The electrophysiology of chloroplast protein import

The involvement of an anion channel in protein translocation across the inner membrane

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The involvement of an anion channel in protein translocation across the inner membrane

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Proefschrift
Ter verkrijging van de graad van doctor
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Voorwoord

Voor u / jou ligt het resultaat van vier jaar werk. Hoewel ik de afgelopen vier jaar niet als werken heb ervaren. Ik heb vier jaar iets mogen doen, waar ik heel veel plezier in heb gehad en ik kreeg er nog voor betaald ook. Mede verantwoordelijk voor het plezier, dat ik heb gehad in het werken aan dit proefschrift zijn alle mensen van het laboratorium voor / de leerstoelgroep Plantenfysiologie. Een aantal van deze mensen verdienen het om hier persoonlijk genoemd te worden.

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Stellingen

1. Een anion kanaal van de chloroplast envelop is betrokken bij chloroplast eiwit import.

   Dit proefschrift (hoofdstuk 2)

2. Het functioneren van een specifiek anion kanaal in de binnenmembraan van de chloroplast envelop is essentieel voor chloroplast eiwit import.

   Dit proefschrift (hoofdstuk 3)

3. Het anion kanaal, dat betrokken is bij chloroplast eiwit import, is onderdeel van de import machinerie van de envelop binnenmembraan.

   Dit proefschrift (hoofdstuk 5)

4. De verplichting voor een promovendus om ten minste zes verdedigbare stellingen bij het proefschrift te voegen, zou tevens een verplichting voor de commissie in moeten houden er ten minste één vraag over te stellen.

5. Een goede methode om de wetenschappelijke literatuur bij te houden is twee uur per dag per trein te reizen.

6. Het voeren van een zogenaamd "zero tolerance" beleid tegen hanggroepjongeren is een vorm van leeftijdsdiscriminatie.

Stellingen behorende bij het proefschrift "The electrophysiology of chloroplast protein import. The involvement of an anion channel in protein translocation across the inner membrane"

Wageningen, 23 november 1999 Paul van den Wijngaard

CONTENTS

Chapter 1

General Introduction

Chloroplasts

Plant cells contain organelles called plastids. Dependent on the tissue type different plastid types are found. In green tissue chloroplasts are present. In these plastids photosynthesis takes place. The chloroplast interior is separated from the cytosol by two membranes, which form the envelope. The interior contains an internal membrane, the thylakoid. The thylakoid membrane is the actual site of photosynthesis. A schematic representation of the chloroplast is shown in Fig. 1. The outer envelope membrane contains large pore like proteins, which allow the passage of solutes with a molecular mass of less than approximately 10 kDa (1). The osmotic barrier between the chloroplast interior (the stroma) and the cytosol is formed by the inner envelope membrane. The intermembrane space between the two envelope membranes remains an unexplored territory. No proteins are yet described to be located in the intermembrane space. The physiological relevance of this compartment is therefore unclear. This is in contrast to the situation in mitochondria, where proteins in the intermembrane space were found to be essential for mitochondrial biogenesis (2-4). The chloroplast stroma however contains different enzymes, as does the thylakoid lumen (the space surrounded by the internal thylakoid membrane). In the chloroplast stroma DNA is also found. This DNA encodes for a small number of the proteins found in the chloroplast. To understand this semi-autonomous nature of the organelle it is important to consider the evolutionary process that has led to its existence. It is thought that an endosymbiotic event between an early eukaryotic cell and a photosynthetic prokaryote was the first onset of the evolution of chloroplasts. In the course of the evolution the majority of the original prokaryote genes were transferred to the nuclear DNA. Along with this gene transfer, the necessity of an

efficient pathway to import the proteins, that were now nuclear encoded and synthesised in the cytosol, arose.

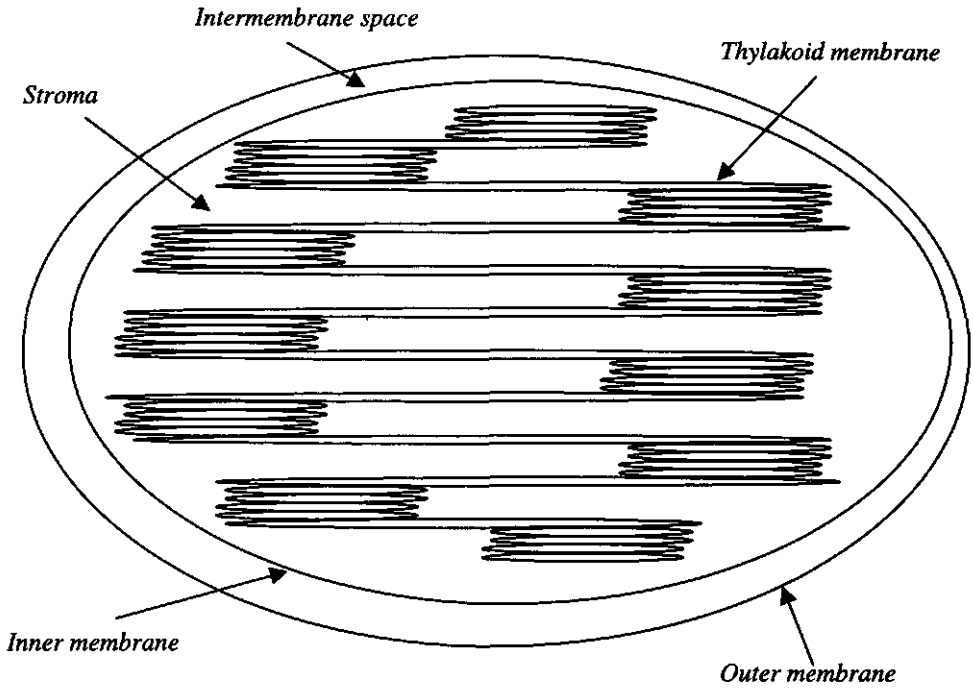

Fig. 1 Schematic representation of a chloroplast

Chloroplast protein import

Chloroplast proteins that are nuclear encoded, and therefore have to be translocated across the envelope, are synthesised as precursors with an N-terminal extension, termed transit sequence. The transit sequence is both necessary and sufficient for protein targeting to the chloroplast (5). After translocation into the stroma the transit sequence is removed from the precursor by a specific peptidase (6). The transit sequence consists of four distinct functional domains. The first domain is

important for the initial binding of the precursor to the chloroplast envelope. This domain contains the extreme N-terminal amino acids of the transit sequence. The second domain, corresponding to amino acids 15 to 25 in the precursor of ferredoxin, is important in full translocation of the precursor. Initial recognition and binding is however unaffected by deletions in this region. The third region, roughly containing amino acids 26 to 38 is relatively unimportant for transit sequence functioning. Deletions in this region do however affect the overall efficiency of import. The last region of the transit sequence plays a role in the final processing of the precursor (i.e. the removal of the transit sequence by the stromal processing peptidase). (7)

The translocation of precursor proteins across the chloroplast envelope is an energy-dependent process. The import of proteins into chloroplasts utilises ATP to meet its energy demand (8). In contrast to the situation in mitochondria there is no need for a proton-motive force in chloroplast protein import (9). The first step in chloroplast protein import is the binding of the precursor protein to the outer surface of the organelle. This binding step was found to require ATP (10) or GTP (11, 12). The site of utilisation of the NTP in the initial binding step is the intermembrane space (11). The subsequent step in protein import is the translocation of the precursor across the outer membrane. This step can only be driven by ATP, that is provided in the intermembrane space (13). Translocation across the inner envelope membrane requires ATP in the chloroplast stroma (9, 13).

The import machinery

It was shown that both chloroplast envelope membranes have independent protein import machineries (13), termed Translocon of the Outer membrane of Chloroplasts (Toc) and Translocon of the Inner membrane of Chloroplasts (Tic), respectively (14).

Several components of both translocons have been identified on a molecular level. A model of protein import is shown in Fig. 2.

The import receptor on the outer surface of the chloroplast was identified as a 86 kDa protein of the outer membrane and termed Toc86 (15-17). Recently, however, it has been found that Toc86 is a proteolytic fragment of a larger protein of 159 kDa (18). Here the name Toc86 will be used, because only work is described where the protein will be digested to the 86 kDa fragment. Toc86 was found to be a GTP binding protein (12). Another GTP binding protein of the chloroplast outer membrane was also found to be involved in protein import, Toc34 (12, 19). It is thought that Toc86 and Toc34 cooperate in the initial binding of the precursor protein to the chloroplast (20). Toc86 and Toc34 are thought to function in a larger multisubunit complex in the chloroplast outer membrane (19, 21). The other constituent of this complex is Toc75 (15, 17, 20). Toc75 has been proposed to be the protein conducting channel of the translocon of the chloroplast outer membrane. It could be shown that the reconstituted Toc75 has ion channel activity (22). Two Hsp70 homologues were also shown to be part of the outer membrane import machinery. Both are associated with the outer membrane. One is facing the cytosol (23, 24) the other one is exposed to the intermembrane space (17, 25).

The function of components involved in translocation of precursors across the inner envelope membrane is less clear. First of all there is Tic22 (20), this protein is a peripheral membrane protein of the inner membrane facing the intermembrane space (26). It is thought to route the precursor protein to the translocation channel of the inner membrane when it has inserted across the outer membrane. Another component of the translocon of the inner membrane is Tic20 (20, 21). This protein is an integral membrane protein of the inner membrane (26) of unknown function.

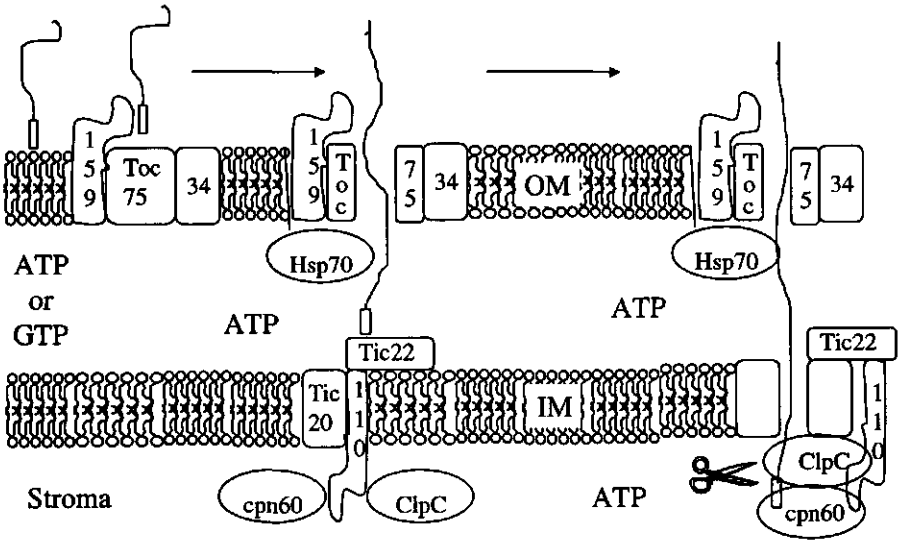

Fig. 2 Model for protein import into chloroplasts

A protein of 55 kDa containing a Rieske-type iron sulphur cluster was also proposed to be part of the Tic complex (27). Finally a integral inner membrane protein of 110 kDa is part of the Tic complex (17, 28, 29). Tic110 contains a large hydrophilic part that faces the chloroplast stroma and is therefore thought to function as a recruiting site for stromal factors (30). Two soluble stromal chaperons are shown to be involved in protein import. These proteins are cpn60 (31) and a Hsp100 homologue, ClpC (27, 29, 32). Both of these stromal proteins were shown to interact with Tic110.

It has been found that a small fraction of the Toc and Tic complexes are preformed in the envelope in the absence of precursor (29, 32). This fraction was shown to form a large Toc-Tic supercomplex, which contains all the components described thusfar (26). Upon interaction with a precursor protein the remainder of the Toc and Tic complexes are assembled. Translocation of the precursor across both the outer and the inner membrane has been shown to occur simultaneously, as illustrated

by the finding that a precursor protein spans both envelope membranes while translocating (33, 34). A role for chloroplast specific lipids in the outer membrane has been suggested in the correct targeting of precursors to the chloroplast and in the initial interaction with the outer surface (35-40). It has also been suggested that interaction of the transit sequence with lipids induces the correct secondary structure of the transit sequence for efficient import (41).

A homologue of Toc75 is also found in the cyanobacterium *Synechocystis* (42). This homologue was shown to have comparable ion channel activity as the higher plant Toc75 (18).These findings support the hypothesis that the chloroplast has evolved from a photosynthetic prokaryote taken up by a primitive eukaryote. It also shows that the protein import machinery used by the chloroplast is an adapted version of a transport system already present in the prokaryotic ancestor.

The patch clamp technique and ion channels

The so called patch clamp technique allows the study of ion transport across a biological membrane on the level of individual ion channels (43). The narrow tip of a heat-polished glass pipette is pressed against a membrane surface. When subsequently suction is applied to the pipette interior, a seal between the membrane and the glass is formed, resulting in a very high electrical resistance (10-100 GΩ). The patch at the tip of the pipette is now electrically isolated from the rest of the membrane and can be voltage clamped. Due to the high resistance the leak current becomes of the same order of magnitude as currents passing through individual ion channels. This configuration is called cell attached patch (CAP, Fig. 3). When the pipette is moved away from the cell, the patch is separated from the rest of the membrane, resulting in an inside out patch (IOP). When additional suction or a short electrical pulse is

applied to the pipette interior in the CAP, the membrane patch is ruptured. The configuration obtained by this procedure is termed whole cell (WC) configuration. In the WC configuration the entire membrane is voltage clamped and the measured currents are the result of the action of the different ion transporters present in the membrane. The WC configuration is also the starting point of the fourth possible patch clamp configuration. Moving the pipette away from the cell in the WC configuration results in the isolation of the ruptured patch from the membrane. This causes a spontaneous fusion of the remaining membranes on the outside of the pipette, resulting in a new patch, called an outside out patch (OOP). The experiments described in this thesis mainly make use of the IOP configuration; a limited number of experiments were performed in the CAP configuration. Three of these four configurations (CAP, IOP, and OOP) allow the current passing through single ion channels to be measured directly.

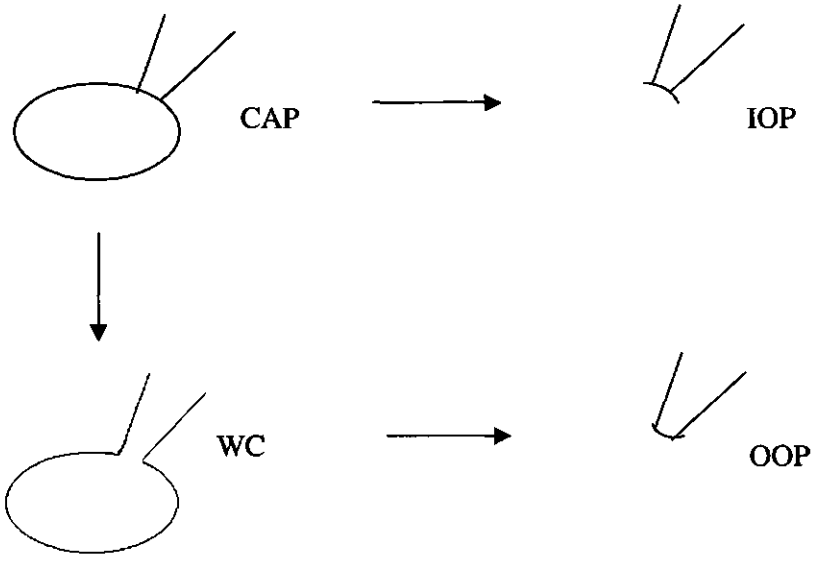

Fig. 3 Different patch clamp configurations

Ion channels are membrane protein structures that allow the passage of ions at rates in the range between 10^6 and 10^8 s^{-1}. The transport is not directly coupled to the input of free energy, i.e. the transport is 'passive'. These properties suggest a simple view of ions diffusing through a pore in the membrane. Ion channels do however show a certain degree of selectivity. This means that a particular ion channel only allows the passage of one particular ion or a class of ions, e.g. K^+ or anions. Selectivity implies, that the channel must contain sites for recognition of the permeating ion, proving the view of a diffusion pore to be too simple. Selectivity for a particular ion varies greatly among different channels. Some channels show a very strict selectivity for one ion, while others hardly show any selectivity at all. Ion selectivity can be used as a characteristic to describe an ion channel.

Another obvious characteristic to describe channels is their conductance. Given the ionic conditions, the conductance of a channel is considered to be fixed. Some ion channels show multiple conductance levels between the non-conducting and the maximum conductance level. Such levels are called sub-conductance levels. A third characteristic of ion channels is their kinetic behaviour. Channels can switch from the closed (non-conducting) to the open (conducting) state almost instantaneously (in a time well below the resolution of the patch clamp technique). After opening or closing the channel can reside in the open or closed state for a limited amount of time. The mechanism of opening and closing of ion channels is called channel gating. Gating can be affected by ligands or by changes in membrane potential.

Ion channels are found in the plasma membranes of cells of all kinds of organisms, such as bacteria (e.g. 44), animals (e.g. 43), and plants (e.g. 45). Furthermore ion channels are observed in membranes of organelles such as mitochondria (e.g. 46), vacuoles (e.g. 47), and chloroplasts (e.g. 48). The

omnipresence of ion channels suggests that they have evolved early in evolution. This illustrates the importance of ion channels for living cells.

Ion channels of the chloroplast envelope

The outer envelope membrane of the chloroplast has been found to contain pore forming activity. This activity can be partly ascribed to a porin like channel of large conductance found in this membrane (1). The single channel conductance of this channel (in 100 mM KCl) was found to be 720 pS. Two other large conductance channels are also described to be present in the chloroplast outer envelope, showing conductances of 520 and 1020 pS, respectively (49, 50). Both of these channels are slightly cation selective. The 520 pS channel was suggested to be related to the Voltage Dependent Anion Channel found in the mitochondrial outer membrane (51). Recently a general solute channel of high conductance was found in the outer chloroplast envelope membrane (52). This channel is also slightly cation selective and it is likely to represent the porin activity found in the outer membrane. Besides this general solute channel highly specialised channels are also present in the outer envelope membrane. One example is Toc75, which is considered to be the protein translocating channel (22). Another specialised channel is a high conductance channel that is very selective for the passage of amino acids (53).

A high conductance (~500 pS) channel was also described to be present in the inner envelope membrane of chloroplasts (50, 54). This channel was shown to be anion selective and strongly voltage dependent. Under physiological conditions the channel is thought to be closed, which is consistent with the permeability barrier function of the inner envelope membrane. An anion channel of smaller conductance (~60 pS) has also been found in the inner membrane (50). The exact function of this

channel is presently not clear. It was also shown that two translocators present in the chloroplast inner envelope membrane can function as ion channels. First of all the triose phosphate / phosphate translocator was shown to have anion channel activity (55). The second translocator that was shown to have ion channel activity is the 2-oxoglutarate / malate translocator (50).The physiological relevance for this translocator ion channel activity remains unclear. A K^+-selective channel with a single channel conductance of approximately 100 pS is also observed in the inner membrane (50, 56-58). This channel is thought to function in the H^+ / K^+ counterflux across the envelope, which is important in the regulation of the stromal pH and photosynthesis. Indirect evidence has been provided for the existence of a Ca^{2+}-selective ion channel in the inner envelope membrane (59).

Scope of this thesis

The study described in this thesis was aimed at identifying and understanding the electrophysiological response of the chloroplast envelope during protein import. For this purpose the patch clamp technique was used. Advantage was taken of the known import characteristics of different deletion mutants of the precursor protein of ferredoxin (7).

Chapter 2 describes the identification of a anion channel of the inner membrane, that is involved in chloroplast protein import. The channel is inactivated by the addition of a precursor protein. The inactivation is shown to be dependent on the availability of ATP and the presence of a functional transit sequence.

In chapter 3 the intermediate step in protein import that leads to the inactivation of the channel described in chapter 2 is unravelled. The channel has been named Protein Import Related Anion Channel (PIRAC). The possible involvement of stromal

factors in channel gating is investigated. Finally this chapter describes the correlation between anion channel activity and protein import by using the anion channel blocker DIDS. It is found that the particular channel described is inactivated upon association of the translocating precursor with Tic. Stromal factors were found to be not involved in channel gating or in channel inactivation during protein import. Blocking the channel with the anion channel blocker DIDS causes an inhibition *of in vitro* protein import.

The experiments described in chapter 4 were aimed at investigating the mechanism of channel inactivation during protein import. Precursor protein induced channel inactivation was found to be caused by the induction of a long lived closed state of the channel. It is found that there is a direct interaction between the precursor protein and the channel. Furthermore the ability of the precursor to another chloroplast protein (the small subunit of Rubisco) was shown to induce an inactivation of PIRAC identical to the inactivation induced by preferredoxin.

In chapter 5 some gating characteristics of the channel are described. Furthermore a possible association of the channel with Tic is investigated using antibodies to a known component of Tic. A first approximation of the pore size of the channel, using the gating characteristics, reveals that the channel dimensions are comparable to the dimensions of the protein conducting channel of the outer envelope membrane. The channel is found to be associated with Tic, based on the ability of antibodies to Tic110 to irreversibly inactivate the channel.

Finally in chapter 6 the characteristics of the channel will be discussed in relation to protein import. Possible roles of this channel in protein translocation will be discussed in view of the experimental evidence provided in the preceding chapters.

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A 50 pS anion channel of the chloroplast envelope is involved in chloroplast protein import

ABSTRACT

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 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 preFd , Δ6-14-preFd, that 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.

INTRODUCTION

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 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 the response of the envelope during protein import. An increase in envelope conductivity during protein import is shown to occur in *Peperomia metallica* chloroplasts (4). This increase was identified using electrophysiological measurements in the whole chloroplast configuration. It was suggested that the opening of protein translocation channels is the cause of the observed response of the envelope.

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 implication 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 to 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 leafs 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 $CaSO_4$. 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 firepolished. Electrodes were filled with buffer containing: 2.5 mM TES/KOH, pH 7.2, 250 mM KCl and 2 mM $CaSO_4$, 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, FRG) or an Axopatch 200B patch clamp amplifier (Axon Instruments, USA). The data were filtered using an 8-pole Bessel filter (Frequency Devices, USA or the internal filter of the Axopatch 200B) at a minimum cut-off frequency of 1 kHz. The filtered data were digitised at 10 kHz using a CED 1401+ (Cambridge Electronic Design, UK). Data were analysed with the Patch and Voltage Clamp Software (Cambridge Electronic Design, UK).

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 at ground, using a 1 M KCl agar bridge. 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. Regarding the high seal resistances it is highly unlikely, that the seals consist of the outer membrane alone, due to the abundance of large pores in the outer membrane (13). No light-induced currents (14) were ever observed directly after making the patch (i.e. in the attached configuration) or after excision of the patch. This indicates that the thylakoid membrane was not included in the patch. The observed current is likely to run mainly across the inner membrane in a seal consisting of a sandwich like structure of the outer and the inner membrane.

A 50 pS anion channel was found to be involved in protein import. The channel could be blocked by the precursor of the stromal protein ferredoxin in the presence of ATP. Preferredoxin also induced a burst-like increase in the envelope conductivity. Activity of other ion channels could be observed only very rarely under the experimental conditions used here.

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.

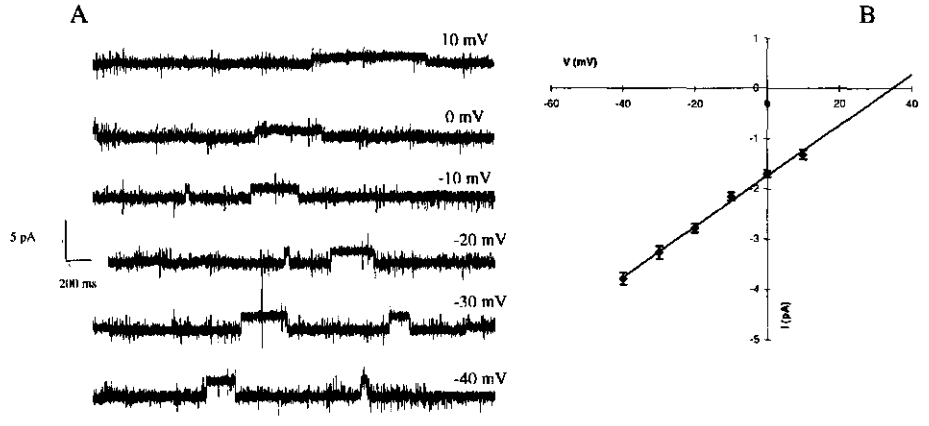

Fig. 1 **A**: Single channel recordings of the 50 pS anion channel of the chloroplast envelope at different holding potentials. The upper level of the current trace is that of the fully closed state. **B**: I/V plot of the channel in the open state in 25/250 mM KCl. Error bars represent the standard deviation of an average of ten experiments on individual chloroplasts.

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 substates, 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, reflecting the distribution of current levels (15), of the whole single channel recording (approximately 2 minutes). The all point amplitude histograms in Fi. 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 it has a considerably larger conductance than the chloroplast envelope channel that is blocked by precursor protein. A further difference is the occurrence of multiple subconductance levels of MCC, while 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 mitochondrial import machinery, namely Tim22 (17).

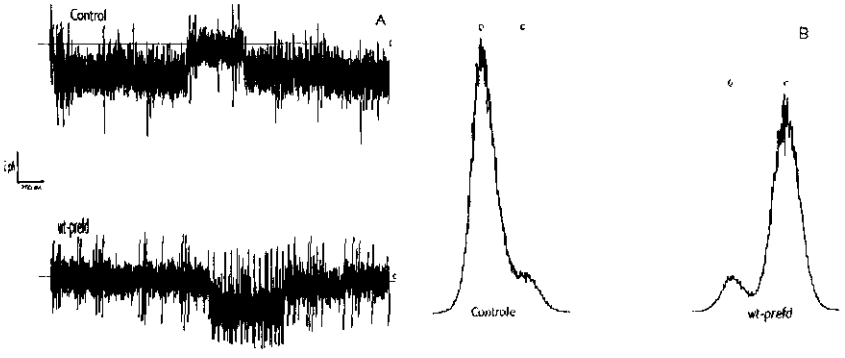

Fig. 2 **A**: Single gating event taken from single channel recordings of the 50 pS anion channel in the absence and in the presence of wt-preFd. V_H = 0 mV. **B**: All point amplitude histograms of the channel

in the presence and in the absence of wt-preFd. V_H = 0 mV. These histograms indicate a decrease in the open time probability (P_o) of the channel from 0.86 to 0.16 in the presence of wt-preFd and ATP. The characters c and o mean closed and open states of the channel respectively

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 (see Fig. 3).

The addition of 0.8 μg/ml of the deletion mutant of preferredoxin, 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 anion channel (see 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 (tpFd) 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 tpFd is however less than that caused by preFd (see Fig. 3).

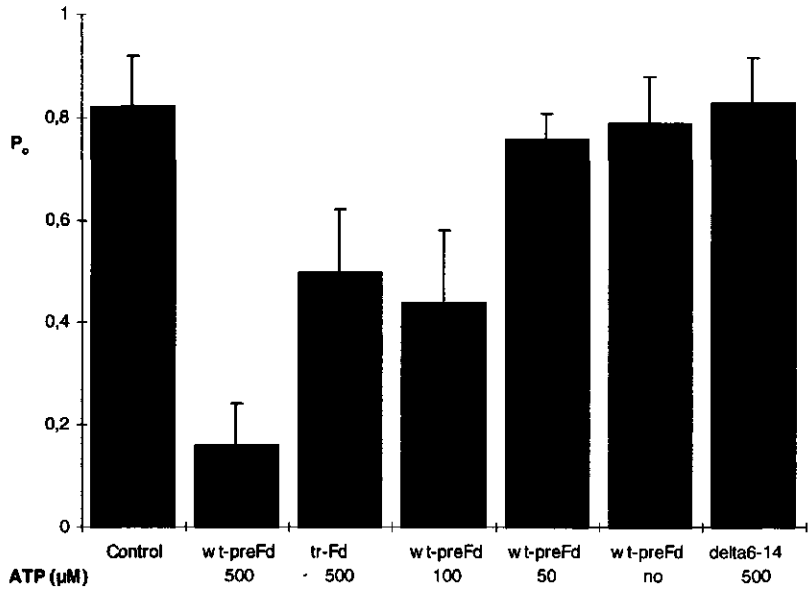

Fig. 3 Open time probability the 50 pS anion channel with different pipette filling solutions and different ATP levels in the bath solution. The error bars represent the standard deviation of the average of observed open time probabilities in single channel recordings with indicated pipette filling solution and ATP level in the bath solution. n refers to the number of recordings with individual patches of different chloroplasts.

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.

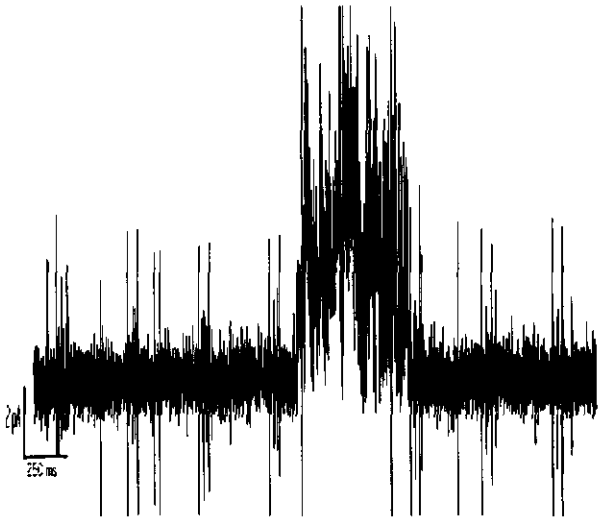

Fig. 4 Typical example of a burst-like increase in envelope membrane conductivity induced by the presence of 0.8 μg/ml wt-preFd in the pipette filling solution, in the presence of 0.5 mM ATP in the bath solution. V_H = 0 mV.

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 dependency of the wt-preFd triggered response on high ATP

concentrations strongly suggest that the response 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 *Peperomia metallica* (4). Recently an increase in permeabillity has also 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 tpFd 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 tpFd is smaller than preFd the decrease in open time probability caused by tpFd 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), while translocation of the precursor requires higher amounts of ATP (19). The dependency of the blockade of the anion channel on low amounts of ATP suggest 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 favours the last possibility as an explanation for the blockade of the channel by preFd.

The dependency 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 which lead to the two effects of protein import on the chloroplast envelope observed.

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Functioning of the protein import related anion channel is essential for protein import

ABSTRACT

The ability of preferredoxin to inactivate a 50 pS anion channel of the chloroplast inner membrane in the presence of an energy source was investigated using single channel recordings. It was found that preferredoxin cannot inactivate the channel when GTP is the only energy source present. From this it is concluded that the precursor has to interact with the Tic complex to be able to inactivate the 50 pS anion channel. The ability of two mutants of preferredoxin, with deletions in their transit sequence, to inactivate the channel was also tested. Both mutants have been shown to have a similar binding affinity for the chloroplast envelope, but only one is able to fully translocate. The mutants were both able to inactivate the channel in a comparable manner. From this it is concluded that full translocation is not necessary for the inactivation of the channel. It is also shown that preferredoxin is capable of inactivating the 50 pS anion channel in the chloroplast attached configuration as was previously found in the inside-out configuration. From this it is concluded that stromal factors do not influence the protein import induced inactivation of the 50 pS anion channel of the chloroplast inner membrane. Finally the effect of the anion channel blocker DIDS on the channel activity and on protein import was investigated. It is found that DIDS blocks the channel. Furthermore the addition of the channel blocker reduces that efficiency of import to 52 %. This leads to the conclusion that correct functioning of the channel is required for protein import.

INTRODUCTION

Chloroplasts are surrounded by two membranes. 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 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 (for recent reviews see Refs. 2 and 3). The two surrounding membranes both have their own import machinery, that can function independent of each other (4). The outer membrane machinery has been termed Toc (translocon of the outer membrane of chloroplasts) and the inner membrane machinery has been named Tic (translocon of the inner membrane of chloroplasts) (5).

The involvement of an anion channel of the chloroplast envelope in protein import was identified in chapter 2. This envelope channel, which is located in the inner membrane, will be called Protein Import Related Anion Channel (PIRAC) here. The PIRAC was shown to be inactivated, i.e. the open probability of the channel (P_O) decreased, by the addition of precursor protein. The inactivation was found to be dependent on ATP and the presence of a functional transit sequence. The exact role of PIRAC in chloroplast protein import is however not known yet.

Several steps can be distinguished in protein import into chloroplasts. These steps are distinguishable on the basis of their different energy requirement (for review see Ref. 2). First of all the precursor protein can associate with the Toc complex on the outer surface of the chloroplast. This step is energy independent and therefore does not require the hydrolysis of NTPs. After association with the outer surface of the chloroplast the precursor can translocate across the outer membrane. This

translocation is dependent on NTP, it can be stimulated by either ATP or GTP. The subsequent association of the precursor with the Tic complex requires the hydrolysis of ATP. Higher amounts of ATP are needed for full translocation across the inner membrane. The final step in chloroplast protein import is the removal of the transit sequence by the processing peptidase in the stroma.

In this report the involvement of PIRAC in chloroplast protein import is studied in more detail. Single channel recordings are made in the inside-out configuration and in the chloroplast attached configuration. To clarify the inactivation of PIRAC during protein import use was made here of two deletion mutants of the precursor of the stromal protein ferredoxin, with known in vitro import characteristics (6). Furthermore the NTP dependence of the precursor induced inactivation of PIRAC was investigated. Finally the effect of the anion channel blocker DIDS on PIRAC and protein import was measured.

EXPERIMENTAL PROCEDURES

Precursor protein

The precursor of the *Silene Pratensis* stromal protein ferredoxin (preFd) was overexpressed in *Escherichia coli* and isolated as described before (6). Two mutant precursors of ferredoxin were used. Both are deletion mutants lacking amino acids of the transit sequence, Δ15-25 lacks amino acids 15 to 25 from the N-terminus and Δ35-42 lacking amino acids 35 to 42 from the N-terminus. The Δ15-25 mutant is shown to be inactive in *in vitro* import into pea chloroplasts. It does however compete with the wild type precursor for binding sites (6). The Δ35-42 deletion mutant can be imported in an *in vitro* import assay; this mutant also competes with the wild type precursor for binding sites (6).

Chloroplast isolation

Chloroplasts for electrophysiological experiments 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 $CaSO_4$. 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. Chloroplast for *in vitro* import experiments were isolated as has been described before (7).

Electrophysiological measurements

For recording the currents across the chloroplast envelope a standard patch clamp technique was used (8). Electrodes were pulled from borosilicate glass by a two step pull, and extensively firepolished. Electrodes were filled with buffer containing: 2.5 mM TES/KOH, pH 7.2, 250 mM KCl and 2 mM $CaSO_4$, leading to a 10-fold KCl gradient. Electrode resistances were found to be typically around 30 MΩ. To clarify the inactivation of PIRAC during protein import, precursor proteins were added to the pipette filling solution (i.e. the outside of the chloroplast envelope). ATP or GTP was added to the bath solution at least 5 minutes before electrophysiological experiments were started.

Currents were measured using an Axopatch 200B patch clamp amplifier (Axon Instruments, USA). The data were filtered at a cut-off frequency of 1 kHz, using an 8-pole Bessel filter (internal filter of the Axopatch 200B). The filtered data were digitized at 10 kHz using a CED 1401+ (Cambridge Electronic Design, UK). Data were analyzed with the Patch and Voltage Clamp Software (Cambridge Electronic Design, UK).

Current recordings were made from inside-out patches, obtained by moving the pipette away from the chloroplast after giga-seal formation, or from chloroplast attached patches (8). Potentials are given with regard to the pipette interior, the bath was kept at ground, using a 250 mM KCl agar bridge. Data are given as mean ± the standard deviation.

In vitro import experiments

Import assays were performed essentially as described before (6). Briefly, ^{3}H-leucine labelled preFd was prepared in an *in vitro* transcription translation. Different concentrations of DIDS were added to the isolated chloroplast suspension. Import experiments were started by adding translation mixture to the chloroplasts. The samples were incubated for 30 minutes at 25°C in the light to allow import.

RESULTS

Regarding the high seal resistances it is highly unlikely, that the seals consist of the outer membrane alone, due to the abundance of large pores in the outer membrane (9). No light-induced currents (10) were ever observed directly after making the patch (i.e. in the attached configuration) or after excision of the patch. This indicates that the thylakoid membrane was not included in the patch. The observed current is likely to run mainly across the inner membrane in a seal consisting of a sandwich like structure of the outer and the inner membrane.

NTP dependence of PIRAC inactivation

The NTP dependence of the precursor mediated inactivation of PIRAC was clarified by adding GTP, together with an equimolar amount of Mg^{2+}, to the pipette

filling solution. The precursor of ferredoxin is unable to inactivate the channel in the presence of GTP. When 0.8 µg/ml wt-preFd was present in the pipette filling solution together with 0.5 mM GTP there was no significant decrease in P_O of PIRAC as compared to the control without preFd in the pipette filling solution. Fig. 1A shows small parts of single channel recordings of PIRAC in the presence of 0.5 mM GTP in the pipette solution and in the absence or presence of 0.8 mg/ml wt-preFd in the pipette filling solution respectively. The P_O was calculated from the all point amplitude histograms of the entire single channel recordings, which are shown in Fig. 1B (11). Preincubation of the isolated chloroplast in bath solution containing 0.5 mM GTP and Mg^{2+} did also not result in a precursor protein induced inactivation of PIRAC (not shown).

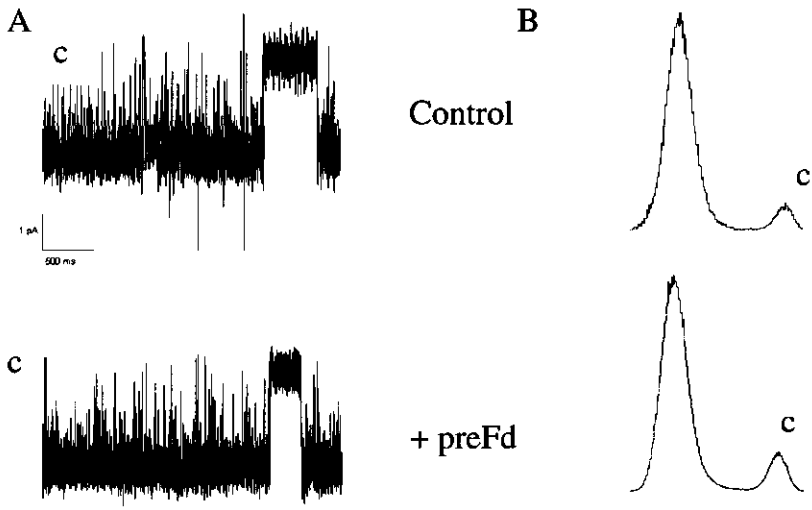

Fig. 1. A: Single channel recordings of PIRAC in the inside-out patch configuration, with 0.5 mM GTP in the pipette filling solution, in the absence and presence of wt-preFd. Recordings were made at a holding potential of -20 mV. **B**: All point amplitude histograms of PIRAC, with 0.5 mM GTP in the pipette filling solution, in the absence and presence of wt-preFd in the pipette filling solution. The

histograms show that wt-preFd is unable of deactivating PIRAC with GTP as an energy source. The character *c* indicates the closed state of the channel.

Deletion mutants

The deletion mutant, Δ15-25-preFd, inactivates PIRAC in the presence of ATP. The P_O is decreased from 0.82±0.1 (n=10) in the control situation to 0.36±0.09 (n=16) with Δ15-25-preFd in the pipette filling solution. Fig. 2A shows parts of single channel recordings of PIRAC in the control situation and with Δ15-25-preFd added to the pipette filling solution. Open probabilities were calculated from all point amplitude histograms of single channel recordings, examples of these are shown in Fig. 2B. The deletion mutant Δ35-42-preFd does also inactivate. The P_O of PIRAC in the presence of Δ35-42-preFd in the pipette filling solution was 0.28±0.11(n=19) (Fig. 3).

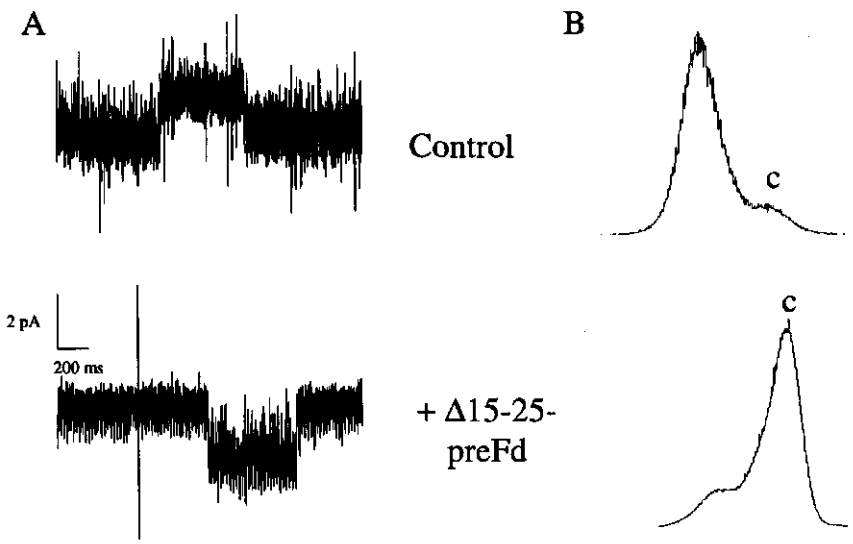

Fig. 2. A: Single channel recordings of PIRAC, in the inside-out patch configuration, with 0.5 mM MgATP in the bath solution, in the absence and presence Δ15-25-preFd in the recording pipette.

Recordings were made at a holding potential of 0 mV. **B**: All point amplitude histograms of single channel recordings of PIRAC in the absence and presence of Δ15-25-preFd. The all point amplitude histograms indicate a decrease in P_O of PIRAC from 0.86 to 0.36 in the presence of Δ15-25-preFd. The characters *c* means closed of PIRAC.

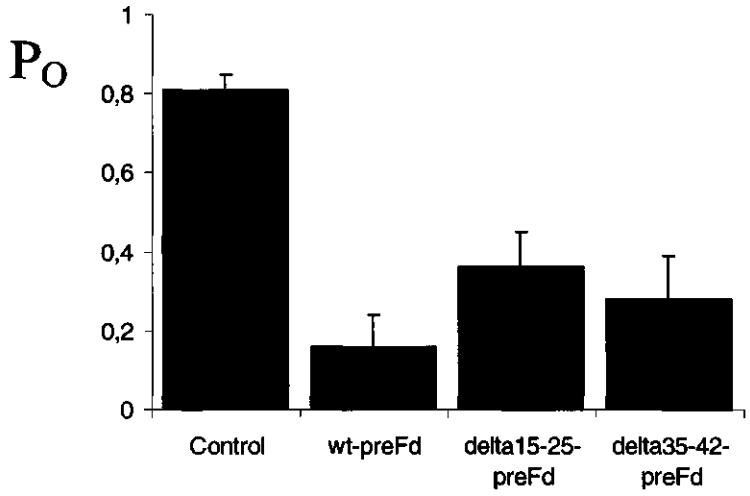

Fig. 3. Open time probability of PIRAC in the absence and presence of different precursor proteins in the pipette filling solutions, respectively. Error bars represent the standard deviation.

Influence of stromal factors

Stromal components are known to be involved in protein import into chloroplasts (12-14). These stromal factors might influence the gating behavior of PIRAC, both in the control situation and during inactivation by a precursor protein. To identify the effect of stromal factors on the gating behavior of PIRAC single channel recording were performed in the 'chloroplast attached' configuration (8). In this configuration the patch of membrane, across which the current is measured, is not excised from the whole membrane and the chloroplast remains intact during the measurement. In the

chloroplast attached configuration the single channel conductance of PIRAC is found to be 50 pS (data not shown) and is identical to the single channel conductance measured in the inside-out configuration (15). The 50 pS single channel conductance of PIRAC in both recording configurations is determined by the 250 mM KCl concentration in the pipette filling solution.

Fig. 4 shows the gating behavior of PIRAC in the chloroplast attached configuration. The influence of preFd on the gating behavior in this configuration is also shown in Fig. 4. In the chloroplast attached configuration the P_O of PIRAC in the control situation is 0.89±0.09 (Fig. 4B). This is identical to the P_O of PIRAC in the inside-out configuration. When 0.8 µg/ml wt-preFd was included in the pipette filling solution the P_O of PIRAC was decreased to 0.27±0.09 (Fig. 4B).

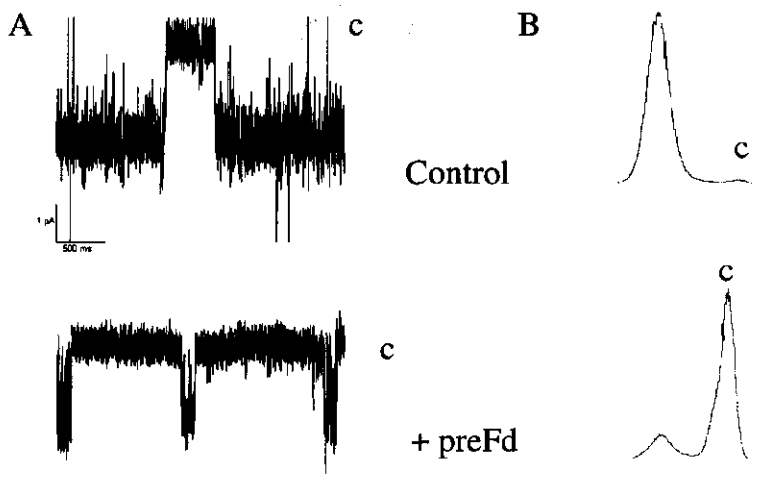

Fig. 4. A: Single channel recordings of PIRAC in the chloroplast attached configuration in the absence and presence of preFd in the pipette filling solution. Recordings were made at a holding potential of –20 mV, with 0.5 mM MgATP in the bath solution. **B**: All point amplitude histograms of PIRAC in the chloroplast attached configuration in the absence and presence of preFd. The histograms show that the

inactivation of PIRAC in the chloroplast attached configuration is similar to that in the inside-out configuration reported earlier (15). The character *c* indicates the closed state of PIRAC.

Effect of DIDS on PIRAC activity

DIDS is a known inhibitor of anion channels (16). From the single channel recordings in the presence of 10 or 50 μM DIDS, shown in Fig. 5, it is clear that DIDS is able to block PIRAC activity. The addition of DIDS to the cytosolic side of the envelope patch changes the gating properties of PIRAC: the long open intervals present in the control situation are transformed into flickering bursts of channel opening. When 10 μM DIDS was added to the pipette filling solution together with preFd, flickering bursts of channel opening were accompanied by long inactive periods of the channel (not shown). This indicates, that DIDS and preFd can interact with PIRAC simultaneously. When a concentration as high as 100 μM of DIDS was used channel activity could only be observed for several tens of seconds after formation of the inside out patch. In this relatively short period of PIRAC activity the channel opened in short flickering burst, typical for DIDS block. After several tens of seconds single channel activity completely disappeared. The disappearance of channel activity, channel rundown, also occurs in control measurements. However in the control situation channel rundown is very rare and never associated with flickering bursts of channel activity. The disappearance of PIRAC activity in the presence of 100 μM DIDS therefore must be ascribed to DIDS block.

Fig. 6 shows the open probability of PIRAC as a function of the holding potential in the absence and presence of 10 μM DIDS, respectively. This figure shows, that in the control situation the P_O is independent of the V_H in the range shown. In the

presence of DIDS however the P_O decreases with increasing V_H. This indicates that PIRAC blocking by DIDS is voltage dependent.

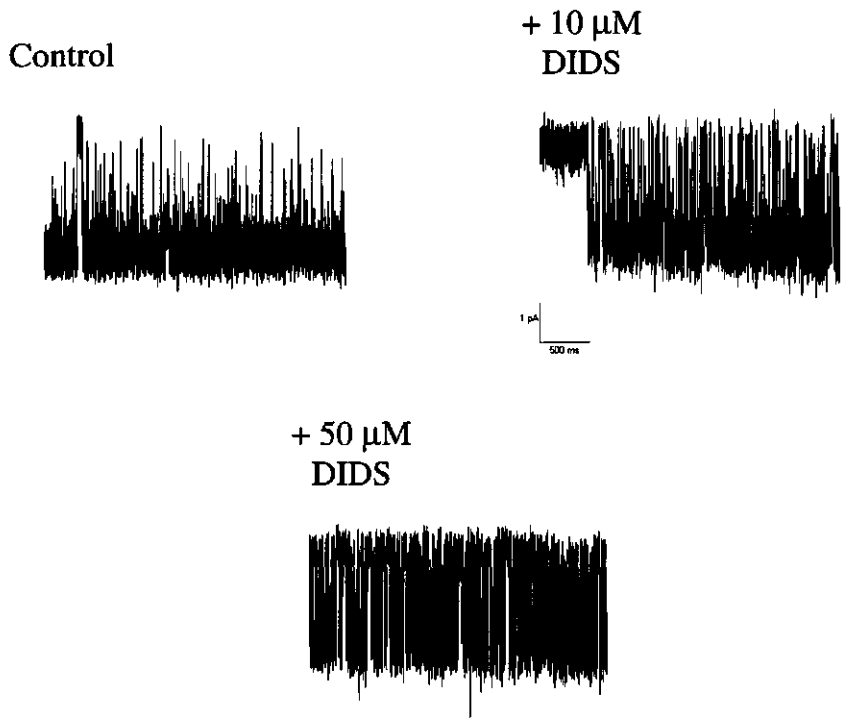

Fig. 5. Single channel recordings of PIRAC in the absence and presence of 10 µM and 50 µM DIDS, respectively. Recordings were made at a holding potential of –20 mV. The single channel recordings show an increase in 'flickering' behavior with increasing DIDS concentration.

Effect of DIDS on in vitro import

Addition of DIDS resulted in a decrease in import efficiency preFd into isolated pea chloroplasts. The import of preFd was decreased to 52% of the import in the control situation by the presence of 100 µM DIDS. The import efficiency of preFd in the presence of different concentrations of DIDS relative to the control situation is

shown in Fig. 7. The figure shows that the DIDS induced decrease in import efficiency is concentration dependent.

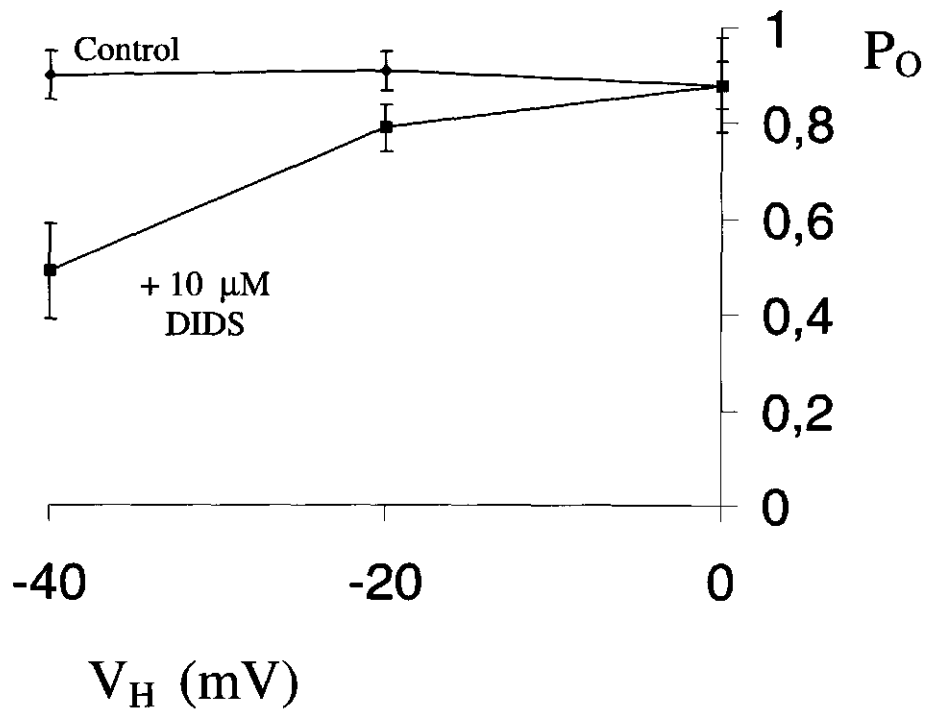

Fig. 6. Open probability of PIRAC as a function of the holding potential in the absence (◆) and presence (■) of 10 µM DIDS, respectively.

DISCUSSION

The binding of a precursor protein to the outer surface of a chloroplast has been found to be stimulated in the presence of GTP (4, 17). The subsequent translocation across the outer membrane (and further steps of protein import) has been shown to require the hydrolysis of ATP (17). However it was suggested recently that GTP can induce the translocation of a precursor protein across the outer membrane as well, but

that for a stable association with the inner membrane ATP is needed (18). Further it was shown before that in the presence of ATP wt-preFd is able of decreasing the P_O of PIRAC in identical experiments as those performed in this study (15). It is shown here, that wt-preFd is unable to decrease the P_O of PIRAC with GTP as an energy source (Fig. 1). From this it can be concluded, that the precursor protein at least has to cross the outer envelope membrane before it is able to inactivate PIRAC. The recent finding that GTP can induce the translocation across the outer membrane (18), together with the results presented here, suggest that the precursor has to interact with the Tic complex before PIRAC is inactivated.

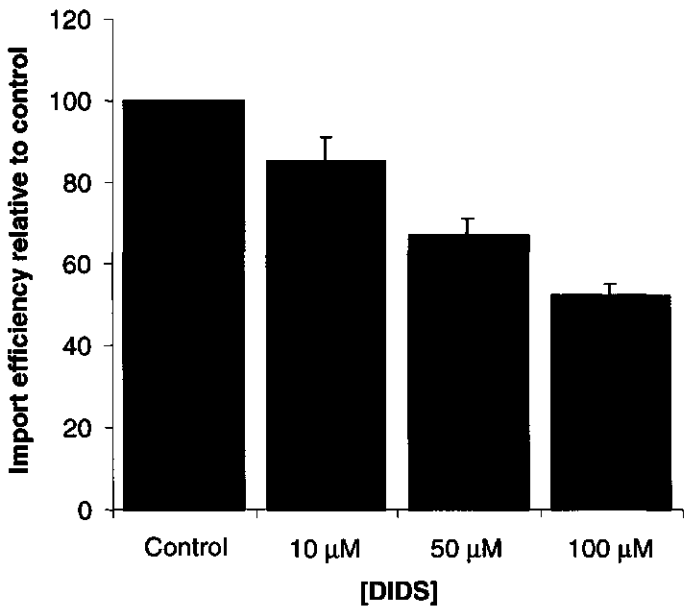

Fig. 7. Import efficiency of isolated chloroplast relative to control in the presence of different concentrations of DIDS.

The fact that a precursor protein has to interact with the components of Tic complex raises the question whether full translocation of the precursor (i.e. the precursor reaching the stroma) is necessary for PIRAC inactivation. To test this the

deletion mutant Δ15-25 of the precursor of the stromal protein ferredoxin was used. In this deletion mutant amino acids 15 to 25 from the N-terminus of the transit sequence are deleted. This mutant was shown to compete with wild type preferredoxin for binding sites on the chloroplast envelope, indicating that it has the ability to interact with an essential part of the chloroplast protein import machinery. The deletion mutant has been found to be unable to fully translocate across the envelope in *in vitro* import experiments (6). It is shown here that Δ15-25-preFd has the ability to inactivate PIRAC (Fig. 2). The inactivation of PIRAC by Δ15-25-preFd indicates that, although interaction with the Tic complex appears to be necessary for PIRAC inactivation, full translocation of the precursor across both envelope membranes is not necessary.

The decrease in P_O caused by Δ15-25-preFd is somewhat lower than the decrease caused by wt-preFd. An explanation for this could be that for the most effective inactivation of PIRAC full translocation of a precursor protein is required. A reduced binding affinity of the deletion mutant as compared to wt-preFd could explain the apparent reduced capability of inactivating PIRAC as well. The deletion mutant Δ35-42-preFd has a binding affinity which is comparable to that of Δ15-25-preFd (6). This mutant however, unlike Δ15-25-preFd, can be imported into chloroplasts, albeit with a lower efficiency than wt-preFd. The decrease in P_O caused by Δ35-42-preFd is comparable the decrease in P_O caused by Δ15-25-preFd (Fig. 3). This suggests, that the reduced ability of both deletion mutants to inactivate PIRAC is caused by their reduced binding affinity as compared to wt-preFd. This indicates that full translocation over the inner envelope membrane is not necessary for PIRAC inactivation.

Stromal components are known to be involved in protein import into chloroplasts (12-14). One of these stromal components, ClpC, is a member of the hsp100 family (13, 14). It was found in *in vitro* import experiments, that upon addition of a precursor protein large complexes of components of both the Toc and Tic complex and ClpC are formed (14). These complexes are thought not to be formed *a priori*, but only during protein import. The inactivation of PIRAC by the precursor of ferredoxin was first identified in the inside-out patch configuration (15), in which stromal components, such as ClpC, are absent. The formation of import complexes including stromal components could influence the inactivation of PIRAC by precursor protein. Single channel recordings in the chloroplast attached configuration showed the P_O of PIRAC to be similar as found in the inside-out single channel recordings. When wt-preFd was added to the pipette filling solution the P_O of PIRAC measured in the chloroplast attached configuration was decreased to a similar extent as in the inside-out patch configuration (Fig. 4). This leads to the conclusion that the presence of stromal factors does not affect the gating behavior of PIRAC in the control situation. Furthermore the presence of stromal factors does not influence the gating behavior under import conditions. This suggests that the formation of import complexes including stromal components does not influence the inactivation of PIRAC by precursor protein.

The results with GTP as an energy source suggest that PIRAC is inactivated upon interaction of the precursor protein with the Tic complex. The experiments with Δ15-25-preFd show that full translocation is not a prerequisite for PIRAC inactivation. These two findings are consistent with the conclusion that stromal factors do not influence the inactivation of PIRAC during protein import. Stromal factors involved in protein import are likely to act when the first part of the precursor (i.e. the transit

sequence) enters the stroma; full translocation (i.e. the precursor reaching the stroma) is however not necessary for PIRAC inactivation.

PIRAC was shown earlier to be an anion channel (15). The blocking effect of DIDS on PIRAC is consistent with this conclusion. DIDS causes a typical flickering block of PIRAC, moreover this flickering block is voltage dependent. This suggests that DIDS induces an open channel block of PIRAC, i.e. DIDS binds in the open pore of the channel. The *in vitro* import experiments show that blocking PIRAC with DIDS reduces the import efficiency of isolated chloroplasts (Fig. 7). This shows that correct functioning of PIRAC is required for protein import into chloroplasts.

In mitochondria the multiple conductance channel (MCC) has been shown to be blocked by a mitochondrial presequence. This channel is therefore thought to be involved in mitochondrial protein import (19). For normal activity of MCC, including the blocking of the channel by a presequence, Tim23 is required (20). Tim23 has been shown to be a component of the import machinery of the mitochondrial inner membrane (21). The protein is suggested to act as the preprotein receptor in this membrane. This suggests a similarity between MCC and PIRAC, both channels are influenced by the addition of a precursor and for both an interaction between the precursor and the inner membrane import machinery appears to be necessary.

The role of PIRAC in chloroplast protein import is not clear yet. PIRAC could be the protein conducting channel of the chloroplast inner membrane. The inactivation of PIRAC as an ion channel by precursor protein in that case would mean the switching of the channel to the protein conducting mode. The transition between the ion channel mode and the protein conducting mode could be triggered by the interaction of the precursor with the Tic complex or by a direct interaction of the precursor with PIRAC. Since the transit sequences of chloroplast precursors in general lack

negatively charged amino acid residues, it is unlikely that the precursor protein passes through the anion channel pore. This would mean, that the ion channel mode and the protein conducting mode of PIRAC use separate pores or that upon switching to the protein conducting mode there is a major change in conformation of the pore. Another possibility is, that PIRAC is part of a larger import complex of the inner membrane. In that case PIRAC would not be the protein conducting channel, but the inactivation of PIRAC by precursor protein could be facilitating the transfer of the precursor to the translocation channel.

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The mechanism of inactivation of a 50 pS envelope anion channel during chloroplast protein import

ABSTRACT

The mechanism of import-competent precursor protein induced inactivation of a 50 pS anion channel of the chloroplast envelope is investigated using single channel recordings. The inactivation by precursor protein is the result of the induction of a long-lived closed state of the channel. The mean duration of this state does not depend on precursor concentration. From this it can be concluded that PIRAC enters the inactive state less frequently when the precursor concentration is lowered, but that the time spent in this state remains the same. Furthermore it was found that the presence of precursor protein also decreases the mean durations of pre-existing open and closed states of the channel. This decrease is found to be dependent on the precursor concentration. From this it is concluded that there is a direct interaction between the precursor protein and a protein complex of which the channel is a constituent. The mean duration of the precursor induced long-lived closed state does not depend on the length of the translocation-competent precursor. This suggests that the duration of import is independent of precursor length.

INTRODUCTION

Chloroplasts are surrounded by the envelope consisting of two membranes. 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 as precursors with an N-terminal extension called transit sequence. The transit sequence is both necessary and sufficient to target a protein to the chloroplast (1). Several components of the chloroplast protein import machinery have been identified (for recent reviews see Refs. 2, 3). The two envelope membranes both have their own import machinery, that can function independent of each other (4). The outer membrane machinery has been termed Toc (translocon of the outer membrane of chloroplasts) and the inner membrane machinery has been named Tic (translocon of the inner membrane of chloroplasts) (5).

Recently the involvement of an anion channel of the chloroplast envelope in protein import was identified (6). This envelope channel, which is located in the inner membrane, will be called Protein Import Related Anion Channel (PIRAC) here. The addition of precursor protein was shown to inactivate PIRAC, i.e. the open probability (P_O) of the channel decreased. The precursor protein induced inactivation was found to be dependent on ATP and the presence of a functional transit sequence. The exact role of PIRAC in chloroplast protein import is not known as yet. The mechanism of PIRAC inactivation by precursor protein during chloroplast protein import is also unclear yet.

In this report the role of PIRAC in protein import and the mechanism of PIRAC inactivation by precursor protein are studied in more detail, using the patch clamp technique. Single channel recordings of PIRAC in the inside-out patch configuration

were performed. Analysis of open and closed time duration distributions are used to clarify the mechanism of PIRAC inactivation during protein import. Furthermore the inactivation of PIRAC induced by different precursor proteins and a transit peptide is used to further analyze the role of PIRAC in chloroplast protein import. It is found that precursor protein and transit peptide induce a long-lived inactive state of PIRAC, the duration of this state is not dependent on precursor length.

EXPERIMENTAL PROCEDURES

Precursor proteins and transit peptide

The precursors of *Silene pratensis* ferredoxin (preFd) and of tobacco small subunit of ribulose-2,5-biphosphate carboxylase/oxygenase (preSSU) were overexpressed in *Escherichia coli* and isolated as described before (7, 8). The transit peptide of preSSU (trSSU) was isolated from a GST fusion system as has been described before (8).

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, 0.5 mM MgATP and 2 mM $CaSO_4$. The sliced preparation was transferred directly to a 1 ml chamber, which was mounted on a light microscope to allow visual selection of single intact chloroplasts.

Electrophysiological measurements

For recording the electrical currents across the chloroplast envelope a standard patch clamp technique was used (9). Electrodes were pulled from borosilicate glass by

a two step pull, and extensively firepolished. Electrodes were filled with buffer containing: 2.5 mM TES/KOH, pH 7.2, 250 mM KCl and 2 mM $CaSO_4$, leading to a 10-fold KCl gradient across the patch. Electrode resistances were found to be typically around 30 MΩ. To clarify the inactivation of PIRAC during protein import, precursor protein or transit peptide was added to the pipette filling solution (i.e. the outside of the chloroplast envelope).

Current recordings were made from inside-out patches, obtained by moving the pipette away from the chloroplast after giga-seal formation, using an Axopatch 200B patch clamp amplifier (Axon Instruments, USA). Potentials are given with regard to the pipette interior, the bath was kept at ground, using a 250 mM KCl agar bridge. The data were filtered at a cut-off frequency of 1 kHz, using an 8-pole Bessel filter (internal filter of the Axopatch 200B). The filtered data were digitized at 10 kHz using a CED 1401+ (Cambridge Electronic Design, UK) interface.

Data were analyzed with the Patch and Voltage Clamp Software (Cambridge Electronic Design, UK). To determine the distributions of open and closed time durations of PIRAC a module was developed in the matrix calculating software Matlab (The Mathworks, Inc., USA). This module uses the 50%-threshold method to identify transitions of the channel between the open and the closed state. Histograms of open and closed time durations were constructed using the square root of the number of events vs. the log binwidth of durations (10). The distributions were fitted with multi-exponential probability density functions using the maximum likelihood method (11). Data are given as mean ± the standard deviation.

RESULTS

The high seal resistances routinely obtained with the chloroplast envelope make it highly unlikely, that the seal consists of the outer membrane alone, due to the abundance of large pores in this membrane (12). No light induced currents (13) were ever observed directly after seal formation (i.e. in the chloroplast attached configuration) or after excision of the patch. This indicates, that the thylakoid membrane was not included in the patch. The measured current is likely to run across the inner envelope membrane in a seal consisting of a sandwich like structure of the outer and the inner membrane, including the intermembrane space.

To further elucidate the mechanism of precursor protein induced inactivation of PIRAC, the distributions of open and closed time durations were determined in the absence and presence of precursor protein. Fig. 1A shows single channel recordings in the absence and presence of preFd, respectively. Comparison of the two recordings suggests, that the inactivation of PIRAC is caused by the induction of a long-lived closed state induced by preFd. In the control situation PIRAC has three distinct closed states, as can be concluded from the distribution of closed time durations shown in Fig.1B. The distribution is best fitted with a mixture of three exponential probability density functions, the line in Fig.1B superimposed on the histogram shows this fit. The time constants of the closed states are: 0.35 ± 0.03, 2.41 ± 0.98 and 29.1 ± 6.8 ms. When preFd is included in the pipette filling solution, a new long-lived closed state is observed. In Fig. 1B the distribution of closed time durations of PIRAC in the presence of preFd is shown. The distribution was fitted with a mixture of four exponential probability density functions. The fit is shown in Fig. 1B as a line

superimposed on the histogram. The time constant of the preFd induced long-lived closed state of PIRAC is 835 ± 109 ms.

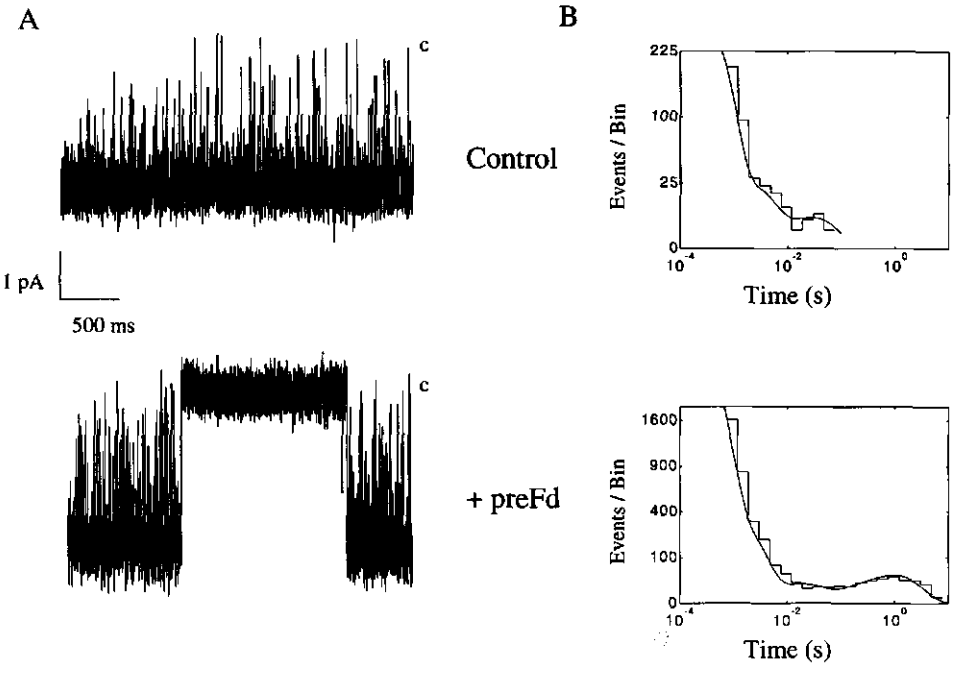

Fig. 1 A: Single channel recordings in the absence and presence of preFd in the pipette filling solution. Recordings were made at a holding potential of –20 mV. **B**: Closed time duration distribution of PIRAC in the absence and presence of preFd in the pipette filling solution respectively. Distributions are plotted on square root vs. log time axes with 5 bins per decade. The distributions show, that the preFd induced inactivation of PIRAC is the result of a long-lived closed state not present in the control measurement.

Another precursor, which is translocated across the chloroplast envelope, preSSU is also able to inactivate PIRAC. Fig. 2A shows single channel recordings of PIRAC in the absence and in the presence of 23 nM and 54 nM preSSU, respectively. From the all point amplitude histograms shown in Fig. 2B the open probability (P_O) of

PIRAC was determined. PreSSU causes a dose-dependent decrease in P_O of PIRAC, in the presence of 23 nM preSSU the P_O decreased from 0.81 ± 0.04 in the control situation to 0.39 ± 0.12. In the presence of 54 nM preSSU the P_O is 0.17 ± 0.08. The mature SSU protein was found not to inactivate PIRAC (not shown). The single channel recordings shown in Fig. 2A suggests, that also the preSSU induced inactivation of PIRAC is caused by the occurrence of a long-lived closed state.

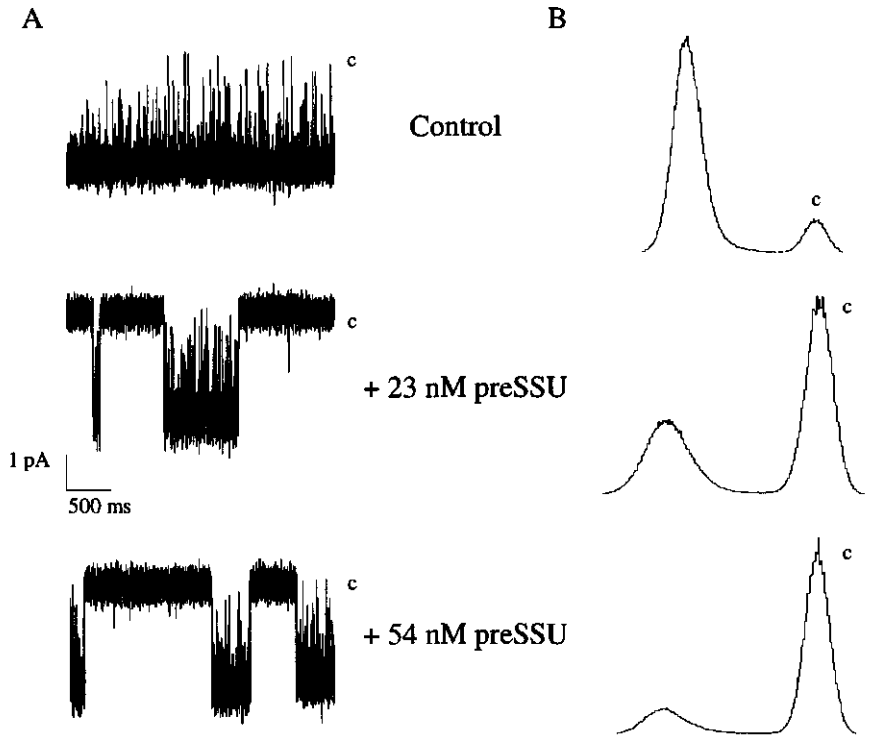

Fig. 2 A: Single channel recording of PIRAC in the absence and presence of two different concentrations of preSSU respectively. Recordings were made at a holding potential of –20 mV. **B**: All point amplitude histograms of single channel recordings of PIRAC in the absence and presence of two different concentrations of preSSU respectively. The histograms indicate a dose dependent decrease in P_O of PIRAC induced by preSSU.

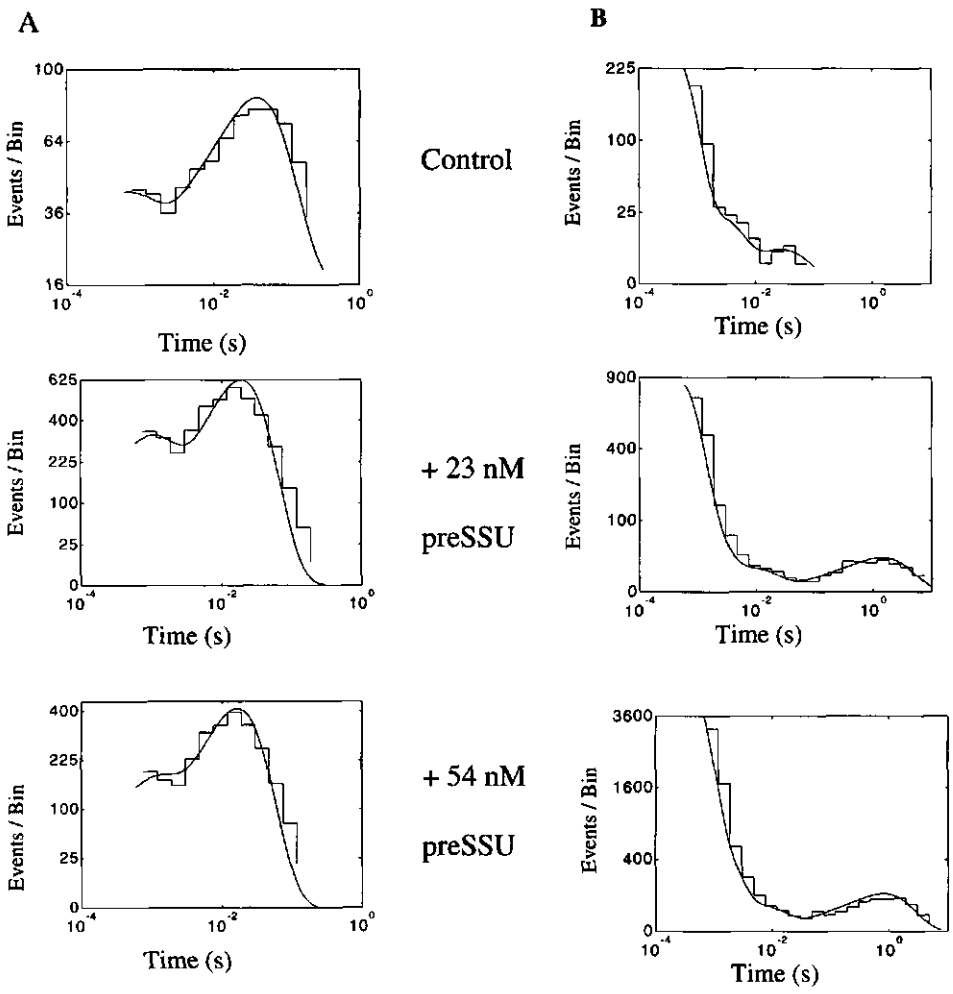

Fig. 3 A: Open time duration distributions of PIRAC in the absence and presence of two different concentrations of preSSU. **B**: Closed time duration distributions of PIRAC in the absence and presence of two different concentrations of preSSU. The closed time duration distribution show the precursor induced long-live closed state.

All distributions are plotted on square root vs. log time axes with 5 bins per decade and were obtained at a holding potential of –20 mV.

In Fig. 3 the open and closed time duration distributions of PIRAC in the absence and in the presence of the two different concentrations of preSSU are shown. The closed time durations distribution illustrated in Fig. 3B clearly shows the precursor protein induced long-lived closed state, also observed in the presence of preFd (Fig. 1B). The open time durations distribution of PIRAC shows, that the channel has two distinct open states. Table I shows the mean durations of the different open states of PIRAC in the control situation and in the presence of 23 nM and 54 nM preSSU, respectively. Mean durations of the closed states are shown in Table II. Table I and Table II show, that preSSU not only induces a long-lived closed state. The mean duration of the other open and closed states is also affected by the presence of preSSU. A concentration dependent decrease in the time constant of open and closed states with a mean duration larger than 1 ms can be observed.

TABLE I

Mean durations of the open states of PIRAC in the absence and presence of preSSU respectively

	Time constants open states (ms)	
	τ_1	τ_2
Control	0.88 ± 0.34	47.2 ± 9.6
23 nM preSSU	0.97 ± 0.12	20.9 ± 3.3
54 nM preSSU	0.92 ± 0.11	16.2 ± 0.3

TABLE II

Mean durations of the closed states of PIRAC in the absence and presence of preSSU respectively

	Time constants closed states (ms)			
	τ_1	τ_2	τ_3	τ_4
Control	0.35 ± 0.03	2.41 ± 0.98	29.1 ± 6.8	-
23 nM preSSU	0.38 ± 0.06	1.60 ± 0.76	11.8 ± 4.5	1432 ± 374
54 nM preSSU	0.33 ± 0.03	0.76 ± 0.16	5.12 ± 1.03	989 ± 214

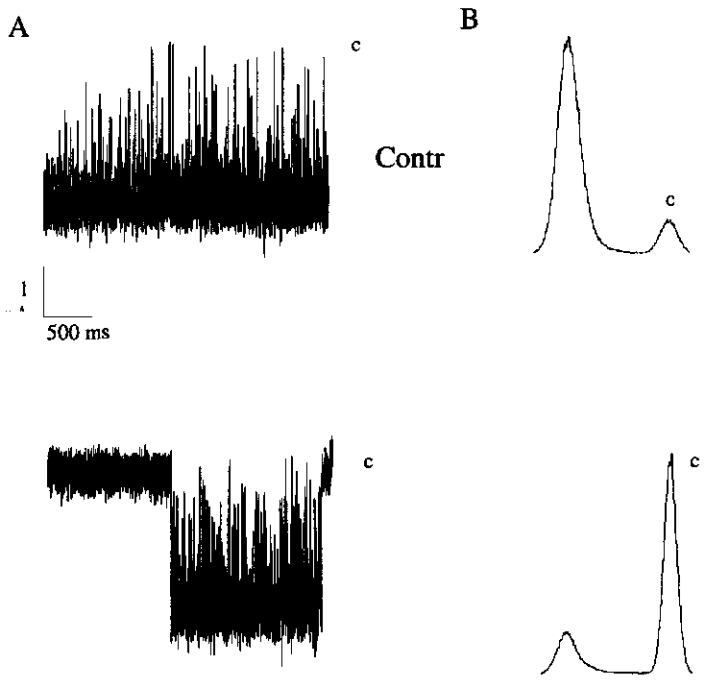

Fig. 4 A: Single channel recordings of PPIRAC in the absence and presence of tpSSU respectively. The recordings were made at a holding potential of –20 mV. **B**: All point amplitude histograms of single channel recordings of PIRAC in the absence and presence of tpSSU respectively. The histograms indicate a decrease in P_O of PIRAC in the presence of tpSSU.

The transit peptide of preSSU also causes inactivation of PIRAC. In Fig 4A single channel recordings are shown in the absence and presence of trSSU respectively. The P_O of PIRAC under these conditions was determined from the all point amplitude histograms shown in Fig. 4B. The addition of trSSU causes a decrease in P_O from 0.81 ± 0.04 in the control situation to 0.25 ± 0.06 in the presence of trSSU. Fig. 5 shows the distribution of closed time durations of PIRAC in the presence of trSSU. From this distribution it can be seen, that trSSU induced PIRAC inactivation is also characterised by a long-lived closed state of the channel.

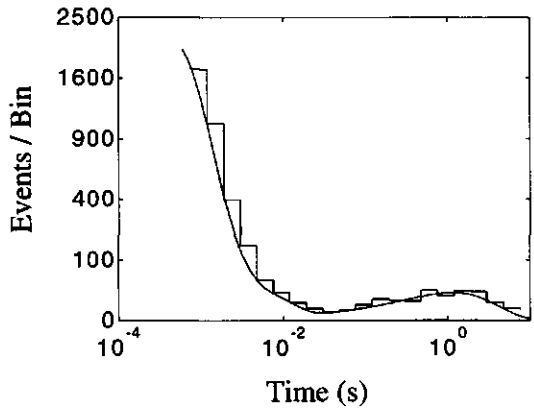

Fig. 5 Closed time duration distribution of PIRAC in the presence of tpSSU. The distribution is plotted on square root vs. log time axes with 5 bins per decade. The distribution shows that also tpSSU induced inactivation of PIRAC is the result of a long-lived closed state. The distribution was determined at a holding potential of –20 mV.

DISCUSSION

The present data demonstrate, that the previously described inactivation of PIRAC during chloroplast protein import (6) is reflected by a precursor protein induced long-lived closed state. This inactive state is never found in the control situation and it is therefore tempting to speculate, that this state is the result of the

binding of a precursor protein to a complex of which the channel is a constituent. Inactivation of PIRAC can be induced by different chloroplast precursor proteins (preFd and preSSU). The decrease in P_O caused by preSSU is identical to the decrease previously described to be caused by preFd (6). It is shown here that the precursor induced PIRAC inactivation is dependent on the precursor concentration in the pipette filling solution (Fig. 2). The mean duration of the precursor induced inactive state of PIRAC is however independent of the precursor concentration (Fig. 3, Table II). From this it can be concluded that PIRAC enters the inactive state less frequently when the precursor concentration is lowered, but that the time spent in this state remains the same.

Furthermore it is shown here, that precursor protein also causes a concentration dependent decrease in the mean duration of different open and closed states identified in the control situation. This suggests that there is a direct interaction between the precursor protein and the channel. This interaction would cause the channel to close and become inactivated for a relatively long period. All time constants of open and closed states of PIRAC, that are present in the control situation are affected by precursor protein, except for the one in the open and closed state, with the shortest mean duration. These states might be too short-lived to be able to interact with the precursor. However, interaction between PIRAC and the precursor might also still occur in these states, but because of their short duration the decrease in time constant is too small and below the time resolution in the measurements described. The fact that PIRAC inactivation by precursor protein can occur in all possible states of the PIRAC channel suggests, that the role of PIRAC in protein import is distinct from its function as an ion channel. It means that there is not one particular state of the

channel that can interact with translocating precursor, but rather that PIRAC interacts with precursor in all possible states of the channel.

The exact role of PIRAC in the protein import process is not clear yet. It is possible that PIRAC represents the protein import channel of the inner envelope membrane. The inactivation of PIRAC by precursor protein could represent a switching of the channel from the ion channel mode to the protein conducting mode. This would mean, that the long-lived closed state represents the time PIRAC spends in the protein conducting mode. This time is then indicative for the duration of translocation of a single precursor protein. The maximum rate of import has been determined for several precursor proteins (14, 15). These V_{max} values show no correlation with overall precursor length. Furthermore it was shown that the V_{max} value of import of a transit peptide is close to the V_{max} of import of a precursor protein (16). The mean durations of the long-lived inactive state induced by different precursors reflects this lack in correlation between V_{max} of import and precursor length. The long-lived the closed states induced by preFd, preSSU, and trSSU respectively all have comparable mean durations. This suggests that the duration of translocation is independent of the length of the precursor protein.

In mitochondria the multiple conductance channel (MCC) has been shown to be blocked by a mitochondrial presequence. This channel is therefore thought to be involved in mitochondrial protein import (17). For normal activity of MCC, including the blocking of the channel by a presequence, Tim23 is required (18). Tim23 has been shown to be a component of the import machinery of the mitochondrial inner membrane (19). In view of the data presented here a similar association between PIRAC and components of the Tic machinery is likely to exist, but this has not yet been investigated. If PIRAC indeed forms a complex with Tic components the

appearance of an inactive state and the decrease in mean durations of the other open and closed states of PIRAC is the result of interaction between the precursor and this complex.

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The envelope anion channel involved in chloroplast protein import is associated with Tic110

ABSTRACT

An anion channel of the chloroplast envelope was previously shown to be involved in protein import. Some gating characteristics of the channel are presented. The pore size of the channel is approximated to be around 6.5 Å. Antibodies raised to Tic110 completely inactivate the protein import related channel. These observations suggest that the channel is associated with the Tic machinery and can function as the protein conducting channel of the inner envelope membrane.

INTRODUCTION

Chloroplasts are organelles surrounded by two membranes. 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 transit sequence. The transit sequence is both necessary and sufficient to target a protein to the chloroplast (1). Several components of the chloroplast import system have been identified (for recent reviews see 2 and 3). The two surrounding membranes both have their own import machinery, that can function independent of each other (4). The outer and the inner membrane machinery have been termed Toc (translocon of the outer membrane of chloroplasts) and Tic (translocon of the inner membrane of chloroplasts), respectively (5). One of the components of Tic has been identified as Tic110 (6, 7). Tic110 is an integral membrane protein of the chloroplast inner envelope membrane. It has a large hydrophilic part, that faces the chloroplast stroma (8).

An anion channel of the chloroplast envelope was shown to be involved in protein import (9). This envelope channel, which is located in the inner membrane, will be called Protein Import Related Anion Channel (PIRAC) here. The PIRAC was shown to be inactivated, i.e. the open probability of the channel (P_O) decreased, by the addition of precursor protein. The inactivation was found to be dependent on ATP and the presence of a functional transit sequence. The exact role of PIRAC in chloroplast protein import is however not known yet.

In this report a further characterization of PIRAC is described. A first approximation of the pore size of PIRAC is made. Furthermore the relationship between PIRAC and the Tic machinery is investigated. It is found that antibodies raised to Tic110 completely inactivate PIRAC.

EXPERIMENTAL PROCEDURES

Chloroplast isolation

Chloroplasts were isolated from pea leaves by cutting them gently with a razor blade in electrolyte solution used in the bath for the electrophysiological measurements, as indicated in the text. The sliced preparation was transferred directly to a 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 (10). Electrodes were pulled from borosilicate glass by a two step pull, and extensively firepolished. Electrodes were filled with electrolyte solution as indicated in the text. Electrode resistances were found to be typically around 30 MΩ.

Currents were measured using an Axopatch 200B patch clamp amplifier (Axon Instruments, USA). The data were filtered at a cut-off frequency of 1 kHz, using an 8-pole Bessel filter (internal filter of the Axopatch 200B). The filtered data were digitized at 10 kHz using a CED 1401+ (Cambridge Electronic Design, UK). Data were analyzed with the Patch and Voltage Clamp Software (Cambridge Electronic Design, UK).

Current recordings were made from inside-out patches, obtained by moving the pipette away from the chloroplast after giga-seal formation (10). Potentials are given with regard to the pipette interior, the bath was kept at ground, using a 250 mM KCl agar bridge.

RESULTS

Regarding the high seal resistances it is highly unlikely, that the seals consist of the outer membrane alone, due to the abundance of large pores in the outer membrane (11). No light-induced currents (12) were ever observed after seal formation (i.e. in the attached configuration) or after excision of the patch. This indicates that the thylakoid membrane under the conditions used here was not included in the patch. The observed current is likely to run mainly across the inner membrane in a seal consisting of a sandwich like structure of the outer and the inner membrane.

Gating properties of PIRAC

The single channel conductance of PIRAC in symmetrical 100 mM KCl is 42 pS. This conductance is calculated from the current voltage relationship, where the open channel current is plotted against the membrane potential. In Fig. 1A single channel recordings of PIRAC in symmetrical 100 mM KCl buffer at different membrane potentials are shown. Fig. 1B shows small parts of the same single channel recordings at a higher time resolution. At positive potentials the channel shows fast transitions between the open and the closed level. This behaviour is known as flickering. At negative potentials flickering is less pronounced as can be judged from Fig. 1B. The single channel recordings of PIRAC (Fig. 1) show no indication for the existence of subconductance levels. Fig. 2 shows the current voltage relationship of PIRAC in 100 mM KCl, each point was taken from at least 5 different single channel recordings. In buffer containing 25 mM KCl the single channel conductance of PIRAC is found to be around 10 pS (not shown). The open probability of PIRAC in symmetrical 100 mM KCl is found to be around 0.85 (not shown).

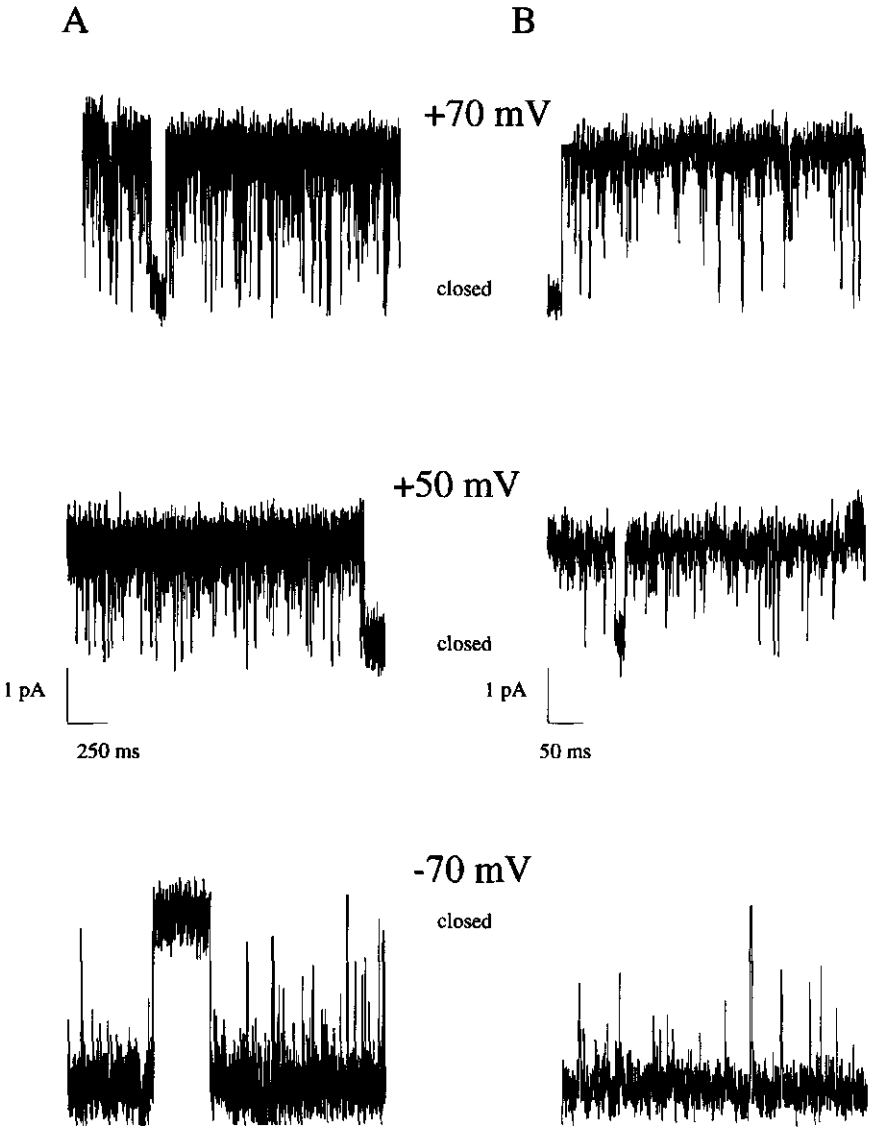

Fig. 1 **A**: Single channel recordings of PIRAC in symmetrical 100 mM KCl at different holding potentials. **B**: Small parts of the recordings shown in **A** displayed at a higher time resolution.

It was shown previously that PIRAC is an anion selective channel (9). To determine the anion selectivity of PIRAC the KCl concentration of the bath solution was lowered from 100 mM to 10 mM. In this 10-fold KCl gradient across the patch, the current-voltage relationship (Fig. 2) shows a reversal potential of +33 mV. Using the Goldman-Hodgkin-Katz equation this reversal potential corresponds to a permeability ratio of P_{Cl^-} / P_{K^+} of 6.6. If PIRAC is considered as a water-filled cylindrical pore, the diameter of the pore would be around 6.5 Å. This estimation is based on the most simple model of channel geometry and is fairly rough (13), but it provides limits for the hydrophilic pore at the narrowest point. The same approximation was used to estimate the pore diameter of the reconstituted Toc75 channel (14). If the resistivity in the channel is assumed to be five times the bulk resistivity (15) a pore diameter of 15 Å is calculated.

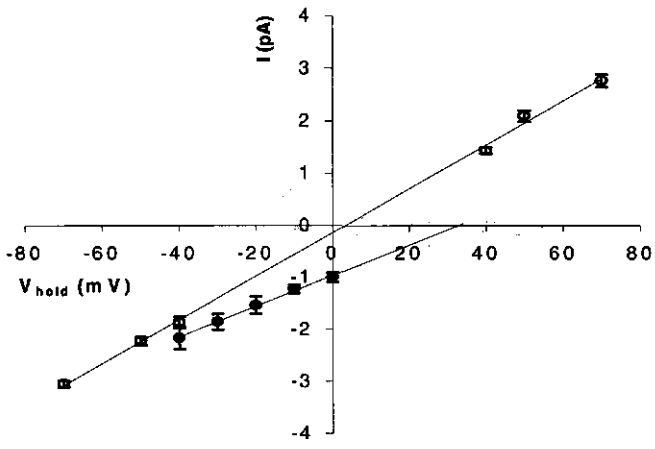

Fig. 2 Current voltage relationship of PIRAC in symmetrical 100 mM KCl (open symbols) and 10/100 mM KCl (closed symbols), respectively

PIRAC is associated with Tic

To identify a possible association of PIRAC with Tic the effect of the addition of antibodies to Tic110 on PIRAC gating was tested. When Tic110 IgG was added to the bath solution PIRAC activity could be observed directly after excision of the patch. After approximately 60 s PIRAC activity was completely lost. In Fig. 3 a single channel recording of PIRAC in the presence of Tic110 IgG is shown. This loss of PIRAC activity in the presence of Tic110 IgG was found in 14 out of 18 single channel recordings. Single channel recordings of PIRAC without antibody in the bath solution very rarely show loss of channel activity due to channel rundown. In the control situation channel rundown is observed in approximately 5% of PIRAC containing patches and occurs in these patches after several hundreds of seconds. The loss of channel activity in the presence of Tic110 IgG can therefore be ascribed to the action of the antibodies.

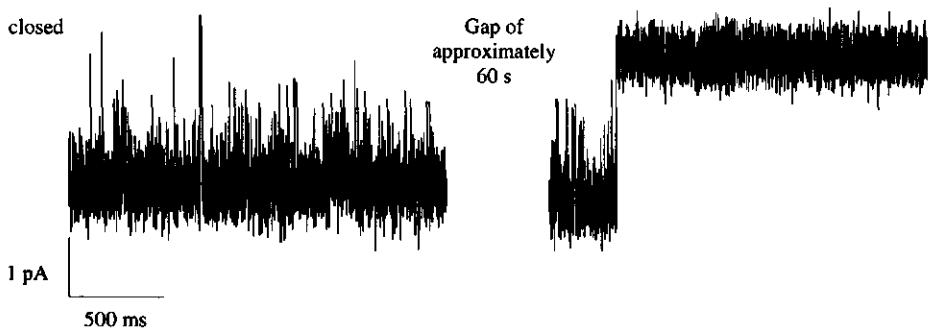

Fig. 3 Single channel recording of PIRAC in the presence of antibodies to Tic110. The bath solution contained 25 mM KCl, the pipette was filled with a 250 mM KCl solution. The holding potential used was –20 mV.

To test the specificity of the antibody effect on PIRAC gating, antibodies against a component of the outer membrane translocon, Toc75, were added to the bath solution as well. In the presence of these antibodies loss of PIRAC activity in single channel recordings was never observed in 10 recordings of 180 s or longer.

DISCUSSION

PIRAC was shown before to be an envelope anion channel with a single channel conductance of 50 pS in 250 mM KCl (9). This value is close to the 42 pS conductance reported here in 100 mM KCl. The value for the single channel conductance of PIRAC in 25 mM KCl is around 10 pS. Thus it appears that below 100 mM KCl the conductance depends linearly on the KCl-concentration. From this it can be concluded that the saturation value of PIRAC single channel conductance is close to 50 pS. Saturation of the single channel conductance in relatively low ionic strength buffers was previously also observed for the reconstituted Toc75 channel (14).The reversal potential found here for a 10-fold KCl gradient (10/100 mM) is in good agreement with what has been found before in 25/250 mM KCl (9). The open probability found in 100 mM KCl is identical to the open probability of PIRAC in 25/250 mM KCl described (9). This indicates that the open probability of PIRAC is not influenced by salt concentration or gradient.

The data presented here demonstrate, that PIRAC is associated with the import machinery of the chloroplast inner envelope membrane. It was shown previously, that PIRAC is inactivated by a translocation-competent precursor protein (9). This inactivation is the result of an interaction between precursor protein and a protein complex of which PIRAC is a constituent (chapter 4). The loss of PIRAC activity

induced by antibodies to Tic110 demonstrates, that PIRAC is associated with Tic110. This component of the chloroplast inner envelope membrane protein import machinery is an integral membrane protein with a large hydrophilic stretch facing the chloroplast stroma (8). It is thought that Tic110 functions as a docking site for stromal chaperones that are involved in protein import. An association of Tic110 and stromal chaperonin 60 was shown to exist in isolated chloroplasts (6). Another stromal chaperone, the Hsp100 homologue ClpC was also shown to interact with Tic110 (16). This suggested role for Tic110 implies that the large hydrophilic part of the protein, that faces the stroma, is located near the stroma-facing exit of the protein translocation channel of the inner envelope membrane.

The inactivation of PIRAC by antibodies to Tic110 shows close similarities with the inactivation of the mitochondrial multiple conductance channel (MCC) by antibodies to Tim23 (17). MCC has been shown to be blocked by a mitochondrial presequence. This channel is therefore thought to be involved in mitochondrial protein import (18).

Because of the inactivation of PIRAC by Tic110 antibodies it seems likely that PIRAC represents the protein conducting channel of the inner membrane. With an approximated pore size of PIRAC of 6.5 Å precursor proteins have to be completely unfolded to be able to pass through the PIRAC pore. The reconstituted protein translocation channel of the outer membrane, Toc75, has been reported to have an approximated pore size of around 8.5 Å (14). This value is based on the same approximation as the one used here for PIRAC. This indicates that the pores of the protein import channels of the outer and inner membrane are of comparable size.

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This work is part of a joined program 'Chloroplast Protein Import' in collaboration with Prof. B. de Kruijff and Prof. P. Weisbeek, supported by the Foundation for Earth and Life Sciences (ALW), with financial aid of the Netherlands Organisation for Scientific Research (NWO). The author likes to thank Mr. W.-A. Rensink for the kind gift of antibodies and Dr. J. Snel and Mr. H. Dassen for useful discussion.

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Chapter 6

General Discussion

Chloroplasts have to import the majority of their proteins from the site of synthesis, the cytosol. These proteins are synthesised with an N-terminal extension, the transit sequence, which is both necessary and sufficient for targeting to the chloroplast (1). The precursor protein is translocated across the chloroplast envelope by two independent translocons located in the outer (Toc) and the inner (Tic) membrane, respectively (2, 3). The aim of the study described in this thesis was to elucidate the electrophysiological response of the chloroplast envelope during protein import. For this purpose single channel recordings of the chloroplast envelope were used. Modulation of channel activity by precursor proteins is described.

The origin of the membrane patch used in the single channel recordings

The outer membrane of the chloroplast envelope contains a large number of pores (4). Regarding the high seal resistances it is therefore highly unlikely that the patch consist of the outer membrane alone. No light-induced currents (5) were ever observed after seal formation (i.e. in the attached configuration) or after excision of the patch. This indicates that the thylakoid membrane under the experimental conditions used was not included in the patch. The observed current is likely to run mainly across the inner membrane in a seal consisting of a sandwich like structure of the outer and the inner membrane.

Electrical identification of a 50 pS anion channel of the chloroplast envelope

Single channel recordings revealed the presence of an anion channel in the chloroplast envelope. The channel was identified as an anion channel by using asymmetric KCl solutions. A 10-fold KCl gradient (25/250 mM) resulted in a reversal

potential (V_{REV}) of the channel of +35 mV (chapter 2). The Nernst-potential for chloride under these conditions would be around +60 mV, leading to the conclusion that the observed channel is more permeable to Cl^- than it is to K^+. The permeability ratio for Cl^- over K^+ was calculated, using the Goldman-Hodgkin-Katz equation to be 6.6 (chapter 5). It is furthermore shown that the channel is sensitive to the anion channel blocker DIDS (chapter 3). Addition of DIDS to the cytosolic side of the patch induces a flickering block of the channel. The effect of DIDS is shown to be voltage dependent. These observations suggest that DIDS binds in the open pore of the channel. Reversible fast open-channel block by DIDS has been described before for animal and plant membrane anion channels (6, 7).

The single channel conductance of the observed anion channel was determined from the current voltage relationship and found to be 50 pS in 250 mM KCl (chapter 2). In 100 mM KCl the single channel conductance is observed to be 42 pS, decreasing the KCl concentration further to 25 mM leads to a linear decrease in single channel conductance to a value of approximately 10 pS (chapter 5). These observations indicate that the saturation value of the single channel conductance is approximately 50 pS and that the conductance is already saturated at 250 mM KCl. Saturation of the single channel conductance at relatively low salt concentrations was also observed for the ion channel activity of the reconstituted Toc75 protein (8). The single channel conductance values can be used to make an approximation of the pore size of the channel. Assuming an increase of the resistivity in the channel pore of a factor five as compared to the resistivity in the bulk solution (9) the pore diameter would be approximately 15 Å (chapter 5).

The anion channel is involved in protein import

It is found that the anion channel of the envelope is involved in chloroplast protein import. The channel is observed to be inactivated by the precursor protein of ferredoxin (preFd) (chapter 2). The 50 pS anion channel is therefore termed Protein Import Related Anion Channel (PIRAC). The inactivation of PIRAC by precursor protein is dependent on the presence of a functional transit sequence. Another prerequisite for protein import, the presence of ATP, is also a prerequisite for the inactivation of PIRAC by precursor protein. It is found that PIRAC inactivation can also be induced by the precursor protein of the small subunit of rubisco (preSSU) (chapter 4). This indicates that PIRAC plays a role in the general import pathway of proteins into the chloroplast.

At what stage in protein import is PIRAC inactivated?

PIRAC is shown to be inactivated by a deletion mutant of the preFd, Δ15-25. This deletion mutant was shown previously to be able to compete with the wild-type precursor for protein import, but cannot be imported (10). This shows that complete translocation of the precursor protein is not required for PIRAC inactivation. It was demonstrated by cross-link experiments in *in vitro* import studies that Δ15-25-preFd does interact with the Tic-complex (Rensink, personal communication).

The interaction of precursor proteins with the outer surface of chloroplast was shown to be stimulated by ATP as well as GTP (11, 12). More recently it was found that GTP can also drive the translocation of precursor protein across the outer membrane (13). Interaction with the Tic machinery however requires the presence of ATP. It is shown in this thesis that PIRAC inactivation by precursor protein requires the presence of ATP (chapter 2). The presence of GTP is not sufficient for precursor

induced PIRAC inactivation (chapter 3). This indicates that interaction of the precursor protein with the outer surface of the chloroplast is not sufficient for PIRAC inactivation.

The inactivation of PIRAC by Δ15-25-preFd and the inability of preFd to inactivate PIRAC in the presence of only GTP indicate that translocation of the precursor across the outer membrane is a prerequisite for PIRAC inactivation. Full translocation of the precursor protein is however not needed. This suggests that PIRAC is inactivated when the precursor protein interacts with the Tic complex. This consistent with a localisation of PIRAC in the inner envelope membrane (see above).

The mechanism of precursor protein induced PIRAC inactivation

It could be demonstrated that the inactivation of PIRAC by precursor protein is the result of a direct interaction between the precursor and the channel. In the control situation PIRAC has three distinct closed and two distinct open states. The states can be distinguished on the basis of their mean durations, as determined from the distributions of open and closed time durations of the channel (chapter 4). It was found that the addition of precursor protein to the cytosolic side decreases the mean duration of two of the closed states and of one of the open states. This decrease in mean duration in the presence of precursor protein proved to be protein concentration dependent. This strongly suggests that there is a direct interaction between PIRAC and the precursor. Furthermore this finding indicates that the precursor can interact with PIRAC in all states of the channel. The decrease of the mean durations of one open and two closed states of PIRAC by the precursor protein illustrates that interaction of the precursor with the channel is not limited to the open channel, but can also occur when the channel is closed. Furthermore precursor protein does not

induce a flickering block as was observed with DIDS (chapter 3). These observations indicate that precursor protein induced inactivation of PIRAC is not the result of an open-channel block.

The interaction between the precursor and PIRAC induces a long-lived closed (inactive) state of the channel. This inactive state could only be observed in the presence of precursor protein and it is this state that is responsible for the decrease in open probability (P_O) induced by precursor. The mean duration of the inactive state was found to be independent of the precursor protein concentration. Overall precursor length did also not affect the mean duration of the precursor protein induced inactive state.

PIRAC is associated with the Translocon of the Inner Membrane

A known component of the Tic complex is Tic110 (14-16). Tic110 is a transmembrane protein of the inner envelope membrane, with two transmembrane segments. A large hydrophilic loop of the protein faces the stroma (17). This stromal loop is thought to function in the recruiting of stromal factors involved in protein import. Addition of antibodies to Tic110 to the stromal side of the patch in single channel recordings resulted in irreversible inactivation of PIRAC (chapter 5). Approximately 60 s after exposure of the patch to the antibodies PIRAC is irreversibly inactivated. This shows that PIRAC is associated with Tic110. The association of PIRAC with the translocon of the inner membrane illustrates the localisation of the channel in the inner membrane.

Protein import and PIRAC

The experimental evidence described in this thesis allows several hypotheses about the role of PIRAC in chloroplast protein import. Three possible functions of PIRAC in the import process will be described below. All hypotheses described will be weighed against the experimental data. An integrating paragraph will compare the different hypotheses and it will describe which of those is more supported by the experimental evidence.

PIRAC as the 'protein conducting' channel of the inner envelope membrane

The most obvious role for PIRAC in protein import would be a function as the 'protein conducting' channel of the chloroplast inner envelope membrane. Several observations described in this thesis support this hypothesis. First of all the direct interaction between the precursor protein and PIRAC leading to channel inactivation (chapter 4). If PIRAC is the 'protein conducting' channel it is obvious that there has to be a direct interaction between the precursor and the channel. The induction of a long-lived inactive state by precursor protein could represent the switching of PIRAC from ion channel mode to protein conducting mode. The association of PIRAC with Tic110 (chapter 5) is consistent with the hypothesis of PIRAC as the 'protein conducting' channel of the Tic complex. Definitive proof for this hypothesis is however still lacking.

If PIRAC is the 'protein conducting' channel of the Tic complex the mean duration of the inactive state could represent the duration of translocation of a precursor across the inner membrane. The maximum rates of import (V_{max})as determined from *in vitro* import studies is independent of the overall precursor length (18, 19). Furthermore it was found that the maximum rate of import of a transit

sequence is comparable to the maximum rate of import of a precursor protein, despite the considerable difference in length (20). The mean duration of the inactive state was also found to be independent of the overall precursor length (chapter 4). Furthermore the mean duration of the inactive state induced by the transit sequence of preSSU is identical to the mean duration of the inactive state induced by the full-length precursor.

It is shown that the interaction between PIRAC and precursor protein can occur in the open state of the channel as well as in two of the closed states and that it does not result in a flickering block (chapter 4). The precursor protein therefore does not bind in the open pore of the channel (see above). An explanation for these observations would be that the interaction of the precursor with a regulatory loop of PIRAC induces the inactivation of the ion channel. This inactivation would involve a change in conformation leading to the activation of the pore for protein translocation. The mean duration of the inactive state in this case would represent the mean duration of activation of the pore for protein translocation and the reversion back to the ion conducting state. This step might represent the rate limiting step in precursor translocation across the envelope and thus determine the V_{max} of import. This would provide an explanation for the lack of correlation between V_{max} and overall precursor length (18, 19) and the fact that the V_{max} of import of a transit sequence is comparable to that of a full-length precursor (20).

A role for PIRAC in activation of the 'protein conducting' channel

PIRAC might also have a regulatory function in protein translocation across the inner envelope membrane. The 'protein conducting' channel of the inner membrane has not been identified on a molecular level yet and therefore the gating properties of

this channel are still unknown. PIRAC inactivation could be important for the activation of the 'protein conducting' channel. The activation of the 'protein conducting' channel of the outer membrane, Toc75, was shown to be dependent on a membrane potential (8). If the same is true for the homologous channel of the inner membrane, PIRAC inactivation could be important to alter the membrane potential of the inner envelope membrane (locally) in order to activate the 'protein conducting' channel. If this is indeed the role of PIRAC in protein import, PIRAC inactivation should lead to a depolarisation of the inner envelope membrane. Protein import into chloroplast does not require the presence of a membrane potential across the envelope (21-23). In the presence of ionophores precursor proteins can be imported into isolated chloroplasts. If activation of the 'protein conducting' channel of the Tic complex would require an electrogenic potential across the inner envelope membrane, this activation would be impossible in the presence of ionophores and protein import would be inhibited.

A role for PIRAC in the regulation of transmembrane potential of the inner membrane

It is known that there is only a very small to negligible membrane potential across the chloroplast envelope (J.H.A. Dassen, personal communication). PIRAC could have a function in maintaining the membrane potential at very low values. The open probability of PIRAC in the absence of precursor protein is approximately 0.8 (chapter 2). This P_O is not dependent on the holding potential applied or on the KCl concentration of the medium (chapter 3 and chapter 5). This indicates that *in vivo* the channel is likely to be in the open state for the larger part of the time. Such a channel with a high P_O could be the way to keep the membrane potential across the envelope close to zero, despite of the large ion fluxes associated with photosynthesis. When a

precursor protein is translocated across the envelope obviously a 'protein conducting' channel has to open to let the precursor pass. The opening of such a channel could be associated with an increase in permeability of the envelope due to ion leakage of the 'protein conducting' channel. An increase in permeability of the envelope has been observed during protein import (24). The inactivation of PIRAC during protein import would then function to compensate the increased permeability of the inner envelope membrane caused by the opening of a 'protein conducting' channel. Single channel recordings in the presence of preferredoxin showed a reversible 'burst-like' increase in permeability of the envelope (chapter 2).

What is the role of PIRAC in protein import?

The experimental data described in this thesis allows several hypothesis about the role of PIRAC in chloroplast protein import (see above). The second hypothesis given above, i.e. the activation of the 'protein conducting' channel by a local polarisation caused by the inactivation of PIRAC is contradicted by the inhibitory effect of DIDS on *in vitro* protein import (chapter 3). The hypothesised polarisation induced by the inactivation of PIRAC would also occur if PIRAC is blocked by DIDS. Blocking of PIRAC would then lead to the activation of the 'protein conducting' channel and import would be unaffected or even stimulated by DIDS. What would be the effect of DIDS on protein import if inactivation of PIRAC during protein import is necessary to compensate for 'ion leakage' of the 'protein conducting' channel? PIRAC blocking by DIDS ultimately also leads to inactivation of the channel and therefor would also compensate for 'ion leakage' of the 'protein conducting' channel. This means that the addition of DIDS would not affect protein import into chloroplasts. One could imagine that binding of DIDS to PIRAC would physically hinder interaction of

precursor protein with the channel and thereby inhibit protein import into chloroplasts. However it has been shown that interaction of DIDS and preFd with PIRAC can occur simultaneously (chapter 3).

This leaves the role of 'protein conducting' channel for PIRAC. This would mean that blocking of the channel with DIDS would prevent preFd from entering the translocation pore. Interaction of preFd with PIRAC is still possible as shown in chapter 3, but the precursor cannot translocate across the inner membrane. This would explain the inhibitory effect of DIDS on protein import. An alternative explanation for the effect of DIDS on protein import and the role of PIRAC in chloroplast protein import can however not be excluded. It could be possible that the addition of DIDS affects other components of the chloroplast translocon leading to the inhibition of protein import.

In conclusion

It can be concluded that a 50 pS anion channel of the envelope (PIRAC) is involved in protein import. PIRAC is associated with the translocon of the inner membrane of the chloroplast. The channel is inactivated during protein import. This inactivation occurs after association of the translocating precursor with Tic. It is the result of a direct interaction of the precursor with PIRAC. The exact function of PIRAC in the translocation process is however not resolved yet.

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Summary

The electrophysiological response of the chloroplast envelope during protein import is investigated by using single channel recordings of the chloroplast envelope. An anion channel localised in the inner envelope membrane is identified. The channel has a single channel conductance of 50 pS (chapter 2 and 5). It is found that this channel is inactivated by precursor protein. The precursor induced inactivation of the channel is dependent on the presence of ATP and a functional transit sequence. A deletion mutant of preferredoxin (preFd), that is defective in *in vitro* import and initial binding, is unable to inactivate the channel (chapter 2). From this it is concluded that the 50 pS anion channel is involved in protein import. The channel is therefore termed Protein Import Related Anion Channel (PIRAC).

Inactivation of PIRAC is shown to require the translocation of precursor protein across the outer membrane and association of the precursor with Tic. In the presence of GTP preFd is unable to inactivate PIRAC (chapter 3). A deletion mutant of preFd (Δ15-25-preFd) is shown to inactivate PIRAC (chapter 3). This deletion mutant can compete with the wild-type precursor for import, but is not imported into the stroma. Furthermore evidence has been presented before that Δ15-25-preFd does interact with Tic. It is therefore concluded that translocation of the precursor across the inner envelope membrane is not required for PIRAC inactivation.

Blocking PIRAC activity with the anion channel blocker DIDS leads to a decrease in import efficiency of isolated pea chloroplasts (chapter 3). This indicates that normal functioning of the channel is required for protein translocation.

It is shown that the precursor protein induces a long-lived closed state of PIRAC, which is not observed in the absence of precursor (chapter 4). The mean duration of

this inactive state is found to be independent of the overall precursor length. In the absence of precursor protein PIRAC is observed to have two distinct open and three distinct closed states (chapter 4). These states can be distinguished on the basis of there mean durations. It is found that the addition of precursor protein decreases the mean duration of one of the open states and two of the closed states (chapter 4). The only states of PIRAC that are unaffected by the addition of precursor are of very short duration (below 1 ms). The precursor induced decrease of the mean duration of PIRAC states is found to be concentration dependent. This leads to the conclusion that there is a direct interaction between the translocating precursor and PIRAC, leading to the switching of the channel to the long-lived inactive state.

It is observed that addition of antibodies to Tic110 at the stromal side of an excised inside out patch irreversibly inactivated PIRAC activity (chapter 5). Tic110 is a known component of the translocon of the inner membrane of the chloroplast envelope. It is an integral membrane protein with a large hydrophilic loop facing the stroma. It is therefore concluded that PIRAC is associated with the translocon of the inner membrane of the chloroplast envelope.

Nederlandse Samenvatting

De elektrofysiologische respons van de chloroplast envelop tijdens eiwit import is gekarakteriseerd met behulp van 'single channel recordings'. Een anion kanaal in de binnenmembraan van de envelop kon worden geïdentificeerd. Dit anion kanaal heeft een 'single channel' geleiding van 50 pS (hoofdstukken 2 en 5). Het genoemde kanaal wordt geïnactiveerd door een precursor eiwit. De precursor geïnduceerde inactivatie van het kanaal is afhankelijk van de aanwezigheid van ATP en een functionele transit sequentie. Een deletiemutant van preferredoxine bleek niet in staat om het kanaal te inactiveren (hoofdstuk 2). Voor deze deletie mutant is beschreven, dat in *in vitro* import experimenten er geen import van deze precursor plaatsvindt. Hieruit is geconcludeerd, dat het beschreven kanaal betrokken is bij eiwit import in de chloroplast. Het kanaal wordt daarom aangeduid als PIRAC ('Protein Import Related Anion Channel').

Aangetoond is, dat voor inactivatie van PIRAC de precursor over de envelop buitenmembraan getransporteerd moet zijn. Tevens is een associatie van de precursor met Tic noodzakelijk voor PIRAC inactivatie. In aanwezigheid van GTP wordt PIRAC niet geïnactiveerd door preFd. (hoofdstuk 3). Een deletie mutant van preFd (Δ15-12-preFd) bleek PIRAC ook te inactiveren. (hoofdstuk 3). Het is bekend, dat deze deletiemutant kan competeren met de 'wild-type' precursor voor import. De deletie mutant wordt daarentegen niet volledig geïmporteerd in het stroma. Verder is eerder aangetoond, dat Δ15-25-preFd tijdens import wel associeert met Tic. Hieruit is geconcludeerd, dat translocatie van de precursor over de envelop binnenmembraan niet noodzakelijk is voor inactivatie van PIRAC.

Blokkering ('blocking') van PIRAC activiteit met de anion kanaal blocker DIDS leidt tot een afname in import efficiëntie van geïsoleerde erwten chloroplasten. Dit

duidt op correct functioneren van PIRAC als een voorwaarde voor chloroplast eiwit import.

Aangetoond is, dat precursor eiwit een gesloten toestand van PIRAC met langere levensduur induceert (hoofdstuk 4). Deze toestand is niet aanwezig in afwezigheid van precursor. De gemiddelde levensduur van deze inactieve toestand bleek onafhankelijk van de lengte van de toegevoegde precursor. In afwezigheid van precursor vertoont PIRAC twee verschillende open en drie verschillende gesloten toestanden (hoofdstuk 4). Deze toestanden kunnen onderscheiden worden op basis van hun gemiddelde levensduur. Het toevoegen van precursor eiwit verlaagt de gemiddelde levensduur van één van de open en twee van de gesloten toestanden. De toestanden van PIRAC die niet beïnvloed worden door precursor eiwit hebben een zeer korte gemiddelde levensduur (minder dan 1 ms). De precursor geïnduceerde verlaging van de gemiddeld levensduur van PIRAC toestanden blijkt concentratie afhankelijk. Hieruit is geconcludeerd, dat er een direct interactie tussen PIRAC en het precursor eiwit is.

Het toevoegen van antilichamen tegen Tic110 aan de stromale zijde van de patch induceert een irreversibele inactivatie van PIRAC (hoofdstuk 5). Tic110 is een eerder aangetoonde component van de translocon van de binnenmembraan. Het is een integraal membraan eiwit met een grote stromale 'loop'. Hieruit is geconcludeerd, dat PIRAC geassocieerd is met het translocon van de binnenmembraan van de chloroplast.

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Curriculum vitae

Paul van den Wijngaard werd geboren op 20 november 1971 te Hilversum. Na het doorlopen van het VWO werd in 1990 begonnen aan de studie Scheikunde aan de Universiteit Utrecht. Het doctoraal examen werd in 1995 behaald met als hoofdvak Biochemie van membranen (prof. dr. B. de Kruijff) en als bijvak Moleculaire farmacologie (dr. D. van Heuven-Nolsen). Aansluitend volgde een aanstelling als onderzoeker in opleiding (OIO) in dienst van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) bij het laboratorium voor plantenfysiologie van de Landbouwuniversiteit Wageningen, hetgeen resulteerde in dit proefschrift. Vanaf september 1999 is hij werkzaam als onderzoeker bij de sectie Plantenfysiologie van de vakgroep Moleculaire ontwikkelingsgenetica van planten van de Vrije Universiteit te Amsterdam.