Pore-forming activity in the outer membrane of the chloroplast envelope

U. Ingo Flügge and Roland Benz

Institut für Pflanzenphysiologie, Universität Göttingen, D-3400 Göttingen and *Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, FRG*

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The permeability properties of the outer membrane of the chloroplast envelope were studied. The exclusion limit for the penetration of molecules through this membrane into the intermembrane space lies between M_r values of about 7000–13000. This is the largest exclusion limit measured so far for pores of the porin type. In addition, the pore-forming activity of the outer membrane of the chloroplast envelope was measured by reconstitution experiments. The pore has a single channel conductance of 0.72 nS, which is a linear function of the salt solution in the aqueous phase. The diameter of the chloroplast porin was calculated to be 2.5–3 nm, which is the largest diameter of all known porin pores, e.g., from mitochondria or Gram-negative bacteria.

Pore-forming protein *Chloroplast envelope* *Porin* *Membrane reconstitution* *Spinach*

1. INTRODUCTION

There exists an hypothesis that the symbiosis of prokaryotic cells led to the evolution of eukaryotic cells [1]. In agreement with this hypothesis chloroplasts have a cell envelope which consists of two distinct membranes like the Gram-negative cyanobacteria [1]. The functional barrier between the chloroplast stroma and cytosol is the inner membrane [2]. This membrane is not permeable for hydrophilic solutes like sorbitol but it contains a variety of different transport systems for the specific transport of substrates such as phosphate, dicarboxylate, nucleotides, glucose and glycerate [3–5]. The outer membrane of the chloroplast envelope is, on the other hand, freely permeable for all these small substrates but not for dextrans of high M_r [2]. This suggests that the outer membrane of the chloroplast envelope has similar molecular sieving properties to the outer membranes of Gram-negative bacteria [6,7] and mitochondria [8–10].

The exclusion limit for the diffusion of hydrophilic molecules through the outer membrane of the chloroplast envelope is not known. Furthermore it is not known if there is a defined pathway for penetration of molecules through this membrane, nor how this pathway might be regulated. We have here examined the penetration of radioactive labeled peptides through the outer membrane of spinach chloroplast into the intermembrane space to elucidate the precise permeability properties of this membrane. In addition, we were able to reconstitute pore-forming activity from the outer membrane of the chloroplast envelope into lipid bilayer membranes. Our results indicate that the diameter of the pore in the outer membrane of the chloroplast envelope is much larger than that estimated for the porin pores in the outer membrane of Gram-negative bacteria [7] and mitochondria [8,9].

2. MATERIALS AND METHODS

Spinach (*Spinacia oleracea*, US hybrid 424, Ferry-Morse Seed Co., Mountain View, CA) was grown in water culture as in [11]. Preparation of intact chloroplasts was as in [2]. Outer envelope

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membranes from intact chloroplasts were prepared as in [12,13].

^3H_2O, [^{14}C]sorbitol, ferro[^{14}C]cyanide, [^{14}C]-sucrose and [^{3}H]inulin were purchased from Amersham Buchler and [^{14}C]formaldehyde from New England Nuclear. S-S lipotropin, insulin β-chain, insulin, aprotinin and lysozyme were obtained from Serva, Heidelberg, and lactoglobulin B, trypsinogen and carbonic anhydrase from Sigma.

Labeling of these substances was performed by reductive alkylation with [^{14}C]formaldehyde and sodium cyanoborohydride as in [14]. Low-M_r components were removed by passage over a Sephadex G-10 column equilibrated with 50 mM ammonium bicarbonate. After lyophilization, the ^{14}C-labeled substances were dissolved in distilled H_2O and stored at −85°C.

Permeability measurements were carried out by silicone layer filtering centrifugation [15,16] in a medium containing 0.3 M sorbitol, 10 mM sucrose, 50 mM Hepes (pH 7.6), 1% bovine serum albumin, 50 mM NaCl, 0.02% mercaptoethanol, 100 mM $MgCl_2$ and chloroplasts equivalent to 0.1 mg chlorophyll per ml.

Optically black lipid bilayer membranes were obtained [17] from a 1–2% (w/v) solution of asolectin (L-α-phosphatidylcholine IV-S from soybean, Sigma) in *n*-decane (purum, Fluka, Buchs). The chamber used for bilayer formation was made from Teflon. The circular hole in the well separating the two aqueous compartments had an area of 0.1 mm^2. The temperature was kept at 25°C throughout. The salts were obtained from Merck (analytical grade, Darmstadt) and Triton X-100 was purchased from Sigma.

Ag/AgCl electrodes were inserted into the aqueous solutions on both sides of the membranes. The current fluctuation experiments were performed using a Keithley 427 preamplifier. The amplified signal was monitored with a 5115 storage oscilloscope and recorded with a tape recorder. The current fluctuations were analyzed as in [18].

3. RESULTS AND DISCUSSION

3.1. *Permeability of the outer membrane of the chloroplast envelope*

Using silicone oil filtering centrifugation as described above the uptake of a number of radioactively labeled substances of various sizes into the intermembrane space was studied. The water-soluble substances had the following M_r values (in parentheses): sorbitol (182), sucrose (342), ferrocyanide (484), S-S lipotropin (794), insulin β-chain (3400), inulin (5200), insulin (5700), aprotinin (6500), lysozyme (14300), lactoglobulin B (18400), trypsinogen (24000), carbonic anhydrase (30000) and bovine serum albumin (66000). Fig.1 shows the cut-off curve of a typical experiment for the penetration of these substances into the intermembrane space. Obviously even aprotinin (M_r 6500) can penetrate the outer membrane completely, whereas peptides with M_r > 13000 are fully retained by the outer envelope membrane. Consequently the exclusion limit for the penetration lies between M_r values of about 7000 to 13000, probably around 9000–10000.

This exclusion limit measured for the pore in the outer membrane of the chloroplast envelope is the largest measured so far for pores of the porin type, e.g., for pores formed by porins from the outer membranes of Gram-negative bacteria [19,20] and mitochondria [8]. The exclusion limit of the OmpF porin pore from *Escherichia coli* outer membrane is about 600 Da [19], whereas that of the protein F pore from *Pseudomonas aeruginosa* outer mem-

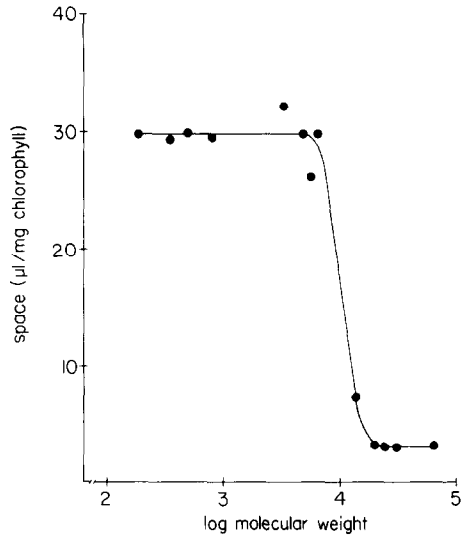

Fig.1. Exclusion limit of the chloroplast pore. For details see text. The values are averages of results obtained in at least 3 experiments. The ^3H_2O space ws 49.6 µl/mg chlorophyll.

brane is around 6000 Da [20]. Pores formed by mitochondrial porin have, on the other hand, a somewhat smaller exclusion limit than the protein F pore of *P. aeruginosa* outer membrane [8]. The comparison of the exclusion limit of the OmpF pore of *E. coli* with that of the pore in the outer membrane of the chloroplast envelope reported here allows a rough estimate to be made of the pore diameter in the latter. The radius r_E of the *E. coli* pore is about 0.6 nm [7,21,22] and since the weight of globular molecules with identical densities is a function of the cube of their radii [23], the radius r_c of the chloroplast pore is given by:

$$r_c^3 = M_c r_E^3 / M_E \quad (1)$$

where M_c and M_E are the exclusion limits of the pore from the outer membrane of the chloroplast envelope and of the Gram-negative bacteria, respectively. If we insert in eq.1 the values for the two exclusion limits (600 for *E. coli* and 10000 for the chloroplast pore), we obtain a radius r_c of approx. 1.5 nm or a diameter of the chloroplast pore around 3 nm. This diameter is much larger than that of the mitochondrial pore (2 nm [21,24]) or that of the F-porin pore of *P. aeruginosa* outer membrane (2.2 nm [25]). Interestingly, this result is also in close agreement with that obtained from lipid bilayer experiments (see below).

3.2. *Lipid bilayer experiments*

Reconstitution experiments in which the outer envelope membrane is first solubilized with a number of ionic and non-ionic detergents and subsequently added to the lipid bilayer membrane proved to be unsuccessful. This might be due to the sensitivity of the pore activity to any type of detergent, an observation which has also been made in the case of the phosphate translocator of the inner envelope membrane [26].

Successful reconstitution of the pore of the outer membrane of the chloroplast envelope was only obtained if the treatment of the outer membrane with detergent was carried out in the presence of the lipid bilayer membrane. For this purpose, a membrane from asolectin/*n*-decane was formed in unbuffered 0.1 M KCl (pH 6). After the membrane was in the optically black state, the outer membranes of the chloroplast envelope were added to a final concentration of 10 μg/ml while stirring. The lipid bilayer membrane did not show any pore activity under these conditions. Triton X-100 was then added (final concentration 0.05 mg/ml) to the same side where the outer membranes had been added. Fig.2 shows that about 3–5 min after addition of the detergent, the membrane current started to increase in a stepwise fashion. The increases in the current were fairly homogeneous as shown by the histogram (fig.3A). A few fluctuations of much smaller size were observed, but these presumably represent a substate of the pore. The single channel conductance of the pore incorporated into lipid bilayers by the above-described methods was considerably larger than that observed in the presence of mitochondrial porin. Fig.3B shows a histogram observed with mitochondrial porin from *Neurospora crassa* under identical conditions to those in [9]. Similar to the mitochondrial pore, a strong voltage dependence is observed with the chloroplast outer membrane pore, which may indicate a similar regulation of this pore as demonstrated for the mitochondrial pore [9,10].

The single channel conductance of the chloroplast pore was a linear function of KCl concentration in the aqueous phase. At 10^{-2} M KCl the single channel conductance was about 80 pS, at 0.1 M KCl in the aqueous phase 720 pS, and at 1 M KCl about 7 nS. This result indicates that the pore does not contain a specific binding site for ions [27]. If we assume that the pore is a hollow cylinder with an aqueous interior which has approximately the same specific conductance as the bulk aqueous phase then we can make a rough estimate of the channel radius r from the average single channel conductance $\bar{A}$ using the relation

$$\bar{A} = \sigma \pi r^2 / l \quad (2)$$

where l is the length of the pore. Assuming a pore length of 7.5 nm (corresponding to a similar thickness of the outer membrane of the chloroplast envelope [28]), the average pore diameter $d = 2r$ may be calculated as being 2.5 nm from the single channel conductance at 1 M KCl (σ = 112 mS/cm). This diameter agrees very well with that estimated above from the exclusion limit if we bear in mind that a number of critical assumptions are inherent in the estimations of both values. Again we want to stress here the point that the single channel conductance of the chloroplast pore is the largest measured thus far for any type of

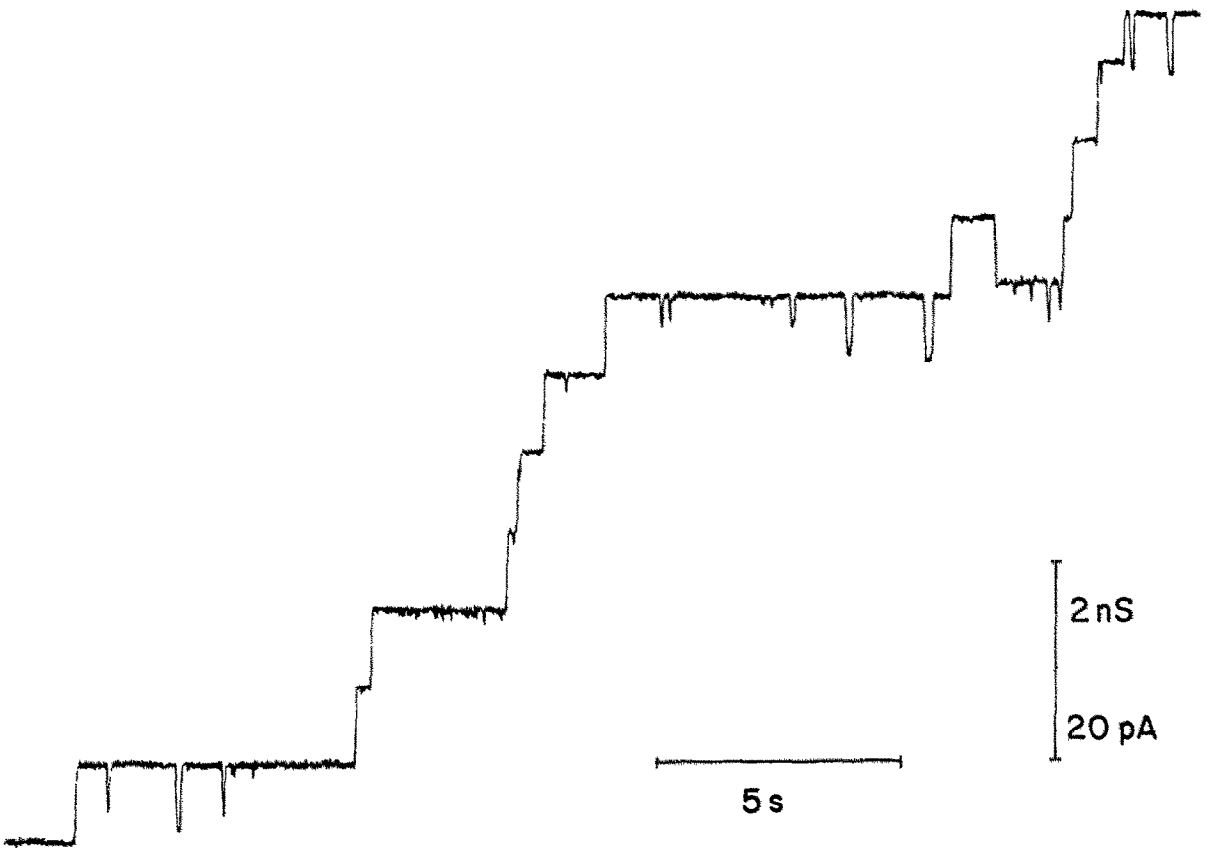

Fig.2. Current fluctuations of a membrane from asolectin/*n*-decane where first outer membrane of the chloroplast envelope in a concentration of 10 μg/ml and then 0.05 mg/ml Triton X-100 was added. The aqueous phase contained unbuffered 0.1 M KCl (pH 6), T = 25°C. The membrane potential was 10 mV.

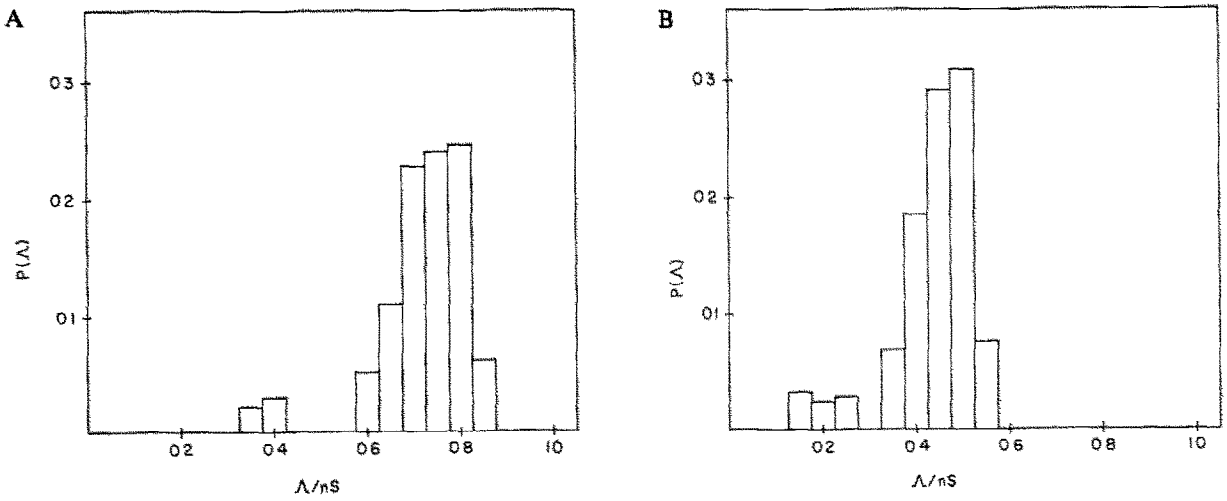

Fig.3. Histogram of current fluctuations observed with membranes from asolectin/*n*-decane in 0.1 M KCl, T = 25°C. The applied membrane potential was 10 mV. (A) Outer membranes of the chloroplast envelope were added in a concentration of 10 μg/ml followed by addition of 0.05 mg/ml Triton X-100. The average single channel conductance $\bar{\Lambda}$ = 0.72 nS was calculated from n = 171 current steps. (B) Mitochondrial porin from *N. crassa* was added in a concentration of 5 ng/ml as in [9]. The average single channel conductance $\bar{\Lambda}$ = 0.45 nS was calculated from 343 current steps.

porin pore. This means that the chloroplast pore has presumably the largest diameter of all known porin pores, a statement which can also be made from the large exclusion limit of this pore.

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