Communication

A 50-Picosiemens Anion Channel of the Chloroplast Envelope Is Involved in Chloroplast Protein Import*

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Single channel recordings were used to investigate the changes on the pea chloroplast envelope during protein import. In the inside-out patch configuration a 50-picosiemens (pS) anion channel of the chloroplast envelope membrane was identified. The open time probability of the channel was decreased by the addition of the wild type precursor protein of ferredoxin (wt-prefd) to the pipette-filling solution in the presence of 0.5 mM ATP. In the absence of ATP or in the presence of 50 μM ATP, wt-prefd did not affect the open time probability of the channel. A deletion mutant of pref, Δ6–14-prefd, which is inactive in in vitro import, was also unable to affect the open time probability of the 50-pS anion channel. In the presence of 100 μM ATP, wt-prefd decreased the open time probability of the channel to a lesser extent, as did the transit peptide alone. It is concluded that the 50-pS anion channel could be part of the protein import machinery of the inner membrane. In addition the precursor protein under import conditions induced burst-like increases of the envelope conductivity. The implication of both responses for the chloroplast protein import process are discussed.

A large part of the chloroplast proteins is nuclear encoded. These proteins are synthesized in the cytosol and have to be imported into the chloroplast. Nuclear encoded chloroplast proteins are therefore synthesized with an N-terminal extension called the transit sequence. The transit sequence is both necessary and sufficient to target a protein to the chloroplast (1). Several components of the chloroplast import machinery have been identified (2, 3). A role for chloroplast envelope channels has been proposed in protein import (2–4). However, little is known about the function of channels in the import process and a number of envelope ion channels (9, 10).

Ion channels in the chloroplast envelope are also thought to be involved in osmoregulation during photosynthesis. Several ion channels of the inner as well as the outer envelope membrane have been identified by reconstitution of the channels in giant liposomes or in planar lipid bilayers (5–8). Direct electrophysiological measurements on isolated chloroplasts have also revealed a number of envelope ion channels (9, 10).

In this report the use of single channel recordings in investigating the role of chloroplast envelope components in protein import is described for the first time. The involvement of a chloroplast envelope anion channel in protein import is identified. A hitherto unidentified electrical response of the chloroplast envelope associated with protein import is also described. The implications of both responses for the chloroplast protein import process are discussed.

EXPERIMENTAL PROCEDURES

Precursor Protein—The precursor of the Silene Pratensis stromal protein ferredoxin (prefd) was overexpressed in Escherichia coli and isolated as described before (11). The mutant precursor of ferredoxin (Δ6–14-prefd) is a deletion mutant lacking amino acids 6–14 from the N terminus of the transit sequence. This mutant is shown to be greatly impaired in initial binding and import efficiency in in vitro import into pea chloroplasts (11). The transit peptide used here is a synthetic peptide.

Chloroplast Isolation—Chloroplasts were isolated from pea leaves by cutting them gently with a razor blade in buffer containing 2.5 mM TES/KOH, pH 7.2, 225 mM sorbitol, 25 mM KCl, and 2 mM CaSO₄. The sliced preparation was transferred directly to a 2-ml chamber, which was mounted on a light microscope to allow visual selection of single intact chloroplasts.

Electrophysiological Measurements—For recording the currents across the chloroplast envelope, a standard patch clamp technique was used (12). Electrodes were pulled from borosilicate glass by a two-step pull and extensively fire polished. Electrodes were filled with buffer containing 2.5 mM TES/KOH, pH 7.2, 250 mM KCl, and 2 mM CaSO₄, leading to a 10-fold KCl gradient. Electrode resistances were found to be typically around 20 MΩ. To identify the electrical response of the chloroplast envelope during protein import, precursor protein or transit peptide was added to the pipette-filling solution. Different amounts of MgATP were added to the bath solution in these experiments.

Currents were measured using an EPC-7 (List-Medical) or an Axopatch 200B patch clamp amplifier (Axon Instruments). The data were filtered using an 8-pole Bessel filter (Frequency Devices, or the internal filter of the Axopatch 200B) at a minimum cut-off frequency of 1 kHz. The filtered data were digitized at 10 kHz using a CED 1401+ (Cambridge Electronic Design). The data were analyzed with the patch and voltage clamp software (Cambridge Electronic Design).

Current recordings were made from inside-out patches obtained by lifting the pipette away from the chloroplast after giga-seal formation (12). Potentials are given with regard to the pipette interior; the bath was kept electrically at ground using a 1 m KCl agar bridge. The data are given as mean ± the standard deviation.

RESULTS AND DISCUSSION

In the inside-out patch configuration single channel, recordings of the pea chloroplast envelope could be obtained. Regardless of the direction of the charge movement across the envelope membrane, the opening of protein translocation channels is the cause of the observed response of the envelope.

The implications of both responses for the chloroplast protein import process are discussed.
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FIG. 1. A, single channel recordings of the 50-pS anion channel of the chloroplast envelope at different holding potentials. The upper level of the current trace is that of the fully closed state. B, I/V plot of the channel in the open state in 25/250 mM KCl. The error bars represent the standard deviation of an average of 10 experiments on individual chloroplasts.

conductance levels of MCC, whereas the channel described in this report completely lacks subconductance levels. In a further comparison between the chloroplast and mitochondrion protein import systems it would be interesting to determine if the anion channel described here is also tightly associated with components of the import machinery. MCC can only function normally if associated with a component of the mitondrial import machinery, namely Tim22 (17).

At a MgATP concentration of 50 μM or in the absence of ATP the anion channel was not affected by the presence of prefd in the pipette-filling solution. In Fig. 3 the open time probabilities of the 50-pS anion channel with different pipette-filling solutions and different ATP levels of the bath solution are represented. When 100 μM MgATP was present in the bath solution, prefd decreased the P_o of the channel to a lesser extent than in the presence of 0.5 mM ATP (Fig. 3).

The addition of 0.8 μg/ml of the deletion mutant of preferre- doxin Δ6–14-prefd to the pipette-filling solution in the presence of 0.5 mM ATP in the bath solution did not block the 50-pS channel.

The 50-pS Anion Channel—An anion selective channel in the chloroplast envelope could be identified in the inside-out patch configuration. Small portions of single channel recordings of this channel at different holding potentials are shown as an example in Fig. 1A. Fig. 1B shows an I/V plot of the channel in 25/250 mM KCl. The single channel conductance of the channel as calculated from the slope of the I/V plot is 50 pS. The selectivity of the channel was derived from the reversal potential (V_R), which is +35 mV in the applied 10-fold KCl gradient. At holding potentials above the V_R, the channel did not show any activity (not shown). The channel is mainly in the fully open state at holding potentials lower than V_R, showing few but relatively long closures. The open time probability of the channel is independent of V_H at holding potentials lower then V_R (data not shown).

Several authors have described anion channels in the chloroplast envelope. The properties of the 50-pS anion channel described here differ from those described before. Most of the anion channels described have a considerably larger single channel conductance than the 50 pS found for the channel described here (7–10). Anion channels with smaller single channel conductance found before all have one or more sub-states, in contrast to the channel described here (6, 8).

Effect of Precursor Protein—In the presence of 0.5 mM MgATP in the bath solution, the 50-pS anion channel could be blocked by 0.8 μg/ml precursor of ferredoxin (prefd) in the pipette-filling solution (i.e. the outside of the envelope). This blockade is concluded from a decrease of the open time probability (P_o) of the channel. The P_o was decreased from 0.86 ± 0.10 (n = 10) in the control situation to 0.16 ± 0.08 (n = 5) in the presence of wt-prefd in the pipette solution and ATP in the bath solution. The data on open time probabilities were calculated from the all point amplitude histograms of single channel recordings of individual chloroplasts. The single channel conductance is not affected by the addition of wt-prefd. In Fig. 2A a single gating event taken from two individual single channel recordings is shown to illustrate the effect of wt-prefd on the 50-pS anion channel. Fig. 2B shows as an example the corresponding all point amplitude histograms of the whole single channel recording (approximately 2 min), reflecting the distribution of current levels (15). The all point amplitude histograms in Fig. 2B show that in the presence of wt-prefd the channel was mainly in the closed state. The single channel recordings illustrate that there were few but relatively long openings in the presence of wt-prefd (Fig. 2A). A flickering block as described for the multiple conductance channel (MCC) of the mitochondrion inner membrane (16) was not observed. In contrast to the channel described here, the MCC is slightly cation-specific and has a considerably larger conductance than the chloroplast envelope channel that is blocked by precursor protein. A further difference is the occurrence of multiple sub-
anion channel (Fig. 3). This deletion mutant of preferredoxin has been shown to be greatly reduced in initial binding and import efficiency (11). These results suggest that the decrease in the open time probability caused by prefd is associated with binding of the precursor to the protein import machinery and subsequent translocation across the chloroplast envelope.

The presence of 1.5 μg/ml ferredoxin transit peptide (trfd) in the pipette-filling solution and 0.5 mM ATP in the bath solution also resulted in a decrease of the open time probability of the anion channel. The decrease caused by trfd is, however, less than that caused by prefd (Fig. 3).

**Burst-like Response**—Addition of 0.8 μg/ml of the transport-competent wt-prefd to the pipette-filling solution also causes transient, burst-like increases in conductivity of the chloroplast envelope. A typical example of such a response at a holding potential of 0 mV is represented in Fig. 4. The burst-like increase in the conductivity of the envelope could be observed in about 50% of the single channel recordings, when wt-prefd was present in the pipette-filling solution and 0.5 mM ATP was present in the bath solution.

The addition of the deletion mutant (0.8 μg/ml), Δ6–14-prefd to the pipette solution in the presence of 0.5 mM ATP does not result in a chloroplast envelope response, like that shown by the wild type precursor (not shown). This implies that only a transport-competent precursor can trigger the observed response. This strongly suggests that the envelope response triggered by wt-prefd is a result either of the binding of the precursor to or of the translocation of the precursor across the envelope.

In the presence of 50 μM or 100 μM ATP, which is needed to support binding in an in vitro import assay, prefd is unable to trigger the envelope response. Also in the absence of MgATP in the bath solution, wt-prefd was unable to trigger the envelope response. The dependence of the wt-prefd-triggered response on high ATP concentrations strongly suggests that the re-
sponse is associated with protein translocation across the envelope and not with binding of the precursor to the chloroplast import machinery.

The wt-prefd-triggered burst-like response of the chloroplast envelope is consistent with the conductivity increase observed in whole chloroplast recordings with the chloroplasts of *P. metallica* (4). Recently an increase in permeability has been described for the mitochondrial envelope in response to a presequence (20).

**Implications for Import**—The 50-pS anion channel might represent the protein import channel of the inner membrane. The decrease in open time probability caused by prefd and trfd in the presence of 0.5 mM ATP could be a consequence of the translocation of the precursor or transit peptide through the channel. Because trfd is smaller than prefd, the decrease in open time probability caused by trfd is smaller than that caused by prefd. It is also possible that binding of the precursor protein to the outer membrane import machinery induces a change in conformation of components of this machinery in the intermembrane space. Due to this change in conformation the 50-pS anion channel of the inner membrane could be blocked. Binding of the precursor to the chloroplast has been shown to require low levels of ATP in the intermembrane space (18), whereas translocation of the precursor requires higher amounts of ATP (19). The dependence of the blockade of the anion channel on low amounts of ATP suggests that this blockade is already induced by the binding of prefd to the chloroplast import machinery and that the subsequent translocation of the precursor protein increases the blockade. This and the fact that the 50-pS anion channel is likely to be a component of the inner membrane favor the last possibility as an explanation for the blockade of the channel by prefd.

The dependence of the burst-like response on higher amounts of ATP suggests that this response is the result of translocation of the precursor protein across the envelope rather than only binding to the import machinery. During this translocation the import machineries of the outer and the inner membrane become linked (21). This process might induce a change in orientation between the outer and the inner membrane, e.g. the formation of a contact site. The burst-like response observed could be caused by such a change in orientation of the outer membrane relative to the inner membrane in the patch during protein import. The use of different deletion mutants of prefd with known import properties (11) can give further insight into the mechanisms that lead to the two effects of protein import on the chloroplast envelope observed.

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