Ion Channels in the Chloroplast Envelope Membrane†

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ABSTRACT: Isolated chloroplast envelope membranes were fused with azolectin liposomes. Ion transport across the membrane of these liposomes was investigated by the patch−clamp technique and in planar bilayers. Our results show that the chloroplast envelope contains voltage-dependent anion- and cation-selective channels as well as anion- and cation-selective pores with high conductances. At least one of the high-conductance pores could be located in the chloroplast outer envelope membrane. The low-conductance chloride channel and the potassium channel showed complex gating behavior with subconductant states. Potassium channel gating was affected by monovalent and divalent cations as well as by millimolar concentrations of ATP. Low concentrations of Cs^+ induced a flickering block. Voltage dependence of the open probability reveals that macroscopic currents of potassium channels are rectified with preferential potassium uptake into the chloroplast. Flux measurements and determinations of the stroma pH of intact chloroplasts confirm the presence of a potassium channel that is regulated by divalent cations (Mg^{2+}) and by ATP. The fully open potassium channel revealed a conductance of $\Lambda \cong 100$ pS in asymmetric KCl (250/20 mM KCl), and the fully open chloride channel revealed a conductance of $\Lambda \cong 60$ ps in 100 mM Tris/HCl. One high-conductance channel, mainly active at holding potentials >60 mV, was slightly selective for glutamate anions ($P_{K^+}/P_{Glu^-} \cong 2$) and revealed fast voltage-dependent gating. This high-conductance channel had a conductance of $\Lambda \cong 540$ pS (in 250/20 mM potassium glutamate) and was closed most of the time. A second type of high-conductance channel, mainly open and active at holding potentials below 30 mV, was slightly selective for cations ($P_{Glu^-}/P_{K^+} \cong 2$) with a conductance of $\Lambda \cong 1.14$ nS (in 250/20 mM potassium glutamate).

Ion channels in the plasma membrane of plant cells and plant organellar membranes are involved in volume regulation, transepithelial transport, and regulation of the membrane potential. The inner envelope membrane of chloroplasts acts as the main osmotic barrier and delimits the chloroplast stroma from the cytoplasm of the plant cell (Douce & Joyard, 1990), while the outer envelope membrane is permeable to macromolecules up to molecular masses of about 10 kDa (Flügge & Benz, 1984). The permeability is mediated by pore-forming proteins, so-called porins (Benz, 1988, 1994; Mannella, 1992; Nikaido, 1992; Gennis, 1989). During photosynthesis, light-driven H^+ gradients are generated between the cytosolic site (pH ~7), the chloroplast stroma (pH ~8), and the thylakoid lumen (pH <6). Ion channels in the chloroplast inner envelope membrane and the thylakoid membrane appear to be involved in the development and regulation of H^+ gradients and membrane potentials across these membranes (Peters & Berkowitz, 1991; Werdan et al., 1975; Enz et al., 1993; Rumberg & Siggel, 1969).

In the chloroplast stroma, concentrations of the physiological important ions are on the order of 150 mM K^+, 50 mM Cl^- (Demmig & Gimmler, 1983), and 5 mM Mg^{2+} (Portis & Heldt, 1976). The steady state membrane potential across the inner chloroplast envelope membrane has been found to be on the order of −100 mV (negative in the stroma; Wu et al., 1991), while the steady state membrane potential across the thylakoid membrane is small (~10 mV positive in the lumen; Rumberg & Siggel, 1969).

Due to the pH optimum (pH ~8) of the key enzymes of the photosynthetic carbon reduction cycle, the rate of light-dependent CO_2 fixation in chloroplasts depends on the maintenance of a high stromal pH (Werdan et al., 1975; Oja et al., 1986). The uphill H^+ flux across the chloroplast envelope is probably driven by a H^+-ATPase and indirectly coupled to the uptake of potassium from the cytosol (Peters & Berkowitz, 1991; Maury et al., 1981; Huber & Maury, 1980; Wu & Berkowitz, 1991, 1992a). Inhibitor and electrophysiological studies with intact chloroplasts and proteoliposomes containing proteins from solubilized chloroplast envelope membranes indicated the presence of a potassium channel in the inner envelope membrane (Wu & Berkowitz, 1991, 1992a; Wang et al., 1993). Very recently, a porin-like channel has been discovered in proteoliposomes containing the purified inner membrane of spinach chloroplasts. This high-conductance channel was slightly selective for anions over cations, revealing a main conductance of $\Lambda \cong 525$ pS (Fuks & Homble, 1995).

The envelope membrane is known to be permeable to chloride (Demmig & Gimmler, 1983), and chloride channels have also been detected in the chloroplast envelope of the green algae *Nitellopsis* and *Eremospheara viridis* by use of

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the patch−clamp technique and ion-selective microelectrodes (Pottosin, 1992; Thaler et al., 1992). It has been postulated that the potassium channel as well as the chloride channel and the H^+-ATPase are important for the regulation of the stromal pH (Berkowitz & Peters, 1993; Peters & Berkowitz, 1991; Douce & Joyard, 1990). In addition to these ion channels, the triose phosphate/phosphate translocator and the 2-oxoglutarate/malate translocator, both being antiport transport systems of the chloroplast inner envelope membrane (Flügge & Heldt, 1991; Menzlaff & Flügge, 1993), have been shown to function also as ion channels. The ion channel activities of these antiporters, however, can only be observed if the concentrations of the physiological substrates are low compared to those of monovalent ions (Schwarz et al., 1994; Schwarz, 1994). Similar to the inner mitochondrial membrane, it is conceivable that the chloroplast inner envelope contains, in addition, a protein-containing channel that is part of the chloroplast protein import machinery (Pfanner et al., 1994; Wienhues & Neupert, 1992; Flügge, 1990; Schnell et al., 1994; Kessler et al., 1994). Our intention was to characterize the electrical properties of ion channels in chloroplast envelope membranes. However, the chloroplasts of most higher plants are rather small, mostly prolate-shaped organelles with the longer axis on the order of 10 μm (Gunning & Steer, 1975) and therefore hardly suitable for direct electrophysiological measurements. For further characterization of ion channels in the chloroplast envelope, we therefore isolated chloroplast envelope membranes and fused them with artificial vesicles. Ion transport across the reconstituted envelope membrane vesicles was investigated by the patch−clamp technique and in planar bilayers. Since our preparation contains both envelope membranes, ion-conducting proteins from the inner as well as from the outer envelope membrane were expected to be active in these vesicles.

Here we report on the identification of two channels with high conductances and on the identification of a low-conductance chloride channel. The potassium channel of the chloroplast inner envelope was characterized with respect to its conductance, voltage-dependent gating, selectivity, and pharmacology.

MATERIALS AND METHODS

Preparation of Chloroplast Envelope Membranes

Preparation of envelope membranes started from intact chloroplasts purified on a Percoll gradient (Douce et al., 1973) followed by a discontinuous sucrose gradient of the membrane fractions (Douce et al., 1973; Flügge & Heldt, 1976). According to marker enzyme tests, no detectable contamination with other organellar membranes is present in this preparations (Mourioux & Douce, 1981).

Fusion of Chloroplast Envelope Membrane Vesicles with Liposomes and Preparation of Giant Vesicles for Patch−Clamp Experiments

Small liposomes were obtained by dissolving 100 mg/mL azolectin (Sigma, type IV-S) in 10 mM Hepes/KOH (pH 6.5) using a Branson sonifier equipped with a microtip. Liposomes were freeze−thawed once and mixed with chloroplast envelope membranes at a ratio of 1:1 (v/v). The chloroplast envelope vesicle/liposome mixture (containing ~200 μg of protein/mL) was frozen in liquid nitrogen and thawed at 4 °C. Giant vesicles suitable for patch−clamp measurements were obtained by a modified dehydration−rehydration procedure according to Criado and Keller (1987). After the mixture had thawed, 5−7 μL was spread on a glass slide and dehydrated at 4 °C for 45−60 min in a 500 mL exsiccator over dry $CaCl_2$. Afterward, 10 μL of the electrolyte solution to be used in the patch−clamp measurements was added to the partially dried sample on the slide. To avoid evaporation, the slide was transferred to a petri dish whose bottom was covered with water-saturated paper. One h later, giant liposomes with incorporated envelope membranes could already be observed. These giant chloroplast envelope membrane/liposome vesicles typically had diameters between 20 and 50 μm. Giant liposomes without fused envelope membranes, when used as controls, did not show any single channel activity under the applied experimental conditions.

Electrophysiological Measurements

Patch−Clamp Measurements. Giant chloroplast envelope membrane/liposome vesicles were placed in a tissue bath mounted on an Olympus IMT-2 inverted microscope, and the vesicles were viewed using phase contrast optics. Single channel current recordings using the patch−clamp technique were performed as described by Hamill et al. (1981). Holding potentials are always referred to the pipette. Gigaohm seals ranging from 5 to 50 GΩ could be achieved by slight suction, once the pipette tip was brought in contact with the membrane. Sealing was apparently affected by the protein content of the vesicles, i.e. the lower the protein concentration, the higher the probability of gigaohm seal formation. The currents were amplified using a PATCH-CLAMP L/M-EPC 7 amplifier (List Medical). Current recordings were digitized at a sampling rate of 36 kHz using a VR 10 digitizer (Instrutech Corp.) and stored on VHS video tapes. For analysis, current recordings were filtered with an eight-pole bessel filter, typically at 1 kHz, digitized at a sampling interval of 0.2 ms with an Axolab 1100 A/D converter (Axon Instruments) and stored on a personal computer.

Planar Lipid Bilayers. Planar lipid bilayers were produced by the painting technique (Mueller et al., 1962). A solution of 50 mg/mL azolectin (Sigma type IV-S) in *n*-decan (analytical grade, Merck) was applied to a hole (100−500 μm diameter) in a Teflon septum, separating the two bath chambers (total volume ~3 mL). Both chambers were equipped with magnetic stirrers. Bilayer formation was monitored optically and by capacitance measurements. The resulting bilayers had a typical capacitance of ≈0.5 $\mu F/cm^2$ and a resistance of >100 GΩ. The noise was 1 pA (rms) at 5 kHz band width. After a stable bilayer was formed in symmetrical solutions of 20 mM KCl and 10 mM Hepes/Tris (pH 7.0), the experimental conditions were changed to asymmetric concentrations. Concentrated solutions of KCl and $CaCl_2$ were added to the cis chamber up to final concentrations of 250 mM KCl and 10 mM $CaCl_2$.

The liposomes were added to the buffer solution in the cis compartment through the tip of a microloader (Eppendorf) so that the liposomes slowly flow directly across the bilayer. If necessary, the solution in the cis chamber was stirred to promote fusion. When proteoliposome preparations contain-

ing about 1−2 mg of protein/mL were directly reconstituted at a final protein concentration of 0.5 mg/mL, we always observed many different channels after fusion into the bilayer. These proteoliposomes were therefore not useful for investigation of single channel activity. However, after dilution of the envelope protein to about 50 μg/mL, we usually observed between one and three active channels (the maximum observed was five). In addition, we checked by the nystatin/ergosterol method (Woodbury & Miller, 1990) that the fusion of about three to ten liposomes into the bilayer was sufficient for incorporation of ion channel activity. Nucleotides (ATP and AMP-PNP) were added as neutral potassium or magnesium salt.

The Ag/AgCl electrodes were connected to the chambers through 1 M KCl agar bridges. The electrode of the trans compartment was directly mounted to the head stage (HS-2A × 10MG) of a current amplifier (GeneClamp 500, Axon Instruments). Reported holding potentials are referred to the trans compartment. The amplified currents were recorded on a modified DAT recorder (Panasonic SV 3700) and stored on DAT tapes.

For analysis, current recordings were low-pass filtered with an eight-pole bessel filter, typically at 1−2 kHz, digitized at a sampling interval of 0.2 ms, fed into an Axolab 1100 A/D converter (Axon Instruments), and stored in a personal computer. For analysis, a Windows-based analysis software developed in our lab (Schwarz et al., 1994) was used in combination with Origin (Microcal Software Inc.).

Measurement of the Stromal pH of Intact Chloroplasts

Chloroplasts from spinach leaves were prepared essentially as described by Mourioux and Douce (1981), except that the homogenization buffer contained 0.33 M sorbitol, 10 mM EDTA, 10 mM $Na_4P_2O_7$, and 5 mM ascorbate (pH 6.5) and that the buffer used for Percoll gradient centrifugation was 0.33 M sorbitol, 10 mM $Na_4P_2O_7$, and 0.1 mM EDTA. Measurements of the stromal pH were performed as described by Heldt et al. (1973). The chloroplasts (0.2 mg of chlorophyl/mL) were resuspended in 0.33 M sorbitol, 50 mM Hepes NaOH (pH 7.6), 0.1 mM EDTA, 5 μM venturicidine, and 0.5 mM [^{14}C]dimethyloxazolidine-2,4-dione (1 μCi/mL), incubated at 20 °C for 5 min in the dark, and then illuminated for 6 min. The chloroplasts were subsequently centrifuged through a layer of silicone oil, and the stromal pH was calculated as in Heldt et al. (1973).

RESULTS

The Chloroplast Envelope Membrane Contains Two Different High-Conductance (Porin-like) Channels. Purified chloroplast envelope membranes were fused with azolectin liposomes. These vesicles were incorporated into planar bilayers, and channel-mediated currents were recorded. Figure 1a shows current recordings at holding potentials (V_{hold}) of +80, −80, and −100 mV, being characteristic of an ion channel with an unusual large conductance. In the right part, the open probability of the fully open channel as a function of V_{hold} is shown. The channel displayed strong flickering with different open channel amplitudes. This is shown in detail in the magnified current trace at $V_{hold} = -100$ mV. Direct transitions from the closed to the fully open state (largest current amplitude) and from the closed state to intermediate states (smaller amplitudes) were observed. The partially open states were also approached from the fully open state. These sequences of current data indicate that the intermediate amplitudes are substates of a single channel rather than amplitudes of simultaneously active channels. This type of channel was observed in four out of fifteen experiments. The zero-current potential of the fully open channel was $E_{rev} = -12$ mV, indicating that the channel has a slight preference for anions over cations. Using the Goldmann−Hodgkin−Katz equation, a selectivity for glutamate over potassium $P_{Glu^-}/P_{K^+} \cong 2$ can be calculated. The slope conductance of the channel was $\Lambda \cong 540$ pS (in 250/20 mM potassium glutamate). The smaller current amplitudes that presumably represent subconductance states of the channel reached about 40 and 15% of the full conductivity. The unusual high conductance of the channel suggests ion permeation through a pore-like structure (Hille, 1992; Benz, 1984).

As is obvious from the plot of P_{open} vs V_{hold} in Figure 1a, the channel is closed mostly at both positive and negative holding potentials below 50 mV, approaching a P_{open} value of $P_{open} \approx 0.2$ at $V_{hold} = \pm 100$ mV. Also, the average mean lifetime of the open channel ($\tau_{open} < 100$ ms, data not shown) was unusually low. These properties indicate that the observed channel may have physiological features different from those of the general diffusion pore (see Discussion). Figure 1b shows current recordings obtained for a second type of high-conductance channel. Activity of this channel with large conductance was observed at low holding potentials. The open probability of this channel was $P_{open} \approx 0.4-0.45$ between V_{hold} −10 mV and V_{hold} = +30 mV and dropped to 0 above $V_{hold} = -20$ mV and $V_{hold} = 50$ mV (details not shown). Only a single conductance state with a slope conductance of $\Lambda \cong 1.14$ nS (in 250/20 mM potassium glutamate) was observed. The reversal potential of this channel in asymmetric potassium glutamate buffers was $E_{rev} = +14$ mV, indicating that this channel was slightly selective for cations.

The Chloroplast Envelope Membrane Contains a Low-Conductance Chloride Channel. We further examined anion channel activity in chloroplast envelope membrane vesicles. As mentioned above, this is hampered by the presence of metabolite translocators of the chloroplast inner envelope membrane (triose phosphate/phosphate translocator and 2-oxoglutarate/malate translocator) that also reveal anion channel activities (Schwarz et al., 1994; Schwarz, 1994). The conductance of the fully open triose phosphate/phosphate translocator channel was $\Lambda \cong 140$ pS in 100 mM KCl, and three additional sublevels were observed (Schwarz et al., 1994). The 2-oxoglutarate/malate translocator revealed a rather small main conductance of $\Lambda \cong 14$ pS in symmetrical 100 mM KCl and was inhibited by the chloride channel inhibitor 5-nitro-2,3-bis[(3-phenylpropyl)amino]benzoate (NPBB) (Schwarz, 1994). In order to obtain a high resolution of anion-selective currents mediated by chloroplast envelope membrane proteins, we employed the patch−clamp technique on giant liposomes containing chloroplast envelope membrane proteins (see Materials and Methods). Figure 2 shows single channel current recordings and the corresponding current−voltage relationships from giant liposomes in symmetrical 100 mM Tris/HCl solution. Under these conditions, ionic currents are mainly carried by chloride ions since $Tris^+$ is a nonpermeant (or only slowly permeant) cation. As is obvious from the traces in Figure 2a, different

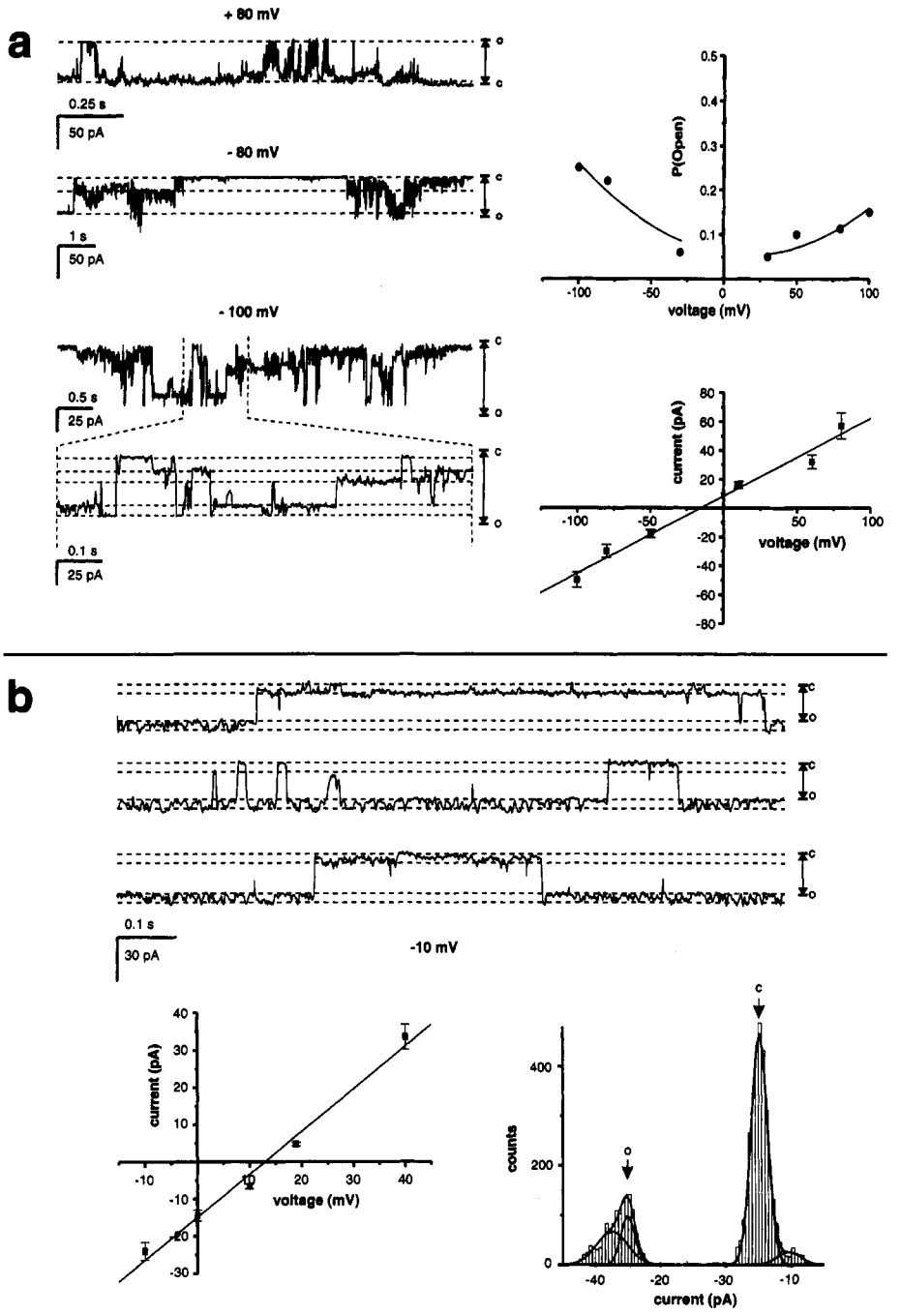

FIGURE 1: Porin-like channel in the chloroplast envelope membrane. (a) Current traces, histogram, and current−voltage relation from a bilayer containing a channel with a main conductance of 540 pS. The enlarged part of the trace at $V_{hold} = -100$ mV shows several current amplitudes and a transition pattern typical for a channel with subconductance states. P_{open} as a function of the voltage was calculated using the all point amplitude histograms from ≥20 s current recordings at the given V_{hold}. Leak current was −3.3 pA at $V_{hold} = -100$ mV. The current voltage relation for the most frequently encountered state reveals a zero-current potential of $V_{rev} = -12$ mV. Bath solutions: cis, 250 mM potassium glutamate, 25 mM calcium glutamate, and 10 mM Hepes/Tris (pH 7.0); trans, 20 mM potassium glutamate and 10 mM Hepes/Tris (pH 7.0). (b) Current traces, current−voltage relation, and amplitude histogram from a bilayer containing a channel with a conductance of 1.1 nS. The traces and the histogram are recorded at $V_{hold} = -10$ mV. The histogram was obtained from a current trace with a duration of 20 s. The small fluctuations can be attributed to a second, simultaneously active channel, as also shown in the histogram. The current−voltage relation shows a zero-current potential of $E_{rev} = +14$ mV, which indicates a slight selectivity for K^+. Bath solutions: cis, 250 mM potassium glutamate, 25 mM calcium glutamate, and 10 mM Hepes/Tris (pH 7.0); trans, 20 mM potassium glutamate and 10 mM Hepes/Tris (pH 7.0).

current amplitudes were observed. The slope conductance from the current−voltage relationship revealed values of $\Lambda \cong 60$ and 27 pS. The current traces showed direct transitions between the two conductance states (details not shown), indicating that the smaller conductance presumably represents a subconductance state of the open channel. Figure 2b shows a data set obtained in a different experiment also carried out with proteoliposomes in symmetrical 100 mM Tris/HCl solution. The current traces again revealed two different current amplitudes, and channel gating was more pronounced at higher positive holding potentials. The current−voltage relationship revealed slope conductances of $\Lambda \cong 6$ and 16 pS. Again, the current recordings showed direct transitions between the two conductance states (details not shown),

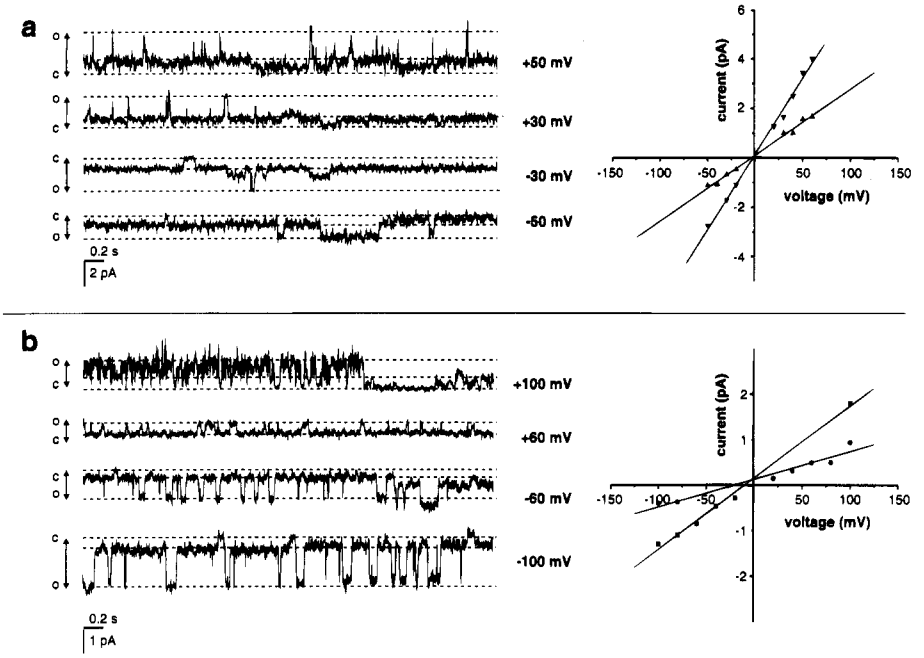

FIGURE 2: Anion channels in the chloroplast envelope membrane. (a) Current traces and current−voltage relation from an excised patch containing a channel with conductant states of 60 and 27 pS. (b) Current traces and current−voltage relation from an excised patch containing a channel with conductant states of 16 and 6 pS. Solutions: pipette and bath, 100 mM Tris/HCl (pH 7.0).

indicating that the smaller conductance may be a subconductant level. The rather low-unit conductance obtained for the channel in Figure 2b is almost identical to that obtained for the reconstituted 2-oxoglutarate/malate translocator in solutions with identical ionic strengths (Schwarz, 1994). We therefore conclude that the conductances of the anion channel shown in Figure 2b are carried by the 2-oxoglutarate/malate translocator, while the higher conductance observed in the experiment shown in Figure 2a can be attributed to a different channel. Since the reconstituted purified triose phosphate/phosphate translocator also revealed unit conductances different from that obtained in Figure 2a and has a nonlinear current−voltage relationship, we propose that the chloroplast inner envelope membrane contains an anion (chloride) channel with a conductance of $\Lambda \cong 60$ pS (see Discussion).

The Chloroplast Envelope Membrane Contains a K^+ Channel. The K^+ channel of the chloroplast inner envelope membrane was characterized in planar bilayers. Purified chloroplast envelope membrane vesicles were fused with azolectin liposomes, and these vesicles were incorporated into planar bilayers. In order to suppress anion channel activities present in most of the bilayers (>80%) after fusion of chloroplast envelope membrane vesicles, currents were mainly recorded in solutions containing potassium glutamate. However, in some experiments, only cation-selective channels were present in the bilayer membrane after fusion and the selectivity of the channel could be determined after perfusion of the bath solutions simply by its zero-current potential. A typical example is illustrated in Figure 3 where single channel recordings in asymmetric KCl solutions (cis, 250 KCl; trans, 20 KCl) at different holding potentials are shown. The figure also includes graphs of the current−voltage relationship and the open probability of the channel at different holding potentials. The reversal potential obtained from the current−voltage relationship in Figure 3 was $E_{rev} = +53$ mV, which is close to the Nernst potential of potassium ($E_{K^+} = +62$ mV). This shows that the observed

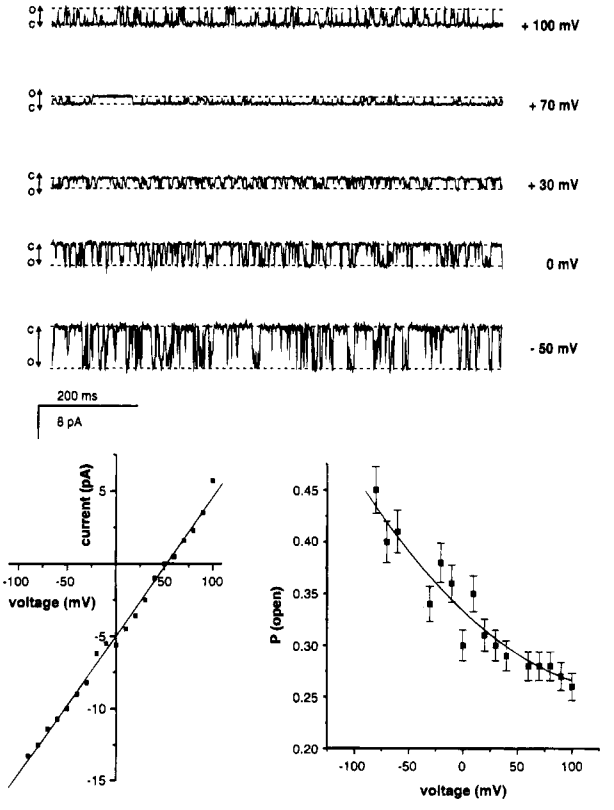

FIGURE 3: Cation-selective channels in the chloroplast envelope membrane. Current traces, current−voltage relation, and open probabilities of a bilayer containing a K^+-selective channel. The zero-current potential of $E_{rev} = +53$ mV is close to the Nernst potential of 63 mV for K^+ ions. Bath solutions: cis, 250 mM KCl, 10 mM $CaCl_2$, and 10 mM Hepes/Tris (pH 7.0); trans, 20 mM KCl and 10 mM Hepes/Tris (pH 7.0). The leak current was <1 pA at $V_{hold} = 0$ mV.

channel was selective for potassium. When K^+ was substituted by Na^+, no significant change in the zero-current potential was observed. This shows that potassium and

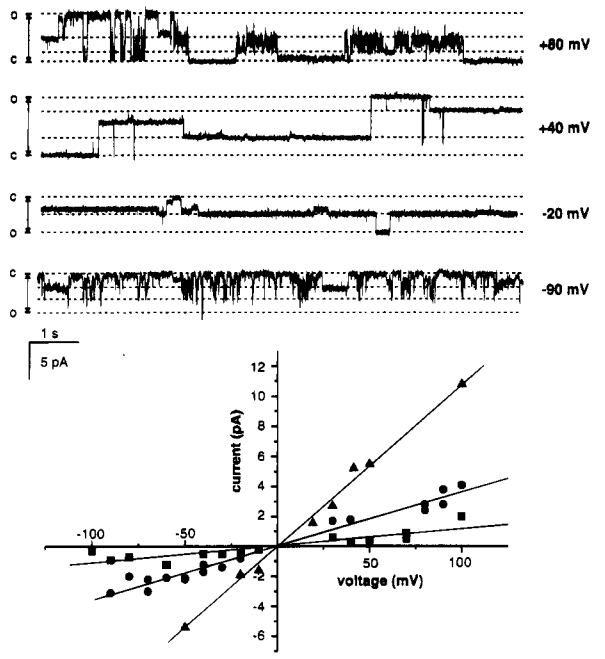

FIGURE 4: Conductance states of cation-selective channels in the chloroplast envelope membrane. Top: current traces from a bilayer containing a cation-conducting channel. The channel displays a complex gating pattern. Bottom: current−voltage relation from this measurement. The data can be grouped into three classes of conductances: $\Lambda \cong 18 \pm 5$ pS, $\Lambda \cong 58 \pm 17$ pS, and $\Lambda \cong 100 \pm 28$ pS. Bath solutions: cis, 250 mM potassium glutamate and 10 mM Hepes/Tris (pH 7.0); trans, 250 mM potassium glutamate, 10 mM calcium glutamate, and 10 mM Hepes/Tris (pH 7.0). The leak current was <1 pA at $V_{hold} = 0$ mV.

sodium are about equally permeable. However, sincepotassium is the most abundant and physiological important cation in chloroplasts (Speer & Kaiser, 1991), the cation-selective channel will be termed K^+ channel in the following. The open probability of the channel was dependent on the magnitude and the direction of the holding potential (see Figure 3). Considering that the measurements were performed in asymmetric buffers ($E_{rev} = +53$ mV), the channel reveals an open probability of $P_{open} \cong 0.45$ at $V_{hold} = -143$ mV and $P_{open} \cong 0.26$ at $V_{hold} = 47$ mV. Thus, the K^+ channel facilitates higher macroscopic currents from the cis to the trans compartment than in the opposite direction, i.e. macroscopic currents will be rectified. From the slope of the *I/V* curve (Figure 3), a conductance of $\Lambda \cong 100$ pS can be calculated.

In most of the measurements, when anion- and cation-selective channels were present in the bilayer membrane, the overall zero-current potentials (E_{rev}) with KCl buffers were typically in the range of −15 to −20 mV, showing that the currents were mainly mediated by anions. In order to decrease anion currents, Cl^- ions in buffer solutions were substituted by the non- or slowly permeable glutamate anion. Under these conditions and with asymmetric buffer solutions, the reversal potentials indeed changed to values between $E_{rev} \cong 0$ and +20 mV.

The Chloroplast Envelope K^+ Channel Reveals Subconductance States. Figure 4 shows current recordings from a bilayer in symmetric potassium glutamate solutions at different holding potentials and the corresponding current−voltage relationship. As is obvious from the current recordings, different open channel amplitudes were observed. The current data can be grouped into three different classes of

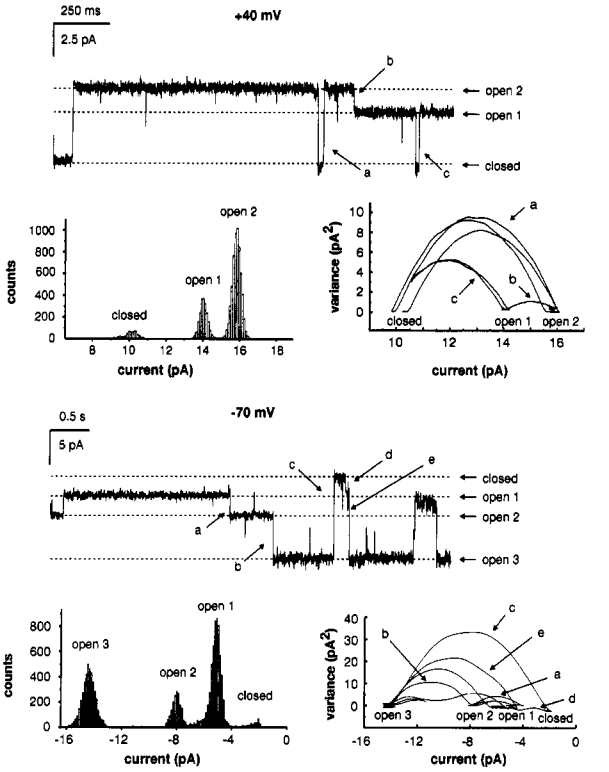

FIGURE 5: Conductance states of cation-selective channels in the chloroplast envelope membrane. Measurements on two different bilayers are shown. Top: current trace and amplitude histogram at $V_{hold} = +40$ mV. The leak current was ≈10 pA at $V_{hold} = 40$ mV. The sequences of transition indicated by arrows were due to subconductant states. Bottom: current trace and amplitude histogram at −70 mV. The leak current was ≈2 pA at $V_{hold} = -70$ mV. The number of open states differs from that of the upper trace. Again, the indicated transitions were due to subconductant states (in particular, c−e). Amplitude histograms and mean−variance plots were obtained from current recordings with a duration of 10 s each.

open channel amplitudes with slope conductances of $\Lambda \cong 18 \pm 5$ pS, $\Lambda \cong 58 \pm 17$ pS, and $\Lambda \cong 100 \pm 28$ pS. Figure 5 shows selected examples of single channel currents with extended time scales, the corresponding amplitude histograms, and mean−variance plots (Patlak, 1988) at holding potentials of $V_{hold} = +40$ and −70 mV. At $V_{hold} = +40$ mV, only two open channel amplitudes were detected, while at $V_{hold} = -70$ mV, three open states of the channel were observed. The course of the current data and the mean variance plots indicate that the smaller amplitudes are due to subconductant levels rather than simultaneous activities of two or more channels; direct transitions between the closed and the fully open state as well as transitions between the close (or open) states and the substates can be observed. Taken together, these data show that the chloroplast inner envelope K^+ channel displays subconductance levels with amplitudes of approximately 20 and 60% of the fully open channel amplitude.

Cs^+ Binding to the Chloroplast Envelope K^+ Channel from the Cis Side of the Bilayer Decreases Channel Open Probability, while Its Permeation Induces a Flickering Block. Figure 6a whose current recordings ($V_{hold} = +50$ mV) and the corresponding all point amplitude histograms from a bilayer containing a K^+ channel in the absence and presence of 100 μM CsCl on the cis side. CsCl obviously decreased channel activity and the overall current amplitude. Closer

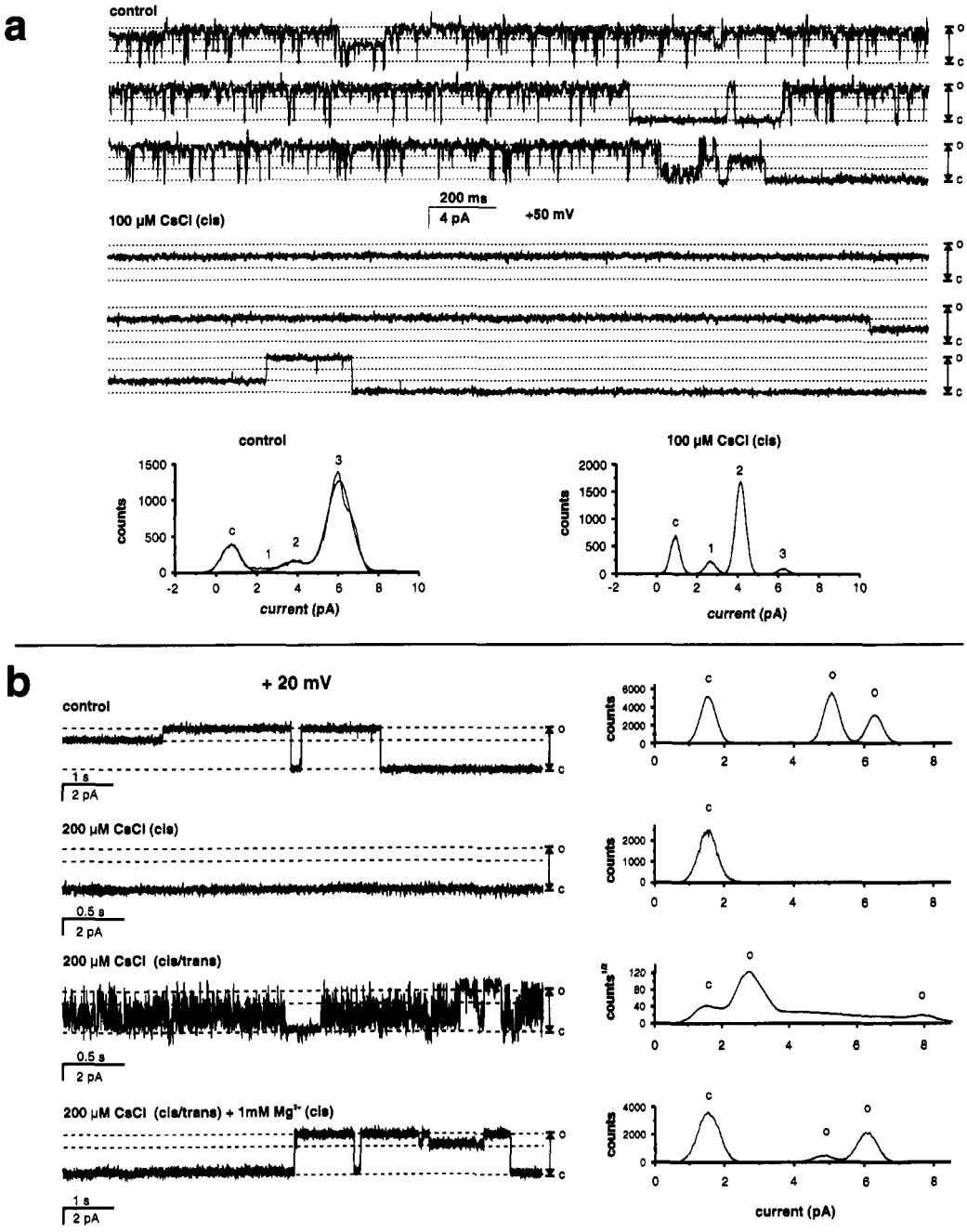

FIGURE 6: (a) Effect of Cs^+ on cation-selective channels in the chloroplast envelope membrane. Current traces and amplitude histograms at $V_{hold} = +50$ mV without or in the presence of 200 μM CsCl on the cis side of the bilayer. Channel activity and the occupancy of the fully open level are decreased by Cs^+. The current traces are part of a continous recording. The time gap between the last trace of the control (lane 3 from top) and the first trace of the measurement with CsCl (lane 4 from top) is 60 s. Bath solutions: cis, 250 mM potassium glutamate, 25 mM calcium glutamate, and 10 mM Hepes/Tris (pH 7.0); trans, 20 mM potassium glutamate and 10 mM Hepes/Tris (pH 7.0). Leak currents were <1 pA at $V_{hold} = 0$ mV. (b) Effect of Cs^+ and Mg^{2+} on cation-selective channels in the chloroplast envelope membrane. Current traces and associated amplitude histograms at $V_{hold} = +20$ mV. First trace and histogram from top control. Second trace: addition of 200 μM CsCl to the cis side closes the channel completely. Third trace: subsequent addition of CsCl to the trans side induces a flickering of the channel. Fourth trace: the effect is reversed by addition of 1 mM Mg^{2+} on the cis side. The current traces are part of a continuous recording. The time gaps between the traces are 60 s. Bath solutions: cis, 250 mM potassium glutamate, 25 mM calcium glutamate, and 10 mM Hepes/Tris (pH 7.0); trans, 20 mM potassium glutamate and 10 mM Hepes/Tris (pH 7.0). Leak currents were <1 pA at $V_{hold} = 0$ mV.

inspection of the current traces and histograms shows that the open probability for the fully open state decreased drastically, although Cs^+ ions on the cis side are, at the applied positive holding potential, not forced to pass the channel. At the same potential, addition of CsCl to the trans side led to a flickering, mainly between the ground state and the 60% sublevel (Figure 6b). Flickering occured also if Cs^+ was added to the cis compartment and Cs^+ permeation was forced from the cis to the trans compartment by negative holding potentials.

The partial channel block by Cs^+ on the cis side is reversed by the addition of 1 mM Mg^{2+} to the same side (Figure 6b), with gating mainly between the closed and fully open state. The reversal of the Cs^+ effect by Mg^{2+} shows that both cations compete for the same binding site(s), although free Mg^{2+} on the cis compartment is not required for channel

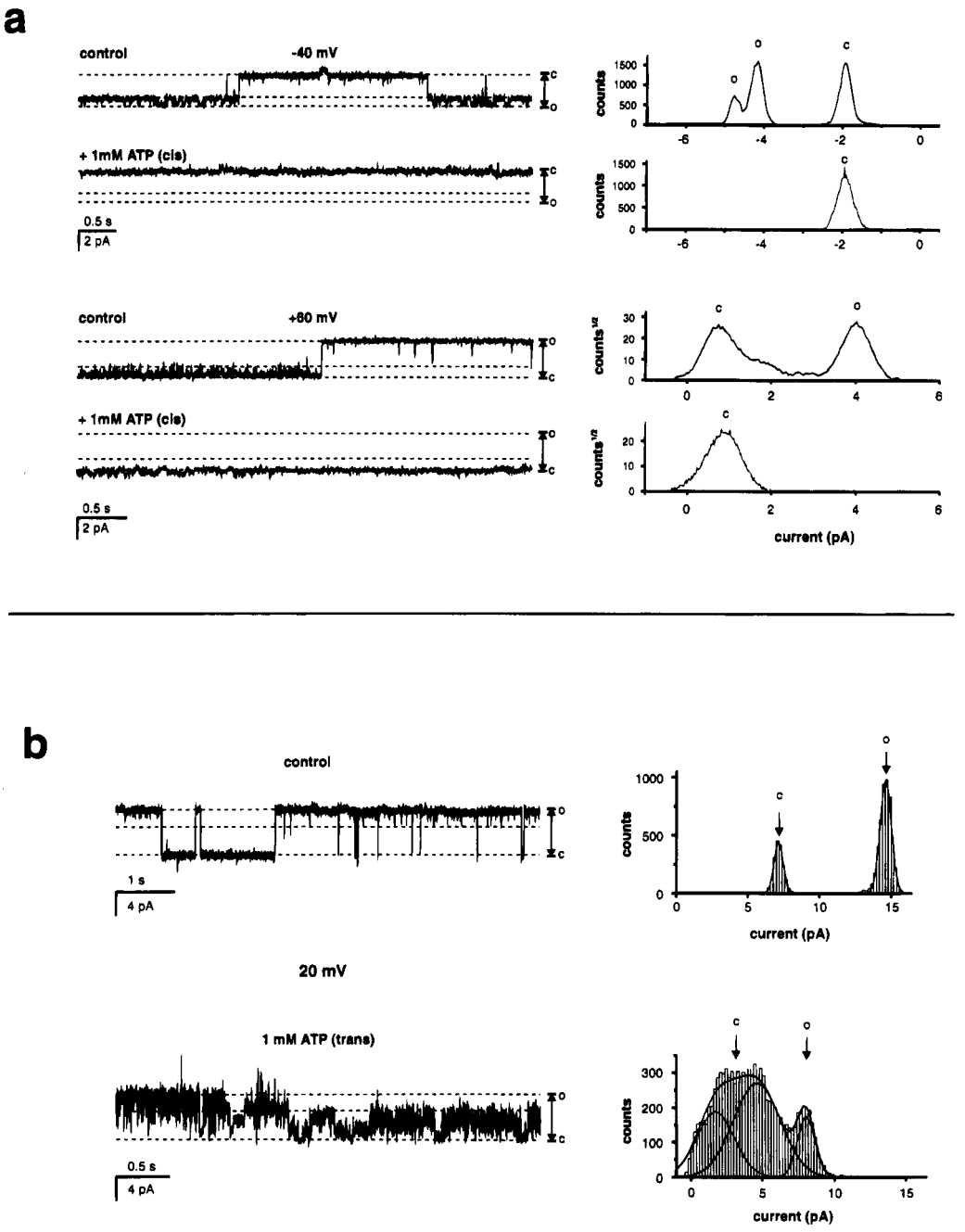

FIGURE 7: Effect of ATP on cation channels in the chloroplast envelope membrane. (a) Current traces and associated amplitude histograms at $V_{hold} = -40$ mV and $V_{hold} = +60$ mV. Addition of 1 mM ATP to the cis side closes the channel completely. Bath solutions: cis, 250 mM potassium glutamate, 25 mM calcium glutamate, and 10 mM Hepes/Tris (pH 7.0); trans, 20 mM potassium glutamate and 10 mM Hepes/Tris (pH 7.0). Leak currents were <1 pA at $V_{hold} = 0$ mV. (b) Current traces and associated amplitude histograms at $V_{hold} = +20$ mV. Addition of 1 mM ATP to the trans side decreases the open probability and induces flickering of the channel. Bath solutions: cis, 250 mM potassium glutamate, 25 mM calcium glutamate, and 10 mM Hepes/Tris (pH 7.0); trans, 20 mM potassium glutamate and 10 mM Hepes/Tris (pH 7.0). Leak currents of the control were ≈ 7 pA at $V_{hold} = 20$ mV.

activity. This effect of Mg^{2+} on channel activity is not due to the control of K^+ flux by screening of negative membrane charges (Guoy Champmann-Stern model; Hille et al., 1975) as proposed for a K^+ channel from the chloroplast envelope membrane (Mi et al., 1994).

These data also show that the chloroplast inner envelope K^+ channel was unidirectionally incorporated into the bilayer membrane. Binding of Cs^+ ions from the cis compartment changes the channel-gating pattern. The probability that the channel occupies its fully open state decreased from $P_{open} \cong 70\%$ to $P_{open} \cong 3\%$ ($n = 3$, see histogram in Figure 6a). Permeation of Cs^+ from either compartment induced a flickering of the channel between the closed state and the 60% amplitude sublevel and between the closed and fully open states. At the given experimental conditions [20 mM K^+ (trans)/250 mM K^+ and 25 mM Ca^{2+} (cis)], the applied Cs^+ concentrations (100−200 μM) are not sufficient to significantly change the surface potential of the bilayer. Therefore, the Cs^+ effect presumably arises from binding of the cation to the channel.

Gating of the Chloroplast Inner Envelope K^+ Channel Is ATP-Dependent. We also investigated the effect of ATP on the activity of the chloroplast K^+ channel. Figure 7a shows single channel current recordings in asymmetric buffers and the corresponding all point amplitude histograms in the absence and presence of 1 mM ATP in the cis compartment

of the bilayer. The bilayer contained one active copy of the K^+ channel, and the leak current was approximately 1 pA. ATP completely blocked the channel ($n = 5$) at both positive and negative holding potentials (see Figure 7a). This effect was reversible ($n = 3$); when ATP was removed from the cis compartment by perfusion, activity of the K^+ channel was recovered (details not shown). ATP, when added from the trans compartment, had a different effect on the currents (Figure 7b). This measurement was conducted on a different bilayer with a leak conductance of about 7 pA ($V_{hold} = 20$ mV). This leak conductance was presumably due to the presence of another open nongating channel. Upon addition of 1 mM ATP, the conductance of the bilayers decreased ($n = 4$), and this effect was accompanied by a change in gating of the K^+ channel. The bilayer shown in Figure 7b also contained one active copy of the chloroplast inner envelope K^+ channel; however, the leak current was rather high. After addition of 1 mM ATP, the leak current (depicted as peak of the closed state) decreased drastically (see Figure 7b) and flickering of the K^+ channel was observed. ATP added to the trans side of the bilayer apparently affected both, the current pathways responsible for the leak currents and the K^+ channel. Again, these results indicate that the chloroplast envelope membrane proteins are unidirectionally incorporated into the bilayer. Moreover, the results show that the K^+ channel of the inner chloroplast envelope is ATP sensitive. The molecular mechanism involved in ATP modulation of the K^+ channel remains unclear. It is not known whether the effect of ATP on channel activity is due to a direct interaction of the nucleotide with the channel protein or due to an indirect effect. Blocking of the channel from the cis compartment closely resembles the characteristics of ATP sensitive inward-rectifying K^+ channels described previously (Cook & Hales, 1984) and may therefore be due to a direct interaction. The ATP effect on the trans compartment may presumably be due to a secondary effect on channel activity either coupled electrically or protein-mediated as observed previously with ATP-dependent channels (Lazdunski et al., 1994; Ashcroft & Ashcroft, 1990; Takano & Noma, 1994).

pH Changes in the Stroma of Illuminated Intact Chloroplasts Can Be Reconciled with the Presence of a K^+ Channel in the Inner Envelope Membrane. Light-dependent H^+ transport from the stroma into the thylakoids leads to an alkalization of the stroma and to the formation of a proton gradient across both the thylakoid and the envelope membranes (Heldt et al., 1973). In the following experiments with intact chloroplasts, the K^+ concentration in the external medium was kept at zero, whereas the stromal K^+ concentration is about 150 mM (Speer & Kaiser, 1991). With a medium pH kept at 7.6, the stromal pH of intact chloroplasts increased to pH 7.96 upon illumination (Figure 8). The presence of nigericin (a H^+/K^+ exchanger) eliminated the pH gradient across the membranes and led to a drastic decrease of the stromal pH. This effect, however, could obviously be reversed by addition of K^+ ions to the external medium (Figure 8a). These experiments show that H^+ fluxes across the envelope membrane are electrically balanced by the transport of K^+ across the envelope membrane as also shown previously (Peters & Berkowitz, 1991). Moreover, we also have conducted control experiments showing that, in the presence of 2 mM Mg^{2+}, which activates the K^+ channel, the K^+ concentration in intact chloroplasts, in the absence of external potassium, is reduced to about 70% of the control value (details not shown). In a second type of experiment, we studied the effect of Mg^{2+} on the stromal pH of illuminated chloroplasts (Figure 8b). All assays contained venturicidine in order to completely prevent light-driven ATP synthesis by the thylakoid H^+-ATPase. Addition of Mg^{2+} to chloroplasts resulted in a decrease of the stromal pH by 0.22 pH units. Mg^{2+}-induced acidification could be abolished by the addition of K^+ and/or ATP. The nonhydrolyzable ATP analog adenyl (β,γ-methylene)diphosphonate (AMP-PCP) displayed almost the same effect as ATP, indicating that hydrolysis of ATP is not required to reverse the Mg^{2+}-induced decrease of the stromal pH. It may be noted that, in the absence of Mg^{2+}, ATP had no effect on the light-induced alkalization of the stromal pH. These observations can be easily explained by the presence of a K^+ channel in the inner envelope membrane. As shown above by electrophysiological studies, the inward-rectifying K^+ channel was activated by Mg^{2+}, probably from the stromal side. Opening of the channel at low membrane potentials (and at low external potassium concentration) will give rise to K^+ efflux that is coupled to a reduced light-driven efflux of H^+. Efflux of K^+ from the stroma can be

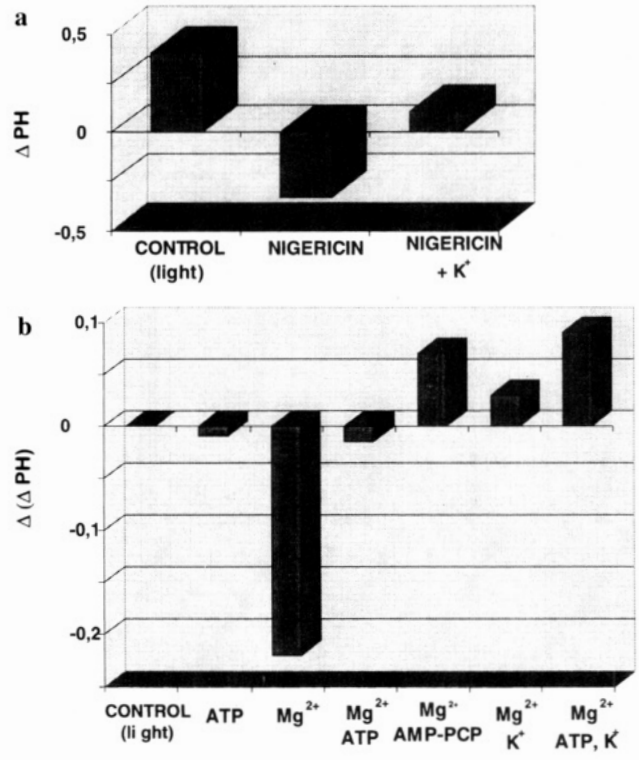

FIGURE 8: pH changes in the stroma of illuminated intact chloroplasts. Measurements of the stromal pH were performed as described in Materials and Methods. Chloroplasts (0.2 mg of chlorophyll/mL) were resuspended in 0.33 M sorbitol, 50 mM Hepes/NaOH (pH 7.6), 0.1 mM EDTA, 5 pM venturicidine, and 0.5 mM [^{14}C]dimethyloxazolidine-2,4-dione (1 μCi/mL), incubated at 20 °C for 5 min in the dark, and then illuminated in the presence of the indicated additions for 6 min. The chloroplasts (aliquots of 0.2 mL) were subsequently centrifuged through a layer of silicone oil. The stromal pH was calculated as described by Heldt et al. (1973). Upper part: nigercin was added to a final concentration of 10 μM, and the externally added K^+ concentration (potassium gluconate) was 60 mM. Lower part: the stromal pH of the illuminated chloroplasts was 7.96 (control). The externally added concentrations were 5 mM (Mg^{2+} as magnesium gluconate), 3 mM (ATP and AMP-PCP, respectively), and 50 mM (potassium gluconate). Both: mean values of at least five experiments.

thermodynamically inhibited by the addition of external K^+, leading to an increased efflux of H^+ and therefore to an increase of the stromal pH. Binding of ATP, probably also from the stromal side, was shown to close the channel. Consequently, K^+ efflux from the stroma is inhibited, and higher ΔpH values can be established. In line with these explanations is the observation that the light-induced uptake of K^+ into intact chloroplasts is reduced to about 70% in the presence of 5 mM Mg^{2+}; the effect is reversed by the presence of ATP or AMP-PCP (data not shown).

DISCUSSION

In order to identify and characterize ion channels in the chloroplast envelope membrane, we have reconstituted purified envelope membranes in planar bilayers and, for patch−clamping, in giant liposomes. The results of our experiments are consistent with the presence of two different high-conductance (porin-like) channels, the presence of a low-conductance anion channel, and the presence of a Mg^{2+}- and ATP-dependent cation channel in the chloroplast envelope membrane.

The outer membranes of bacteria, mitochondria, and chloroplasts are permeable to solutes up to molecular masses of several kilodaltons. Transport across these membranes is facilitated by pore-forming proteins called porins (Schmid et al., 1992; Colombini, 1989; Benz, 1988; Flügge & Benz, 1984). These porins located in the outer membranes are proposed to form transmembrane channels, thus providing a free diffusion pathway for solutes following their electrochemical gradients. Extensive studies on structure and function of bacterial porins and on so-called mitochondrial VDACs (voltage-dependent anion channels) from the outer membrane have provided a detailed picture on their molecular structures and function (Moran & Sorgato, 1992; Zizi et al., 1994; Benz et al., 1993; Blachlydyson et al., 1993; Blumenthal et al., 1993; Thomas et al., 1993; Mannella et al., 1992; Peng et al., 1992; Schmid et al., 1992). Only a few reports on pore-forming proteins from the chloroplast outer envelope membrane exist. In planar bilayers containing reconstituted chloroplast envelope membrane proteins, a large conductance channel ($\Lambda \cong 720$ pS, in 100 mM KCl) was found (Flügge & Benz, 1984). Recently, weak anion- and cation-selective large conductance pathways ($\Lambda \cong 512$ pS and $\Lambda \cong 1016$ pS in 100 mM KCl) have been detected in the chloroplast envelope membrane by direct patch−clamping of chloroplasts from green algae *Nitellopsis obtusa* (Pottosin, 1992, 1993). It has been suggested that the weakly anion-selective channel with a conductivity of $\Lambda \cong 512$ pS may be related to the mitochondrial VDAC (Pottosin, 1993). More recently, a weakly anion-selective high-conductance channel with a single channel conductance of $\Lambda \cong 525$ pS has been discovered in proteoliposomes containing purified inner envelope membranes from spinach chloroplasts (Fuks & Homble, 1995). We also observed a high-conductance channel with a slope conductance of $\Lambda \cong 540$ pS (trans 20/cis 250 mM potassium glutamate) which was slightly anion-selective ($P_{Glu^-}/P_{K^+} \approx 2$).

This channel was mainly active at higher holding potentials ($|V_m|$) 60 mV), displaying brief flickering toward the partially open and fully open states. The characteristics of this channel closely resemble those described by Fuks and Homble (1995) for the anion-selective high-conductance channel; presumably, both channels are identical. The voltage dependence of the channel is rather unusual for porin channels (Benz, 1994) and indicates that this channel is probably not a general diffusion pore. It is therefore tempting to speculate that this large conductance channel may be part of the proposed chloroplast protein import channel (Schnell et al., 1994; Kessler et al., 1994).

In addition, we also observed a weakly cation-selective channel ($P_{K^+}/P_{Glu^-} \approx 2$) with a large conductance of $\Lambda \cong 1.14$ nS in asymmetric electrolytes (trans 20/cis 250 mM potassium glutamate). The channel was mainly open at holding potentials below 30 mV and closed at higher voltages. These properties resembled closely those described for the large cation-selective channel in the chloroplast envelope membrane obtained by direct patch−clamping of chloroplasts (Pottosin, 1992). Moreover, the large conductance and the voltage dependence of this channel are very similar to those of other pore channels, suggesting that this channel can be assigned to a general diffusion type of porin channel from the outer chloroplast envelope (Pottosin, 1992; Mannella et al., 1992; Benz, 1994). The weak cation-selective conductance of $\Lambda \cong 1.14$ nS in asymmetric (trans 20/cis 250 mM potassium glutamate) solutions is on the same order of magnitude as observed previously for a chloroplast porin (Flügge & Benz, 1984).

Besides these high-conductance channels, we detected low-conductance channels, mainly permeable to chloride. However, since we previously observed anion channel activities associated with the purified triose phosphate/phosphate translocator and the purified 2-oxoglutarate/malate translocator from the chloroplast inner envelope membrane (Schwarz et al., 1994; Schwarz, 1994), it is not possible to assign this activity unambiguously to a specific anion channel protein. But the conductance of 60 pS, which we frequently observed in the chloroplast envelope membrane ($n = 5$), did not appear in measurements with the purified translocator proteins. We therefore propose that this activity could be attributed to the presence of a specific anion channel protein in the chloroplast inner envelope membrane. The conductance of this channel is large enough to account for the rather high chloride permeability of the chloroplast envelope membrane (Demmig & Gimmler, 1983).

In most bilayer experiments with reconstituted chloroplast envelope membranes (15 out of 20), an active cation-selective channel was found. This channel revealed a conductance of $\Lambda \cong 100$ pS in the fully open state. Subconductance levels with about 60 and 20% of the fully open channel amplitude were observed. Studies with intact chloroplasts as well as with reconstituted chloroplast envelope membranes by Berkowitz and co-workers have demonstrated the presence of a specific K^+ transport pathway in chloroplast inner envelope membranes (Berkowitz & Peters, 1993; Peters & Berkowitz, 1991; Mi et al., 1994; Wu et al., 1991; Wu & Berkowitz, 1991, 1992a,b; Wang et al., 1993). More recently, they observed a K^+ channel in planar bilayers after fusion of chloroplast envelope vesicles (Mi et al., 1994). This channel revealed a slope conductance of $\Lambda \cong 160$ pS, and no voltage dependence of channel gating was observed. In contrast, we observed voltage-dependent gating of the channel, and the open probability of the channel revealed a marked voltage dependence decreasing from $P_{open} \cong 0.44$ at $V_{hold} = -90$ mV to $P_{open} \cong 0.26$ at $V_{hold} = +100$ mV. However, considering the different experimental protocols,

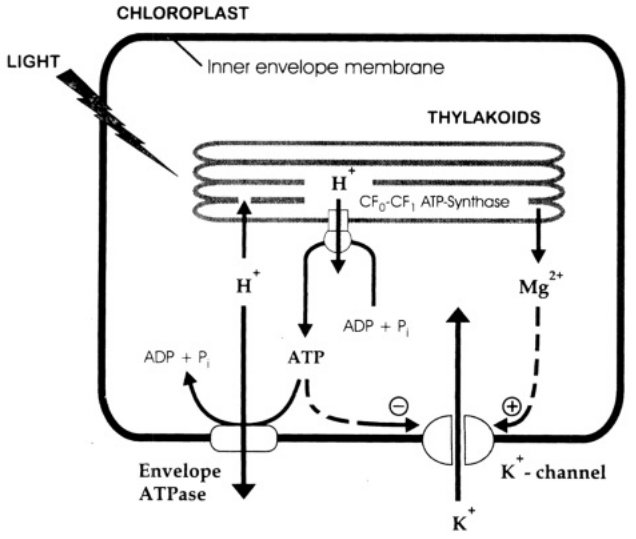

FIGURE 9: Proposed model for the energy-dependent K^+ and H^+ fluxes across the chloroplast envelope membrane. For details, see the text.

it is likely that the K^+ channels described in this paper and the one described previously by Mi et al. (1994) are constituted by the same protein.

As a major physiological function, the chloroplast inner envelope cation-selective channel will provide K^+ uptake to electrically balance the stationary ΔpH across the envelope (Werdan et al., 1975). During illumination, the inside negatively polarized membrane potential of chloroplasts hyperpolarizes ($\approx$100 mV inside negative) due to H^+ pumping by the ATPase of the inner envelope membrane (Demmig & Gimmler, 1983; Wu et al., 1991; Douce et al., 1973). This H^+ extrusion is balanced by K^+ influx, and the cation-selective channel (K^+ channel) should therefore preferentially allow K^+ uptake. Indeed, we have observed that the open probability of the K^+ channel was significantly higher at negative holding potentials (Figure 3). We reproducibly observed rectification from the trans to the cis compartment in the bilayer setup which corresponds to an inward rectification of potassium currents into the chloroplasts. Obviously, the K^+ channel has been incorporated into the bilayer in a preferential orientation with the cis compartment corresponding to the stromal side of the chloroplasts. The detected high-affinity cation binding site for Cs^+ or Mg^{2+} that was located exclusively in the cis compartment and that may play a role in modulation of channel activity is therefore located within the chloroplasts. Another modulator of channel activity was ATP that, when added from the cis compartment, completely blocked the channel. We therefore conclude that the K^+ channel of the chloroplast inner envelope membrane is an inwardly rectifying channel containing, at the stromal side of the chloroplasts, a high-affinity binding site for cations and also a low-affinity binding site for ATP. The possible physiological role of the K^+ channel of the chloroplast inner envelope membrane during photosynthesis is outlined in Figure 9. Illumination of chloroplasts results in an increase of stromal ATP produced by photophosphorylation. Also, the concentration of Mg^{2+} increases due to the light-dependent release of Mg^{2+} from thylakoid membranes (Hind et al., 1974; Portis, 1981). The light-induced pH gradient across the envelope membrane that is due to the proton uptake into the thylakoids is assumed to be maintained by the action of an envelope membrane H^+-ATPase (Douce et al., 1973; Berkowitz & Peters, 1993). H^+ efflux is counterbalanced by the influx of K^+, mediated by the envelope membrane K^+ channel. At elevated levels of Mg^{2+}, the K^+ channel is activated, leading to the increase of K^+ influx coupled to H^+ efflux. In contrast, the open probability of the K^+ channel is decreased by millimolar concentrations of ATP which will thermodynamically decrease H^+ efflux from the chloroplasts. Consequently, the stromal pH is lowered, leading a reduced rate of light-dependent CO_2 fixation. The effect of Mg^{2+}-activated K^+ currents on changes of the stromal pH and the opposite effect of ATP could also be confirmed by direct pH measurements as shown in Figure 8. The light-induced alkalization of the chloroplast stroma and the maintenance of the pH gradient across both the thylakoid and the envelope membrane are essential for high production rates of photoassimilates. Thus, the opposing modulation of the K^+ channel by Mg^{2+} and ATP may play a crucial role in the regulation of photosynthesis.

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