The Envelope Anion Channel Involved in Chloroplast Protein Import Is Associated with Tic110*

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Paul W. J. van den Wijngaard and Wim J. Vredenberg
From the Laboratory of Plant Physiology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

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 estimated 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.

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 the transit sequence. The transit sequence is both necessary and sufficient to target a protein to the chloroplast (1). Several components of the chloroplast import system have been identified (for recent reviews see Refs. 2 and 3). The two surrounding membranes both have their own import machinery that function independently 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, which 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) was 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, however, is not known yet.

In this report a further characterization of PIRAC is described. An initial 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—A standard patch clamp technique was used to record the currents across the chloroplast envelope (10). Electrodes were pulled from borosilicate glass by a two-step pull and extensively fire-polished. Electrodes were filled with electrolyte solution as indicated in the text. Electrode resistances were found to be typically around 30 megohms.

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

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

**Results**

The seal resistances obtained in inside-out patches of the chloroplast envelope were typically between 15 and 30 gigohms. Because of the abundance of large pores in the outer envelope membrane (11), it is highly unlikely that the seals consist of the outer membrane alone. Moreover, in over 2000 successful seals no (changes in) current signals associated with gating activity of the pores were ever observed. It is conceivable that the seal consists of a sandwich-like structure of the outer and the inner membranes. In this configuration gating of the outer membrane pores would cause shifts in the base-line current because of the opening and closing of the pore. Such shifts in base-line currents were never observed in any of the successful seals, which argues against the presence of the outer membrane in the patch. In addition no light-induced currents (12) were ever observed after seal formation (i.e., in the attached configuration) or after excision of the patch. This finding indicates that the thylakoid membrane under the conditions used here was not included in the patch. The patch is therefore likely to consist of the inner membrane alone.

The possibility cannot be excluded, however, that the outer membrane pores are silent in a patch consisting of both the outer and inner membrane tightly compressed. Such a sandwich-like patch would give rise to a higher capacitance seal as compared with a seal with only one membrane present. When patch pipettes identical to those used in the present chloroplast experiments were applied to obtain seals of the vacuolar membrane in the inside-out configuration, the capacitive current induced by a stepwise change in holding potential was not significantly different (data not shown). This finding indicates that the membrane sizes or the thickness of the seals obtained...
from the chloroplast envelope or the vacuole membrane are identical, thus arguing for a seal consisting of the inner envelope membrane alone. In single-channel recordings presented here, the current runs across the inner membrane. Because only the effect of an antibody added to the stromal side of the patch is described, the data are not affected by the presence or absence of the outer membrane in the patch.

**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 levels. This behavior 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 five 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).

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 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 $P_{\text{Cl}} / P_{\text{K}}$ 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 simplest 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.

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 of 18 single-channel recordings. Single-channel recordings of PIRAC without antibody in the bath solution very rarely show loss of channel activity because of 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.

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 has been shown 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 concentration dependency 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 also observed previously 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 as described elsewhere (9). This correspondence 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.² 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, which 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 multiparticle conductance channel by antibodies to Tim23 (17). The multiple conductance channel 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 approximate PIRAC pore size of 6.5 Å, precursor proteins have to be completely unfolded to pass through the PIRAC pore. The reconstituted protein translocation channel of the outer membrane, Toc75, has been reported to have an approximate pore size of 8.5 Å (14). This value is based on the same approximation as the one used for PIRAC in this study, which indicates that the pores of the protein import channels of the outer and inner membranes are of comparable sizes.

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REFERENCES

1. De Boer, A. D., and Weisbeek, P. J. (1991) Biochim. Biophys. Acta 1071, 221–253
2. Scott, S. V., and Theg, S. M. (1996) J. Cell Biol. 132, 63–75
3. Heins, L., Collinson, I., and Soll, J. (1998) Trends Plant Sci. 3, 56–61
4. Fuku, B., and Schnell, D. J. (1997) Plant Physiol. 114, 405–410
5. Schnell, D. J., Blohel, G., Keesstra, K., Kessler, F., Ko, K., and Soll, J. (1997) Trends Plant Sci. 3, 56–61
6. De Boer, A. D., and Weisbeek, P. J. (1991) Biochim. Biophys. Acta 1071, 221–253
7. Heins, L., Collinson, I., and Soll, J. (1998) Trends Plant Sci. 3, 56–61
8. Scott, S. V., and Theg, S. M. (1996) J. Cell Biol. 132, 63–75
9. Schnell, D. J., Blohel, G., Keesstra, K., Kessler, F., Ko, K., and Soll, J. (1997) Trends Plant Sci. 3, 56–61
10. Fuku, B., and Schnell, D. J. (1997) Plant Physiol. 114, 405–410

² P. W. J. van den Wijngaard, unpublished results.
6. Kessler, F., and Blobel, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7684–7689
7. Lübeck, J., Soll, J., Akita, M., Nielsen, E., and Keegstra, K. (1996) EMBO J. 15, 4230–4238
8. Jackson, D. T., Froehlich, J. E., and Keegstra, K. (1998) J. Biol. Chem. 273, 16583–16588
9. Van den Wingnaard, P. W. J., and Vredenberg, W. J. (1997) J. Biol. Chem. 272, 29430–29433
10. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) Pflügers Arch. Eur. J. Physiol. 391, 85–100
11. Flügge, U., and Benz, R. (1984) FEBS Lett. 169, 85–89
12. Vredenberg, W., Bulychev, A., Dassen, H., Snel, J., Van Vourthuysen, T. (1995) Biochim. Biophys. Acta 1230, 77–80
13. Hille, B. (1992) in Ionic Channels of Excitable Membranes, 2nd Ed., pp. 294–298, Sinauer Associates, Sunderland, MA
14. Hinnah, S. C., Hill, K., Wagner, H., Schlicher, T., and Soll, J. (1997) EMBO J. 16, 7351–7360
15. Smart, O. S., Breed, J., Smith, G. R., and Sansom, M. S. P. (1997) Biophys. J. 72, 1109–1126
16. Nielsen, E., Akita, M., Davila-Aponte, J., and Keegstra, K. (1997) EMBO J. 16, 935–946
17. Lohret, T. A., Jensen, R. E., and Kinnally, K. W. (1997) J. Cell Biol. 137, 377–386
18. Lohret, T. A., and Kinnally, K. W. (1995) J. Biol. Chem. 270, 15950–15953