively charged residues regulate the gating process.

EXPERIMENTAL PROCEDURES

Purification of Chloroplast Envelope Membranes

All steps of the purification were carried out at 0–5°C. Crude chloroplasts were obtained from 3 to 4 kg spinach (*Spinacia oleracea* L.) leaves and purified by isopycnic centrifugation using Percoll gradients as described by Douce and Joyard (1982). At this step of purification, protease inhibitors (1 mM PMSF, 1 mM benzamidine, and 0.5 mM amino caproic acid) were added to prevent any protein degradation. Purified intact chloroplasts were lysed in hypotonic medium, and envelope membranes were purified from the lysate by centrifugation on sucrose gradients (Douce and Joyard, 1982). Envelope membranes were stored (in liquid nitrogen) in 50 mM MOPS-NaOH, pH 7.8, in the presence of protease inhibitors (1 mM benzamidine, 1 mM amino caproic acid) and 1 mM DTT.

Solubilization of Membrane Proteins

Protease inhibitors (1 mM benzamidine, 1 mM amino caproic acid) and 1 mM DTT were added to all solutions. An aliquot of envelope membranes corresponding to 8 mg of protein was washed in 150 mM KCl, 25 mM tricine-KOH (pH 7), and centrifuged for 1 h at 125,000 $\times$ g, 4°C. The pellet was resuspended in 3 mL of the same solution containing 1% (v/v) Genapol X-080. The mixture was incubated for 1 h at 4°C under gentle stirring. Solubilized proteins were recovered by centrifugation for 1 h at 125,000 $\times$ g. The pellet, containing the insoluble proteins, was discarded. Then, 750 μL of solubilized proteins were mixed with 260 mg sucrose, 250 μL saline solution (150 mM KCl, 25 mM tricine-KOH, pH 7) and 250 mg dry HTP Biogel (BioRad). This mixture was incubated for 30 min at 4°C, under continuous stirring. At the end of the incubation, HTP was pelleted for 5 min at 12,000 $\times$ g and the supernatant was collected and used for channel reconstitution in planar lipid bilayers.

Electrophysiological Measurements

Planar lipid bilayers were formed, at room temperature, from 1% w/v Diphytanoylphosphatidylcholine (Avanti Polar Lipids, AL) dissolved in n-decane, by using the Mueller–Rudin technique (Fuks and Homblé, 1995). Lipids were painted over a 300-μm hole drilled in a styrene copolymer partition separating two chambers. Each chamber was connected to the recording circuit through Ag/AgCl electrodes and a bridge of 1 M KCl, 2% agar. The cis chamber was connected to the headstage input of a BLM-120 amplifier (BioLogic, France) whereas the trans chamber was held at ground. Electrical potential differences were defined as cis with respect to trans.

The currents were sampled and digitized at 1 kHz, and filtered at a cut-off frequency of 300 Hz (five pole Tchebichev filter). Data acquisition and analysis were

performed using the PAT V7.0 program (courtesy of J. Dempster, University of Strathclyde, Strathclyde, UK). The amplitude of the single-channel current was determined from total amplitude histograms fitted with Gaussian functions. The open probability (P_o) of the fully open state was estimated from the fraction of time spent in the fully open state during voltage pulse lasting 50 s.

Reconstitution of a channel into a planar lipid bilayer was achieved by addition of an aliquot of the protein sample to the trans side of the bilayer under continuous stirring. Experiments containing only one channel were used for the analysis. All solutions of triple distilled water were 0.2 μm millipore filtered and they were buffered with 10 mM Hepes at pH 7.5.

Chemical Modification

An aliquot of succinic anhydride dissolved in DMSO (200-mM stock solution) was added on both side of the membrane to get a final concentration of 800 μM. The solution of each compartment was then vigorously stirred during 1 min. TNBS was solubilized in distilled water (50-mM stock solution).

RESULTS

Because several channels coexist in the outer envelope membrane, different channels can be reconstituted

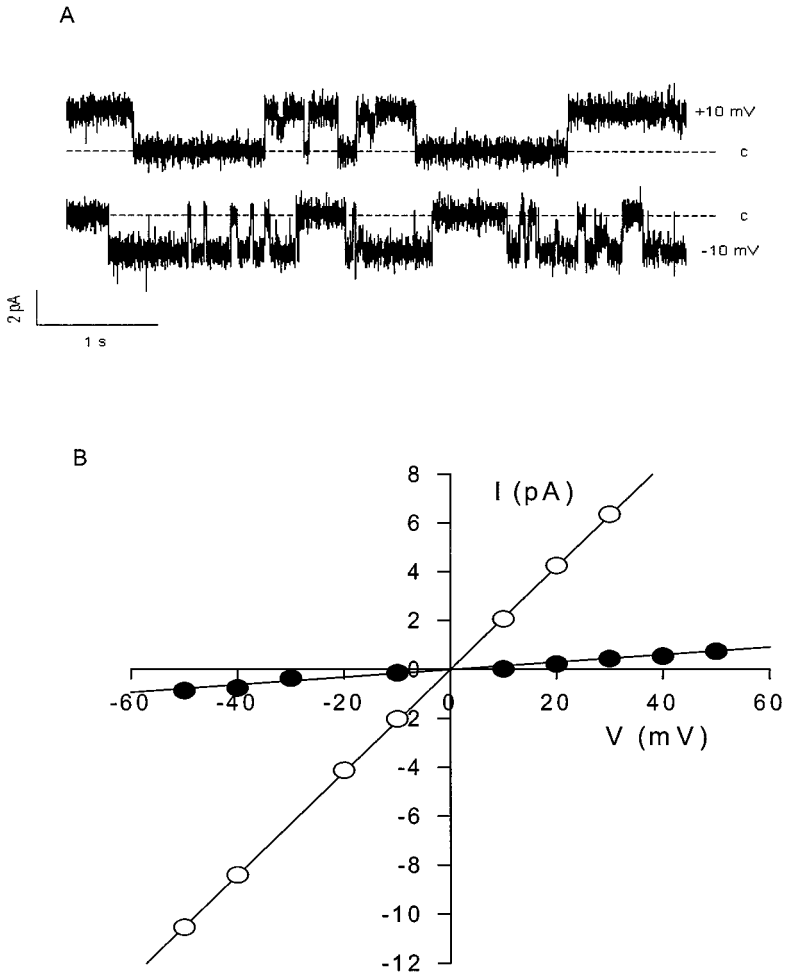

Fig. 1. Single channel recordings. (A) Continuous current recordings of a single channel. The holding voltage is indicated on the right of each trace. The dashed line indicates the closed state. (B) Current–voltage relationship of the two main conducting states. Data were fitted by a linear regression with $r^2 > 0.99$. The planar lipid bilayer was formed from diphytanoylPC in symmetrical conditions (150 mM KCl, 10 mM Hepes, pH 7.5).

simultaneously in a planar lipid bilayer when native membrane fractions are used. Here, the envelope proteins from spinach chloroplasts were solubilized in Genapol X-080 and equilibrated with HTP (see Materials and Methods Section). The recovered fraction was added to the trans side of a planar lipid bilayer and only one kind of channel was detected. All the results reported hereafter concern single-channel experiments.

The Channel is Active at Physiological Salt Concentrations

We first measured the dependence of the single-channel conductance on salt concentration in symmetrical solutions of KCl, pH 7.5. Figure 1(A) shows single-channel current fluctuations of the channel reconstituted in a planar lipid bilayer formed from diphytanoylphosphatidylcholine in 150 mM KCl. Applied voltages are indicated on the left of each trace. Channel activity was detected at both positive and negative voltages. Beside the fully open state, several substates were detected at all voltages tested. In symmetrical 150 mM KCl, two main conductance levels were reproducibly resolved, 222 $\pm$ 3 pS ($n = 15$) and 28 $\pm$ 2 pS ($n = 15$). But experiments done in symmetrical 1 M KCl to increase the signal resolution indicated that at least four substates of conductance could exist. The current–voltage curve of the two main conductance levels recorded in symmetrical 150 mM KCl was linear in the voltage range tested (Fig. 1(B)). Recordings were limited to voltages lower than $\pm$60 mV because the open probability was close to zero outside this voltage range. The single-channel conductance of the fully open state was measured in symmetrical KCl concentrations ranging from 150 mM to 1 M (Fig. 2). The current–voltage curves corresponding to salt concentrations of 1 M, 650 mM, 300 mM, and 150 mM are fitted by a linear relationship (Fig. 2(A)). Single-channel conductances of 908 $\pm$ 16 pS ($n = 7$), 698 $\pm$ 20 pS ($n = 7$), 375 $\pm$ 11 pS ($n = 12$), and 222 $\pm$ 3 pS ($n = 15$), respectively, were calculated from the slope of the regression lines. The relationship between the fully open conductance and the activity is sublinear (Fig. 2(B)). No saturation of the conductance was observed indicating the presence of a binding site with a weak apparent affinity for anions ($K_m = 1.5$ M).

Selectivity

To investigate the selectivity, a triangular voltage wave ($\pm$50 smV, 10 mHz) was applied to the planar lipid bilayer. This permits to get simultaneously the single-channel conductance and its selectivity. As a control, the current–voltage relationship of the open channel was determined in a symmetrical 150 mM KCl solution. Then, an aliquot of concentrated KCl was added to the cis compartment to increase its concentration and the current–voltage curve was measured in asymmetrical conditions. Figure 3 shows the current flowing through the fully open state of a channel reconstituted in a diphytanoylphosphatidylcholine bilayer membrane

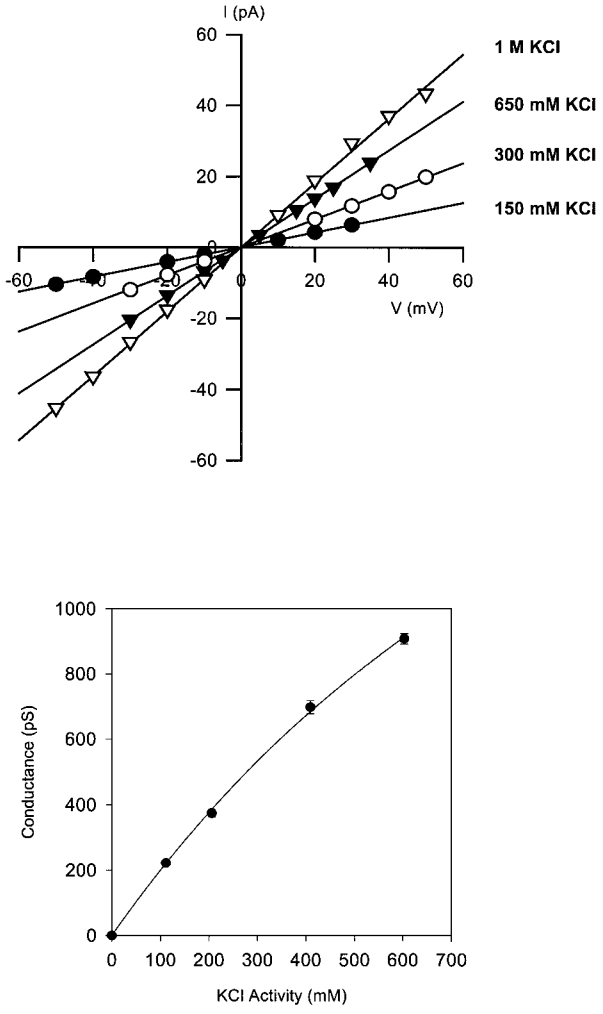

Fig. 2. Effect of the salt activity on the conductance of the fully open state. (A) Current–voltage relationship of the fully open state. Data were recorded in KCl activity of 111.6 mM, 206.4 mM, 410.5 mM, and 604.0 mM corresponding to a concentration of 150 mM, 300 mM, 650 mM, and 1 M respectively, on both sides of the bilayer. Data were fitted by a linear regression with $r^2 > 0.99$. (B) Single-channel conductance–activity relationship in symmetrical KCl solutions. The data were fitted by a Michaelis–Menten equation $\gamma = \gamma_{max}[S]/(K_m + [S])$ where γ is the single-channel conductance, γ_{max} is the maximal conductance, $[S]$ is the activity of KCl and K_m is the Michaelis constant. For some values, the error bars are smaller than the data symbols. The dashed line corresponds to the slope at the origin.

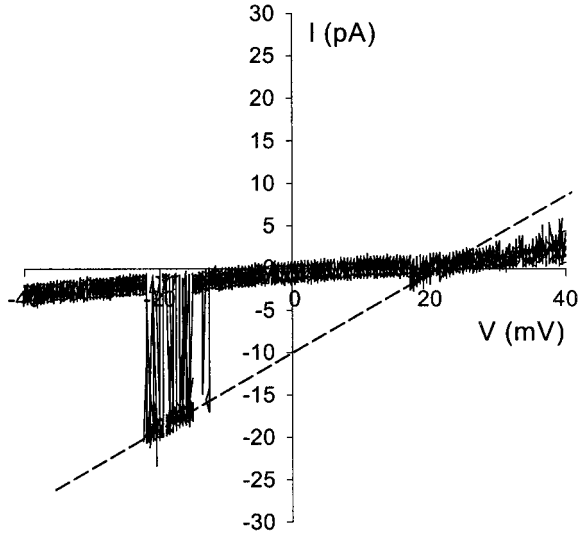

Fig. 3. Single-channel current–voltage relationship recorded in asymmetrical KCll solution: 540/150 mM (cis/trans). A 10 mHz triangular voltage wave of ±40 mV was applied to the planar lipid bilayer. The dashed line indicates the current–voltage relationship of the fully open state. Its reversal potential is +20 mV. Data were filtered at 60 Hz and sampled at 125 Hz.

in the presence of a 540/150 mM (cis/trans) KCl concentration gradient. Under these experimental conditions, anion currents reverse at positive voltages and cation currents reverse at negative voltages. The conductance of the fully open state is about 500 pS in the presence of this KCl gradient and it has an extrapolated reversal potential of +20 mV. The selectivity calculated using the Goldman–Hodgkin–Katz equation gives a permeability ratio $P_{Cl}/P_K = 4.8 \pm 0.1 (n = 9)$. The low conducting state has a reversal potential close to 0 mV indicating that this substate has no ion selectivity.

Effect of Ionic Strength

The open probability, P_o, displayed a symmetrical bell-shaped voltage-dependence centered on 0 mV. Average P_o was close to zero at electrical potential differences larger than ±30 mV. The magnitude of P_o was found to depend on the KCl concentrations (Fig. 4). To get the maximal value of P_o the data of Fig. 4(A) were fitted with a Gaussian function. As shown in Fig. 4(B), there is a linear relationship between the maximal value of P_o and the KCl activity.

Chemical Modification

Above pH 7.0, succinic anhydride reacts with positively charged amino group and forms a carboxylic group

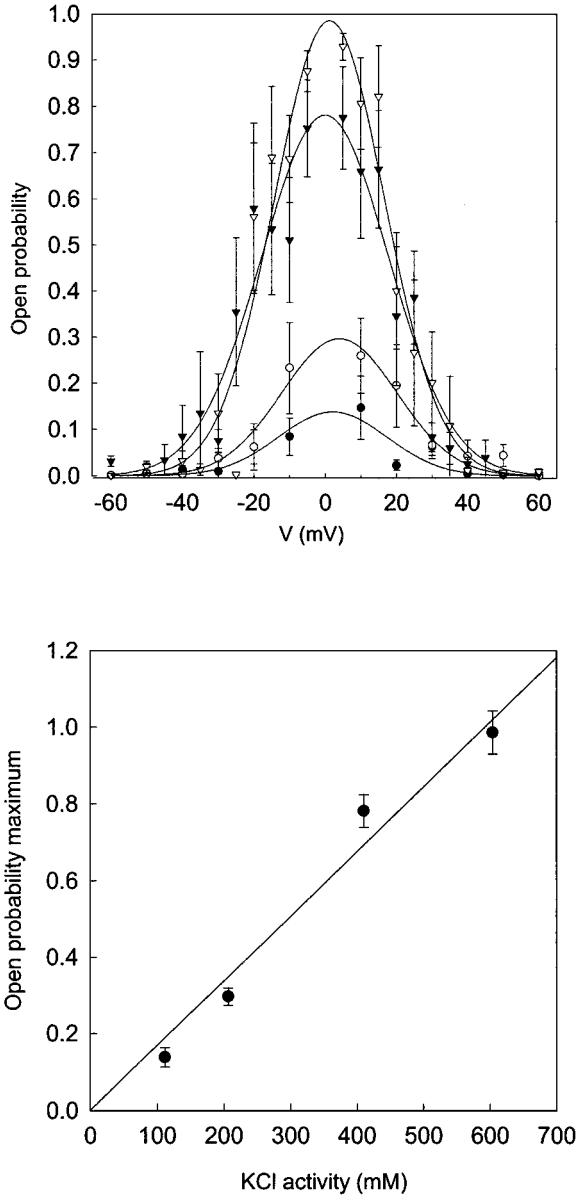

Fig. 4. Effect of salt activity on the open probability. (A) Open probability of the fully open state at various voltages recorded at different KCl concentrations (● 150 mM; ○, 300 mM, ▲ 650 mM, ∇ 1 M). The mean data were fitted by a Gaussian function. (B) Corresponding relationship between the maximum of the open probability curve and the salt activity. Data were fitted by a linear regression with $r^2 > 0.98$.

(Lundblad, 1991). Therefore, the succinylation reaction provides a way to probe the role of positively charged residues on the functional properties of the channel. As shown in Fig. 5, in the presence of succinic anhydride on both side of the membrane, the channel spent more time in its open state and the conductance of the fully open state decreased from 223.0 ± 8.7 to 119.0 ± 15.0 pS. Moreover,

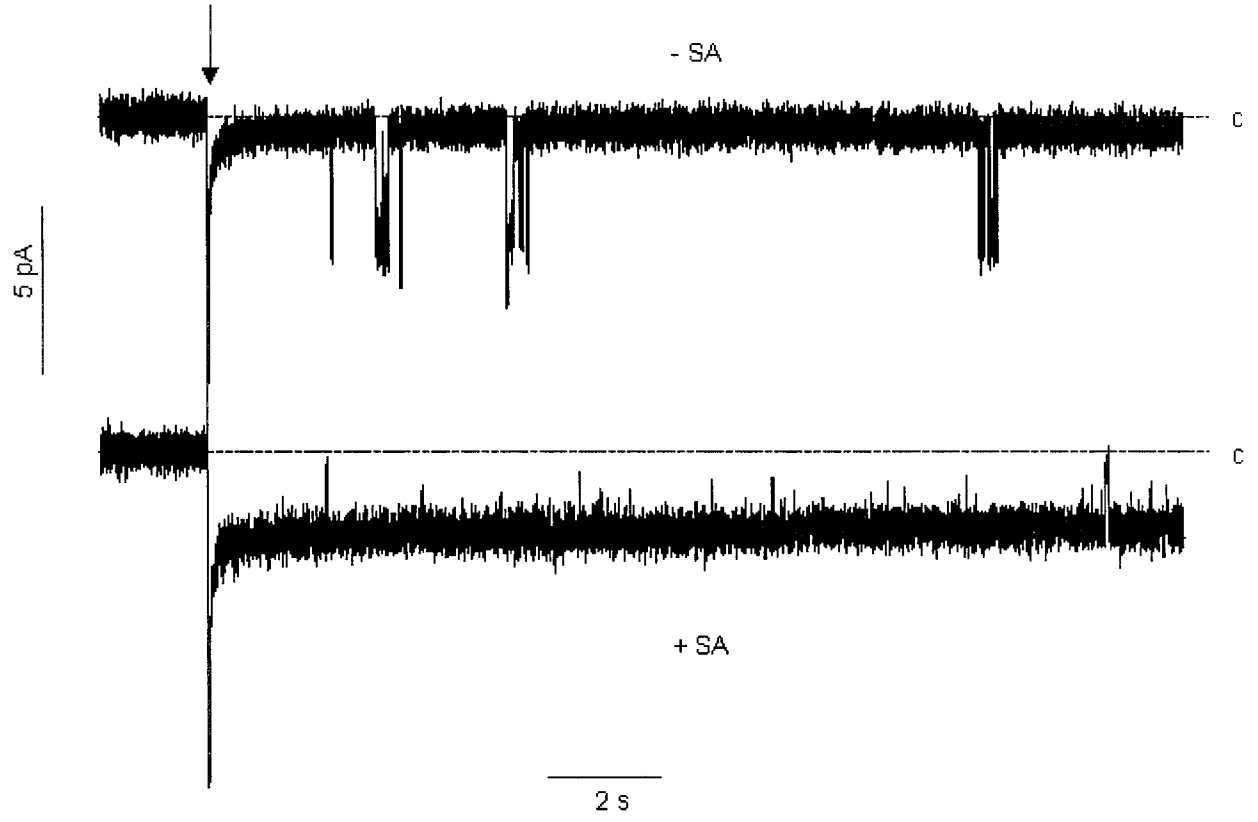

Fig. 5. Effect of succinic anhydride on the channel activity. Single channel currents recorded in 150 mM KCl in the absence and in the presence of 0.8 mM succinic anhydride on both side of the membrane. The arrow marks the time when the voltage is shifted from 0 to −20 mV. The dashed line indicates the closed state (c).

the succinylated anion channel lost its voltage-dependence (Fig. 6(A)). The selectivity of the anion channel was not affected after succinylation ($P_{Cl}/P_K = 5.0 \pm 0.2$ ($n = 4$)). Similar results were obtained in the presence of 1mM TNBS, a reagent that converts positively charged residues into neutral residues (Fig. 6(B)). In the presence of ATP the channel conductance decreased at negative voltages (Fig. 7(A)). This regulation is similar to that described for the recombinant OEP21 channel from pea (Bolter *et al.*, 1999). Moreover, fast flickering between open and closed states occurred in the presence of ATP. Addition of ATP to succinylated channel gave similar results (Fig. 7(B)).

DISCUSSION

The transport of inorganic anions into the chloroplast is essential for the plant metabolism. Some anions such as nitrite, sulfate and phosphate are metabolized in the chloroplast whereas others, like chloride, are essential for photosynthesis. In fact, Cl^- is known to bind to the photosystem II where it is required for the water oxidizing cycle. The Cl^- anion is also known to balance the light-driven H^+ flux through the thylakoid membrane with a stoechiometry Cl^-/H^+ of 0.5 (Hind *et al.*, 1974; Schonknecht *et al.*, 1992). Anion channels exist in both outer and inner chloroplast envelope. A cDNA encoding an anion channel from the outer envelope membrane has been cloned from pea (Bolter *et al.*, 1999). Our results suggest that this channel also exists in spinach chloroplasts. As a matter of fact, the single channel conductance, the selectivity, the bell-shaped voltage-dependence, the existence of several subconducting states and the ATP regulation indicate that the channel studied in our work is identical to the pea channel. However, our results provide a series of new information concerning the functioning of this channel. Up to now, there was no evidence indicating that this protein could work "in vivo" as a channel since the recombinant protein was inactive in physiological conditions. Here, we describe a simple procedure to reconstitute the spinach anion channel in a planar lipid bilayer reproducibly. Under these conditions, the envelope channel was active as single channel. We also demonstrated that the channel is active under physiological conditions prevailing within the plant

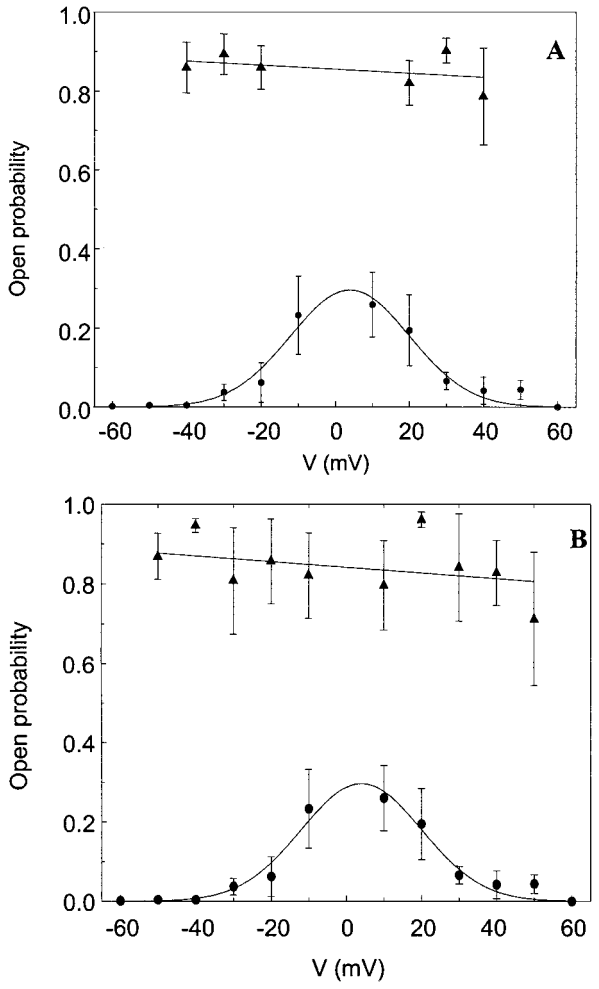

Fig. 6. Effect of the chemical modification of positively charged residues on the open probability of the channel. The experiments were carried on A) in the presence (▲) or in the absence (●) of succinic anhydride or B) in the presence (▲) or in the absence (●) of TNBS.

mesophyll cells and we were able to study in more details the regulation of the gating process.

The double envelope membrane of the chloroplast must control the anion exchange between the cytoplasm and the chloroplast. Till now, it has been generally assumed that the inner envelope membrane is the main selective barrier for solute translocation and that the outer envelope membrane is freely permeable. This statement is mainly based on the identification of specific carriers in the inner envelope membrane whereas an unselective large conductance (7 nS in 1 M KCl) allowing the diffusion of molecules with a molecular weight up to 10 kDa was measured in the outer envelope membrane (Flügge and Benz, 1984). Recently, a 950 pS (in 1 M KCl) anion selective channel was found in the outer envelope membrane of pea chloroplast (Bolter *et al.*, 1999). The coexistence of selective and unselective channels in the outer envelope membrane is not consistent with the concept of unselective free diffusion associated to this membrane. Rather, it suggests that the diffusion of solute through the outer envelope membrane is regulated. Our results are consistent with this hypothesis. It has been shown previously that the anion selective channel activity belongs to the OEP21 protein (Bolter *et al.*, 1999). Experiments done with the OEP21 heterologously expressed in *E. coli* indicated that the channel activity could only be observed at concentrated salt solutions (>250 mM). This raises the question of the role of this protein in vivo because the salt concentration in both cytoplasm and chloroplast is significantly lower than that used in these experiments. Moreover, as suggested by Schwarz *et al.* (1994), at high ionic strength, membrane proteins like carriers could behave like channels. Therefore, further experiments using physiological ionic concentrations were required to elucidate the function of the anion selective channel of the outer envelope membrane. Our results indicate that the channel is still active at physiological concentrations and that its activation increases with the ionic strength of the solution. This suggests that the channel could be involved in osmoregulation of the chloroplast. In response to a water stress, there is an osmotic adjustment of plant cells consisting in an increase of solute concentration. Organic compounds such as proline, sugar, sorbitol and glycine betaine, which do not interfere with enzyme functions, are the main solutes accumulated in the cytosol. In well-watered plants, the osmotic potential, ψ_s, is about -1 MPa and this value decreases to about -2 MPa in moderately water-stressed crop plants. As the chloroplast envelope has a low modulus of elasticity (Nobel, 1969) and chloroplasts behave like a near perfect osmometer (Kaiser *et al.*, 1981), chloroplasts dehydrate when ψ_s decreases which inhibits photosynthesis (Berkowitz and Gibbs, 1983a,b; Teraza *et al.*, 1999). Therefore, activation of ion channels could permit an osmotic adjustment between the chloroplast stroma and the cytosol that would reduce inhibitory effects associated with an increase of the stroma solute concentration.

Since the channel is anion selective, positively charged residues could play a significant role on the functional properties of the channel. We used chemical reagents to modify positively charged residues. The primary amine of the lateral chain of arginine residues belongs to a guanido group that is highly unreactive due to resonance. Therefore, succinic anhydride will react with the lysin-ε-amino groups and the N-terminal α-amino group. Our results show that succinylation strongly increase the open probability but does not affect the channel selectivity. This suggests that the voltage sensor could be formed of lysine residues, the selective filter being either

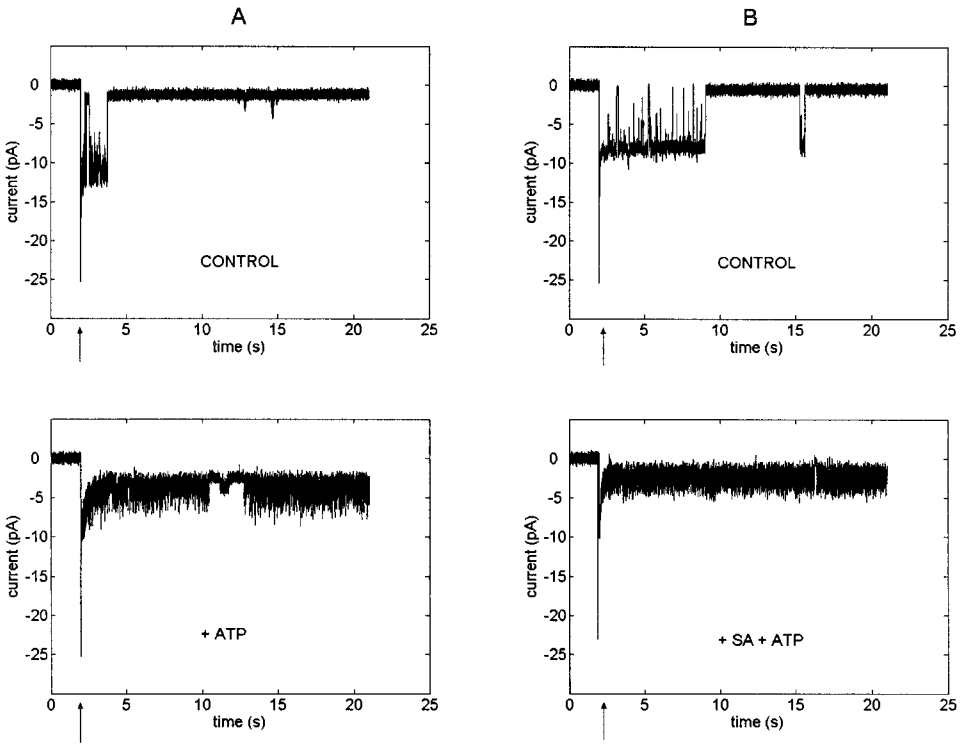

Fig. 7. Effect of 1 mM ATP on single channel current fluctuations recorded in 300 mM KCl buffered with 10 mM HEPES at pH 7.5 in the absence (A) or in the presence (B) of 0.8 mM succinic anhydride. The initial peak of current (indicated by the arrow) corresponds to the capacitive current occurring when the voltage was shifted from 0 to −30 mV.

not available for the succinylation reaction and/or formed of arginine residues. The TNBS neutralizes the free amino group whereas the succinic anhydride reverses its charge. Thus, as both TNBS and succinic anhydride have the same effects, we can conclude that the negative charge arising after succinylation is not responsible for the observed effects. Succinylation did not affect the ATP-regulated channel conductance suggesting that the ATP binding site and the voltage sensor belong to different domain of the protein.

Up to now, no active transport (electroneutral or electrogenic) has been found in the chloroplast outer envelope membrane. Thus, we can reasonably assume that the electric potential difference across this membrane must be close to 0 mV and therefore, the open probability of the anion channel will have its maximal value. At contact sites, the inner and outer envelope membranes are contiguous and there is a capacitive coupling between the two membranes like between capacitors. Therefore, the electric potential difference of the outer envelope membrane will be equal (but opposite in sign) to that of the inner envelope membrane (about 100 mV) and the anion channel will be closed. Thus, the two states of the channel might coexist in the chloroplast outer envelope membrane. The channel will be mainly closed at the contact site and in its fully open state beyond the contact site.

It has been previously suggested that HPO_4^{2-} would be the main ion flowing through the anion channel of the chloroplast inner envelope (Bolter *et al.*, 1999). In the absence of knowledge about the concentration of anions in the intermembrane space, it is difficult to calculate rigorously which anion will have the largest flux through the channel. The use of the concentration in both cytoplasm and stroma indicates that the net flux of Cl^- through the channel could be larger than that of HPO_4^{2-}. It is so, because the chloride concentration on both sides of the envelope and the chloride concentration gradient across the envelope are larger than those for HPO_4^{2-}, even though the permeability ratio $P_{Pi}/P_{Cl} = 4$ (Bolter *et al.*, 1999). The Cl^- concentration is about 10 mM in the cytoplasm and about 50 mM in the chloroplast stroma (Demmig and Gimmler, 1983; Hope and Walker, 1975). In *Chara* the Cl^- concentration of the stroma can be as high as 100 mM (Hope and Walker, 1975). Using the data of Stitt *et al.* (1980) for the phosphate concentration (7 mM in the stroma and 5–10 mM in the cytoplasm), the flux ratio

J_{Cl}/J_P will range between 3 and 5. Thus, the chloride flux through the channel should be larger than the phosphate flux, and this will be true even if we account for a P_i concentration as low as 0.5 mM in the stroma (Bligny *et al.*, 1990). At Cl^- concentrations prevailing in vivo, the open probability of the channel will be rather low ($P_o < 0.2$). Thus, despite its large unitary conductance, the channel will not allow a large ion flux in physiological conditions. The Cl^- flux through the chloroplast envelope is about 1.95 nmol s^{-1} (mg chlorophyll)$^{-1}$ (Fuks and Homblé, 1999). Using a conductance of 100 pS and a $P_o = 0.2$, this flux could be carried by a few copies of the anion channel in the outer envelope membrane, if we assume that Cl^- is the main anion transported through this channel.

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