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Precursor-mediated opening of translocation pores in chloroplast envelopes

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Abstract Direct electrical measurements on native chloroplast envelopes reveal that a full-length chloroplast precursor protein causes an increase in the conductivity of the envelope membranes, due to its transit sequence. The conductivity is not influenced by a truncated precursor protein incapable of efficient translocation, suggesting precursor-mediated opening of translocation pores in chloroplast envelopes.

Key words: Chloroplast protein import; Transit peptide; Patch pipette; Envelope conductivity; Protein channel

1. Introduction

Protein targeting in eukaryotic cells requires within each target organelle the presence of a translocation machinery, which is capable of the specific recognition and subsequent membrane translocation of precursor proteins that carry topogenic information for that organelle. In the case of higher plant chloroplasts, the presence of a cleavable transit sequence in the precursor ensures the ATP-dependent recognition and translocation steps [1]. In several studies, components of the chloroplast envelope have been identified, which are believed to be involved in the initial recognition steps [2,3]. However, very little is known about how the membrane responds during precursor protein translocation. The large size of chloroplasts make them, among protein translocation systems, uniquely suitable for direct electrophysiological studies.

2. Experimental

2.1. Determination of voltage–current curve on whole chloroplasts of Peperomea metallica

Chloroplasts of *P. metallica* were isolated by cutting a leaf section and visual selection of the organelle in a 2 ml chamber containing the following buffer: 25 mM HEPES/KOH, pH 7.5, 0.33 M sorbitol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.2 mg/ml bovine serum albumin, 0.5 mM dithiothreitol and 0.5 mM MgATP. Micropipettes were prepared from borosilicate glass, fire polished and filled with the buffer [4]. The electrode resistance typically was 4–12 MΩ and was found to be independent of voltage in the ranges used. Suction was accomplished with a negative pressure equivalent to a water column of 3–10 cm. Currents were measured with an EPC-7 patch-clamp amplifier. A cycle time of 10 s was used to measure voltage–current relationships [4].

2.2. Protein import competition experiments

In vitro translation was performed in a wheat germ lysate as described [5]. To achieve efficient expression of both wild-type and truncated precursor of the chloroplast protein ferredoxin from *Silene pratensis* (prefd) in *Escherichia coli* [6], unique *Nco*I sites were created at the 5′ end of these sequences. The truncated prefd (prefd-Δ7) has a deletion of 7 amino acids from the C-terminus of the transit sequence. Mutagenesis was performed on plasmids pFDS22 and pFDS-t7 [6]. The *Nco*I–*Bam*HI inserts containing the coding sequences were cloned in vector pET11-D, and strain BL21 (DE3) was used as a host for the expression [7]. Both prefd and prefd-Δ7 were purified by the same protocol [5]. Chloroplast isolation, import experiments, SDS-PAGE and quantification were performed as described [5]. Wild-type or truncated purified precursors were mixed with tritium-labeled in vitro synthesized wild-type precursor in the import buffer prior to the addition of chloroplasts. After a 20 min incubation at 25°C in the light, the amounts of translocated radiolabeled precursor were determined [8].

2.3. Measurement of the envelope resistance of P. metallica chloroplasts upon addition of precursor or transit peptide

For a continuous recording of the envelope resistance under voltage-clamp conditions (holding potential varying from +10 to +30 V), a sine wave voltage (10 mV/100 Hz) was applied and the output current was fed into a lock-in amplifier that was adjusted to a phase shift at which the output signal was insensitive to on–off switching, a capacity condensation circuit [9]. Precursor of ferredoxin and truncated precursor were first diluted 10-fold out of 8 M urea and then added to the chamber to the indicated concentrations. The final urea concentration was always kept below 8 mM. The transit peptide of 47 amino acids corresponding to the transit sequence of wild-type precursor ferredoxin from *S. pratensis* (prepared and handled as described in [8]), was added directly from water.

3. Results and discussion

To measure conductivity changes across chloroplast envelopes, we employed suction electrodes. The size of *Peperomea metallica* chloroplasts allows the visual selection of an intact organelle. Since in protein import the precursor enters from the outside, we pursued the whole chloroplast configuration presented in Fig. 1 for our measurements. In this configuration single channels are not expected to be measured, due to the abundance of translocators in the inner envelope membrane and the size and number of pores in the outer membrane [10,11]. After bringing the pipette into contact with a chloroplast by slight suction, the seal resistance typically stabilized within 10–20 min at values between 40 and 200 MΩ. Light flashes had no immediate effect on the membrane current, indicating an interaction of the pipette with the envelope only. At the same time, as expected for this configuration, the chloroplast stroma was found to be freely accessible to the membrane-

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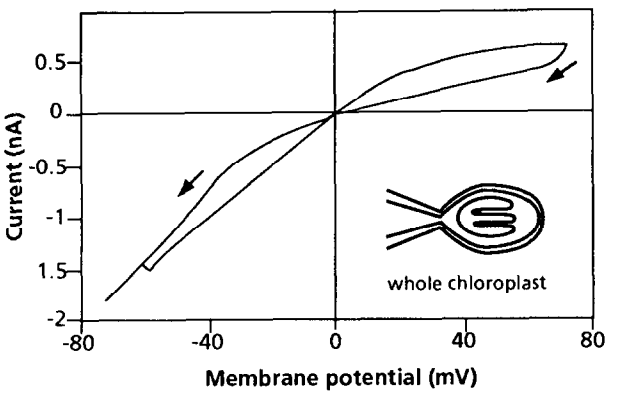

Fig. 1. Voltage–current curve measured on a whole chloroplast of *P. metallica*. The insert shows a diagram of the recording configuration.

impermeable fluorescent dye Lucifer yellow when this dye was included in the pipette initially (not shown). The *I–V* curves measured in this configuration were non-linear and had a convex shape (Fig. 1), with a characteristic hysteresis in current traces for down-rising and up-rising potential scans. Other patch configurations (i.e. the chloroplast attached or isolated patch configurations) were found to possess concave or linear shapes of their *I-V* curves.

Having established that it is possible to directly measure currents across the chloroplast envelope, we selected the chloroplast protein ferredoxin for studying the influence of a precursor on the membrane conductance. This precursor follows the general import pathway [5], and, upon dilution from 8 M urea, it can be translocated into chloroplasts [5].

To be able to assess the specificity of the pursued changes in electrical properties of the envelope, we included in this study the mutant precursor prefd-Δ7. In vitro translation–translocation experiments showed that this deletion greatly reduced the initial binding and import efficiency [6], suggesting that this part of the transit sequence plays an important role in chloroplast recognition [6]. The electrophysiological experiments require purified proteins. Fig. 2 shows that purified prefd-Δ7 is not able to inhibit the import of radiolabeled wild-type precursor into pea chloroplasts, and is thus a suitable control protein in our experiments.

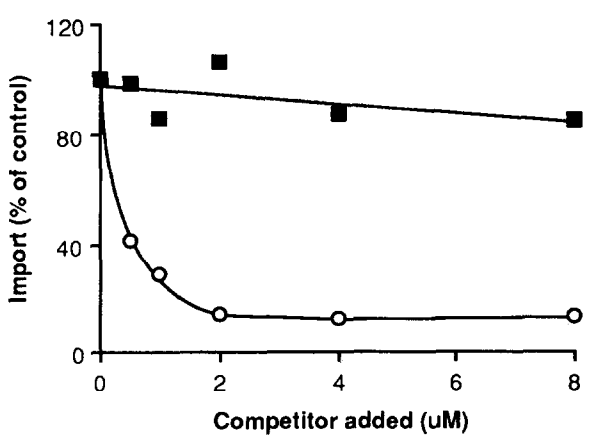

Fig. 2. Import competition of purified full-length and truncated precursor. The points represent the import of radiolabeled wild-type precursor. Final concentrations of unlabeled wild-type (○) or truncated (■) purified precursor are indicated. Each point represents the average of three experiments. Import of the radiolabeled wild-type precursor in the absence of competitor protein was set at 100%.

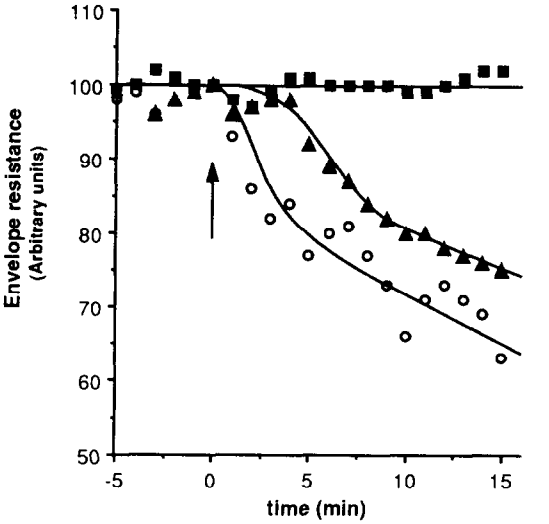

Fig. 3. Changes in the envelope resistance of *P. metallica* chloroplasts upon addition of (○) wild-type (0.75 μg/ml), or (■) truncated precursor (0.8 μg/ml) or (▲) transit peptide (5 μg/ml). Additions were done at time point zero. All points are the average of at least three representative experiments.

Addition of the purified wild-type ferredoxin precursor, at a concentration similar to the estimated K_m for import [12], to the medium induced a decrease in the envelope resistance by 35–40% within 15 min (see Fig. 3 and Table 1). A short lag phase typically preceded this decrease in envelope resistance. Fig. 3 and Table 1 also show that addition of prefd-Δ7 had no measurable effect on the resistance of the chloroplast envelope attached to the pipette, demonstrating a remarkable specificity of this effect, which thus requires a functional transit sequence. To directly test the role of the transit sequence in triggering the increase in envelope conductance, we also supplied chloroplasts with a synthetic peptide, corresponding to the transit sequence. Interestingly, addition of the transit peptide alone, at a concentration similar to the one that gives half-maximum inhibition in an import experiment [8], also led to a decrease in envelope resistance (Fig. 3 and Table 1), emphasizing that the transit sequence is inducing the changes in membrane properties.

The observed decrease in resistance of the envelope of *P. metallica* chloroplasts in the presence of full-length precursor and isolated transit peptide must be due to an increased ion

Table 1
Changes in envelope resistance of *P. metallica* upon addition of wild-type (WT), truncated precursor (TruncP) or transit peptide (TransP)

Time (min)	WT (n = 7)	TruncP (n = 4)	TransP (n = 3)
0	100	100	100
5	77 ± 17	101 ± 3	92 ± 10
10	66 ± 23	99 ± 2	80 ± 7
15	62 ± 17	102 ± 2	75 ± 8

Additions were done at time t = 0. Values are the average for the indicated number of experiments (n) and are given with their standard error. Control values at t = 0 are set at 100. Absolute values for individual chloroplasts were in the range between 50 and 90 MΩ. Additions were done at concentrations (in μg/ml) of 0.75 (WT), 0.8 (TruncP), and 5 (TransP), respectively.

permeability of the envelope. The changes in conductivity can, in principle, be due to the action of translocators in the inner envelope membrane [10], which may act to shuttle metabolites in response to protein translocation. However, the conductivity change is also seen in response to the isolated transit peptide. This peptide was shown in our lab to enter the general import pathway, however, it is not fully translocated into the stroma, but rather accumulates as a late translocation intermediate in the envelope (R. van 't Hof et al., in preparation). Therefore, we strongly favor the interpretation that an increased diffusion through protein conducting structures in the envelope causes the observed increase of current. The estimates for the number of translocation sites per chloroplast are in the range of approximately one thousand [1]. If we assume this number as an estimate for the amount of protein translocating structures (pores) then the conductance of one such pore opened by the transit sequence would be in the range of 10 pS. The only other electrical measurement on putative protein conducting channels reported so far made use of complex reconstitution protocols and led to much larger estimates for the channel conductance [13,14].

In conclusion, the present study demonstrates the feasibility of directly measuring the changes in electrical properties of intact chloroplast envelopes engaged in protein translocation processes, which opens up a new avenue through which the mechanism of membrane translocation of precursor protein can be studied.

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