The Mechanism of Inactivation of a 50-pS Envelope Anion Channel during Chloroplast Protein Import

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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 the protein import related anion channel 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 preexisting 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 an 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 a transit sequence. The transit sequence is both necessary and sufficient to target a protein to the chloroplast (De Boer and Weisbeek, 1991). Several components of the chloroplast protein import machinery have been identified (for recent reviews see Fuks and Schnell, 1997; Heins et al., 1998). The two envelope membranes each have their own import machineries, which can function independently of each other (Scott and Theg, 1996). 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) (Schnell et al., 1997).

Recently the involvement of an anion channel of the chloroplast envelope in protein import was identified (van den Wijngaard and Vredenberg, 1997). This envelope channel, which is located in the inner membrane, will be called the protein import related anion channel (PIRAC) here. The channel was shown to have a single-channel conductance of 50 pS in 250 mM KCl (van den Wijngaard and Vredenberg, 1997). The addition of precursor protein was shown to inactivate the 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 (van den Wijngaard and Vredenberg, 1997). The exact role of the PIRAC in chloroplast protein import is not yet known. The mechanism of PIRAC inactivation by precursor protein during chloroplast protein import is also still unclear.

In this report the role of the 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 the 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 the PIRAC induced by different precursor proteins and a transit peptide is used to further analyze the role of the PIRAC in chloroplast protein import. It is found that precursor protein and transit peptide induce a long-lived inactive state of the PIRAC; the duration of this state is not dependent on precursor length.

MATERIALS AND METHODS

Precursor proteins and transit peptide

The precursors of *Silene pratensis* ferredoxin (preFd) and of the tobacco small subunit of ribulose-2,5-biphosphate carboxylase/oxygenase (preSSU) were overexpressed in *Escherichia coli* and isolated as described before (Pilon et al., 1995; Pinnaduwage and Bruce, 1996). The transit peptide of preSSU (tpSSU) was isolated from a GST fusion system as has been described before (Pinnaduwage and Bruce, 1996).

Chloroplast isolation

Chloroplasts were isolated from pea leaves by cutting them gently with a razor blade in buffer containing 2.5 mM

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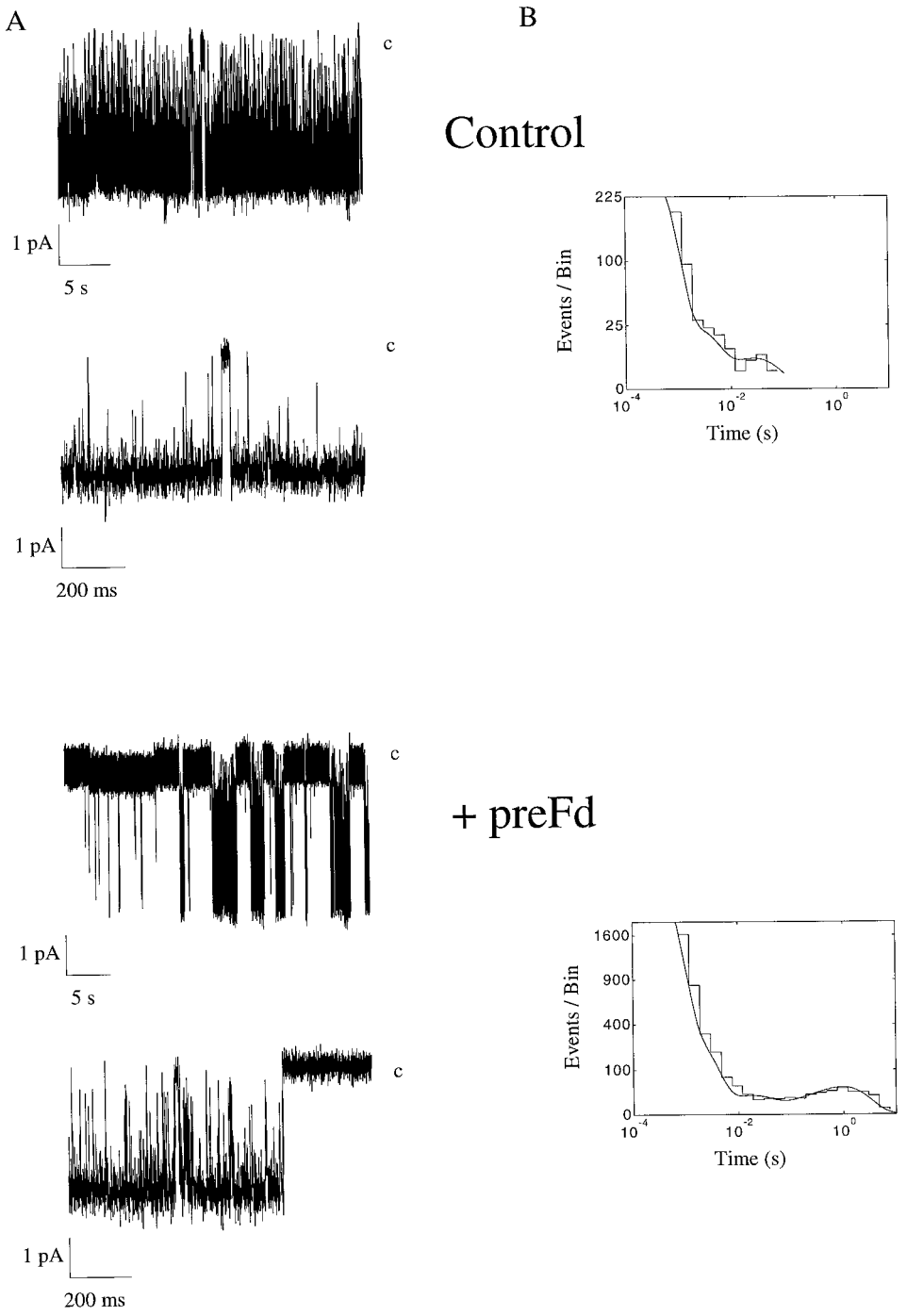

FIGURE 1 (*A*) Single-channel recordings in the absence and presence of preFd in the pipette filling solution. Parts of the same recording are shown at different time scales. Recordings were made at a holding potential of −20 mV. (*B*) Closed time duration distribution of PIRAC in the absence and presence, respectively, of preFd in the pipette filling solution. Distributions are plotted on square root versus log time axes with five 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.

N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid/KOH (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

A standard patch-clamp technique was used to record the electrical currents across the chloroplast envelope (Hamill et al., 1981). Electrodes were pulled from borosilicate glass by a two-step pull and extensively fire-polished. Electrodes were filled with buffer containing 2.5 mM TES/KOH (pH 7.2), 250 mM KCl, and 2 mM $CaSO_4$, 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 the 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

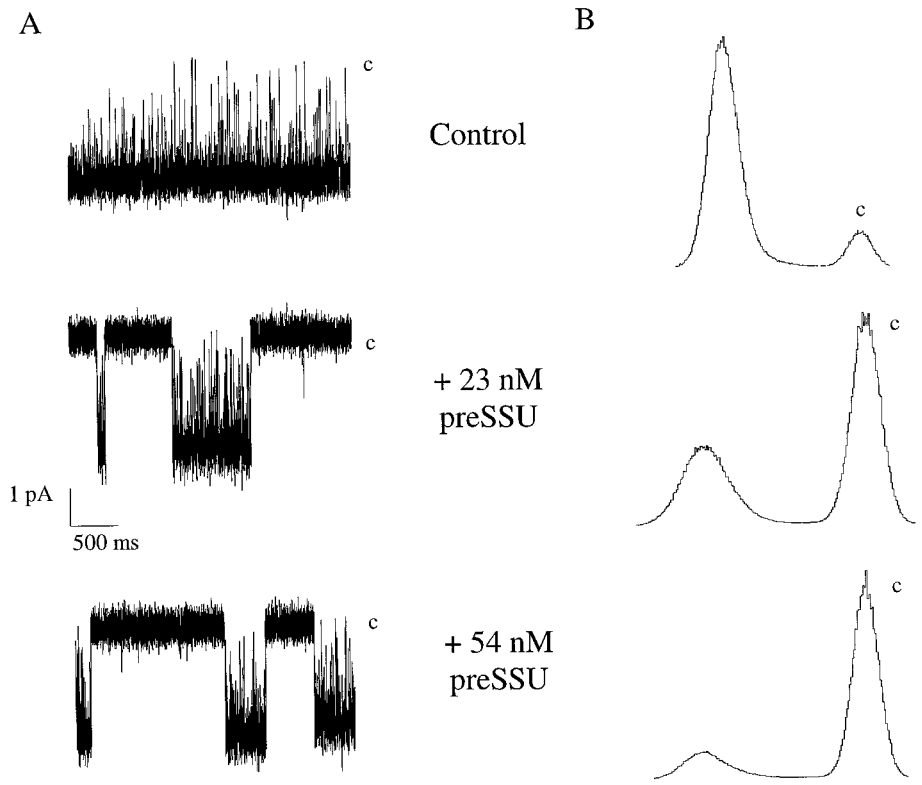

FIGURE 2 (*A*) Single-channel recording of PIRAC in the absence and presence, respectively, of two different concentrations of preSSU. Recordings were made at a holding potential of −20 mV. (*B*) All point amplitude histograms of single-channel recordings of PIRAC in the absence and presence, respectively,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

after giga-seal formation, using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Potentials are given with regard to the pipette interior; the bath was kept at ground, using a 250 mM KCl agar bridge. In this study the chloroplast envelope is treated as one permeability barrier, and the sign convention of single endomembranes is used (Bertl et al., 1992). The stroma is treated as outside, and cation flow from cytosol to stroma is regarded as positive current; conversely, anion flow from cytosol to stroma is treated as negative current. The data were filtered at a cutoff 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, Cambridge, MA) interface.

Data were analyzed with the Patch and Voltage Clamp Software (Cambridge Electronic Design). To determine the distributions of open and closed time durations of the PIRAC, a module was developed in the matrix calculating software Matlab (The Mathworks, Natick, MA). This module uses the 50% threshold method to identify transitions of the channel between the open and closed states. Histograms of open and closed time durations were constructed using the square root of the number of events versus the log binwidth of durations (Sigworth and Sine, 1987). The distributions were fitted with multiexponential probability density functions, using the maximum likelihood method (Colquhoun and Sigworth, 1992). 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, because of the abundance of large pores in this membrane (Flügge and Benz, 1984). No light-induced currents (Vredenberg et al., 1995) 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 inner membranes, including the intermembrane space.

To further elucidate the mechanism of precursor protein-induced inactivation of the PIRAC, the distributions of open and closed time durations were determined in the absence and presence of precursor protein. Fig. 1 *A* shows single-channel recordings in the absence and presence, respectively, of preFd. Single-channel recordings are shown with different time scales to allow a visual impression of the different dwell-time components found. Comparison of the two recordings suggests that the inactivation of the PIRAC is caused by the induction of a long-lived closed state induced by preFd. In the control situation the PIRAC has three distinct closed states, as can be concluded from the distribution of closed time durations shown in Fig. 1 *B*. The distribution is best fitted with a mixture of three exponential

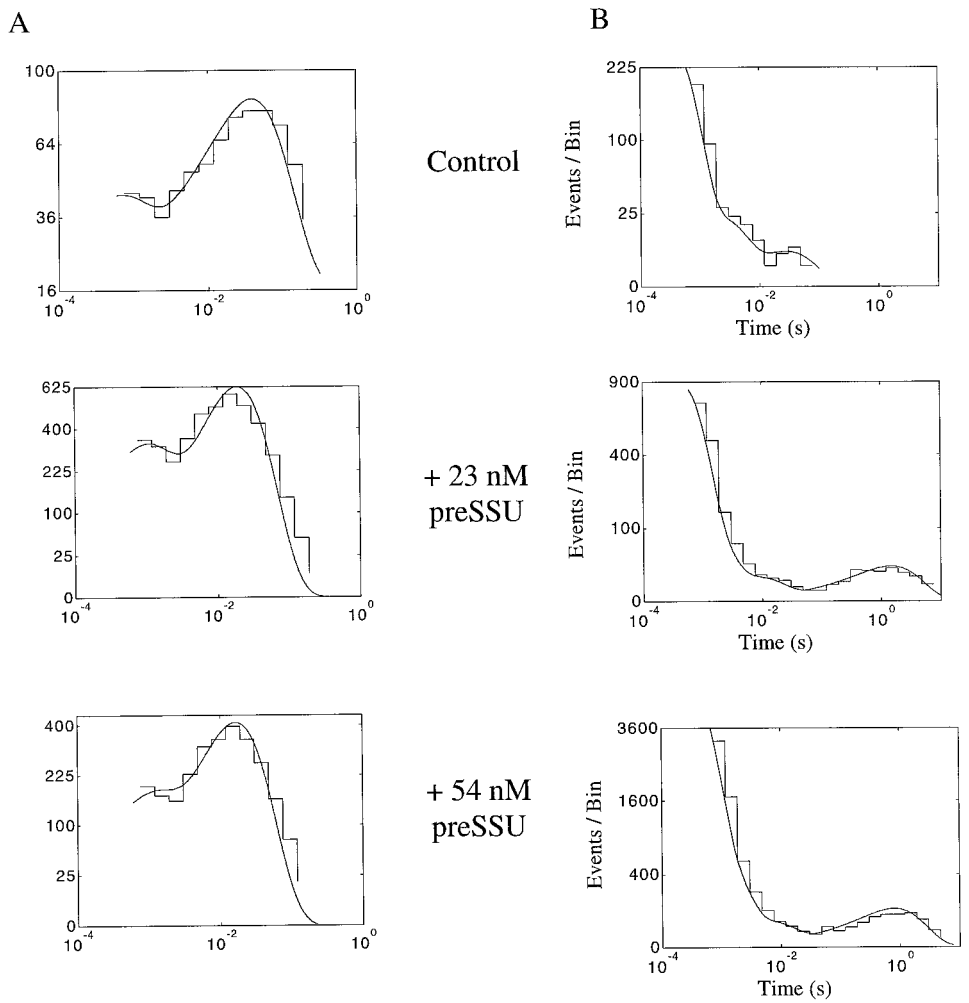

FIGURE 3 (*A*) Open time duration distributions of PIRAC in the absence and presence of two different concentrations of preSSU. (*B*) Closed time duration distributions of PIRAC in the absence and presence of two different concentrations of preSSU. The closed time duration distribution shows the precursor induced long-lived closed state. All distributions are plotted on square root versus log time axes with five bins per decade and were obtained at a holding potential of −20 mV.

probability density functions; the line in Fig. 1 *B* superimposed on the histogram shows this fit. The time constants of the closed states are 0.35, 2.41, and 29.1. When preFd is included in the pipette filling solution, a new long-lived closed state is observed. In Fig. 1 *B* the distribution of closed time durations of the 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. 1 *B* as a line superimposed on the histogram. The time constant of the preFd-induced long-lived closed state of the PIRAC is 835 ms.

preSSU, another precursor, which is translocated across the chloroplast envelope, is also able to inactivate the PIRAC. Fig. 2 *A* shows single-channel recordings of the 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. 2 *B* the open probability (P_O) of the PIRAC was determined. PreSSU causes a dose-dependent decrease in P_O of the PIRAC, in the presence of 23 nM preSSU the P_O decreased from 0.81 in the control situation to 0.39. In the presence of 54 nM preSSU the P_O is 0.17. The mature SSU protein was found not to inactivate the PIRAC (not shown). The single-channel recordings shown in Fig. 2 *A* suggest that the preSSU-induced inactivation of the PIRAC is also caused by the occurrence of a long-lived closed state. In Fig. 3 the open and closed time duration distributions of the PIRAC in the absence and in the presence of the two different concentrations of preSSU are

TABLE 1 Mean durations of the open states of the PIRAC in the absence and presence, respectively, of preSSU

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

	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

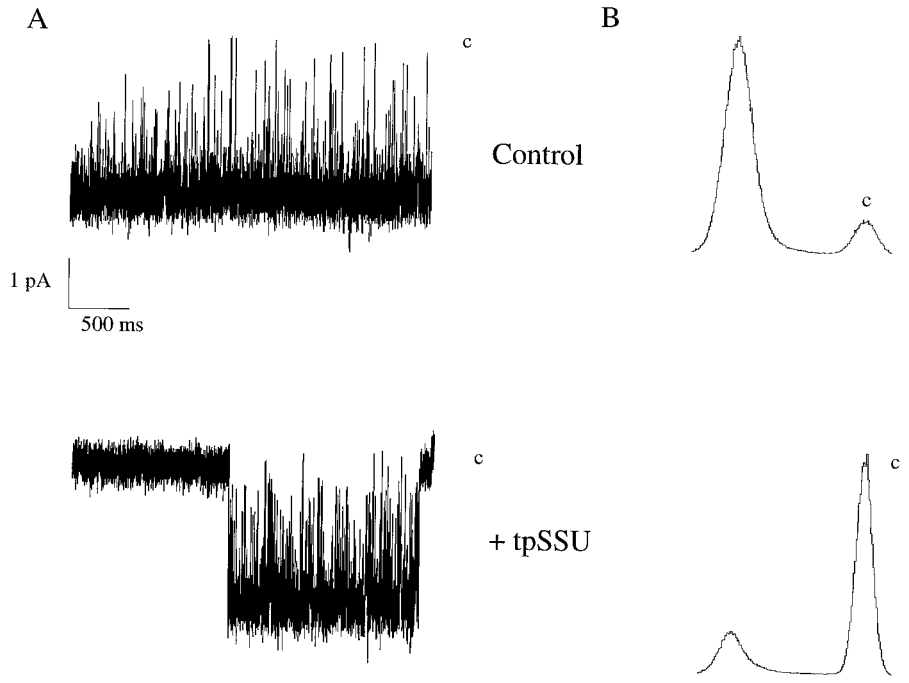

FIGURE 4 (*A*) Single-channel recordings of PIRAC in the absence and presence, respectively, of tpSSU. The recordings were made at a holding potential of −20 mV. (*B*) All point amplitude histograms of single-channel recordings of PIRAC in the absence and presence, respectively, of tpSSU. The histograms indicate a decrease in P_O of PIRAC in the presence of tpSSU.

shown. The closed time duration distribution illustrated in Fig. 3 *B* clearly shows the precursor protein-induced long-lived closed state, also observed in the presence of preFd (Fig. 1 *B*). The open time duration distribution of the PIRAC shows that the channel has two distinct open states. Table 1 shows the mean durations of the different open states of the 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 2. Tables 1 and 2 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.

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

DISCUSSION

The present data demonstrate that the previously described inactivation of the PIRAC during chloroplast protein import (van den Wijngaard and Vredenberg, 1997) 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 the 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 (van den Wijngaard and Vredenberg, 1997). 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 the PIRAC, however, is independent of the precursor concentration (Fig. 3 and Ta-

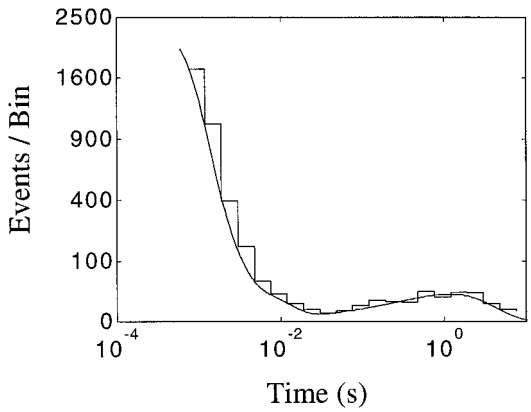

FIGURE 5 Closed time duration distribution of PIRAC in the presence of tpSSU. The distribution is plotted on square root versus log time axes with five bins per decade. The distribution shows that tpSSU-induced inactivation of PIRAC is also the result of a long-lived closed state. The distribution was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

ble 2). From this it can be concluded that the 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 the PIRAC that are present in the control situation are affected by precursor protein, except for the one in the open and closed states, with the shortest mean duration. These states might be too short-lived to be able to interact with the precursor. However, interaction between the PIRAC and the precursor might also still occur in these states, but because of their short duration the decrease in the time constant is too small and is 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 suggests that the role of the 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 the PIRAC interacts with precursor in all possible states of the channel.

The exact role of the PIRAC in the protein import process is not yet clear. It is possible that the PIRAC represents the protein import channel of the inner envelope membrane. The inactivation of the 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 the PIRAC spends in the protein-conducting mode. This time is then indicative of the duration of translocation of a single precursor protein. The maximum rate of import has been determined for several precursor proteins (Pilon et al., 1992; Cline et al., 1993). 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 (van't Hof and de Kruijff, 1995). 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 closed states induced by preFd, preSSU, and tpSSU, 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 (Lohret and Kinnally, 1995). For normal activity of MCC, including the blocking of the channel by a presequence, Tim23 is required (Lohret et al., 1997). Tim23 has been shown to be a component of the import machinery of the mitochondrial inner membrane (Bauer et al., 1996). In view of the data presented here, a similar association between the 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 are the result of interaction between the precursor and this complex.

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