The flux of phosphorylated carbohydrates, the major export products of chloroplasts, is regulated at the level of the inner and presumably also at the level of the outer membrane. This is achieved through modulation of the outer membrane Oep21 channel currents and tuning of its ion selectivity. Refined analysis of the Oep21 channel properties by biochemical and electrophysiological methods revealed a channel formed by eight β-strands with a wider pore vestibule of $d_{\text{vest}} \sim 2.4 \text{ nm}$ at the intermembrane site and a narrower filter pore of $d_{\text{filter}} \sim 1 \text{ nm}$. The Oep21 pore contains two high affinity sites for ATP, one located at a relative transmembrane electrical distance $\delta = 0.56$ and the second close to the vestibule at the intermembrane site. The ATP-dependent current block and reduction in anion selectivity of the Oep21 channel is relieved by the competitive binding of phosphorylated metabolic intermediates like 3-phosphoglycerate and glycerinaldehyde 3-phosphate. Deletion of a C-terminal putative FX,K binding motif in Oep21 decreased the capability of the channel to tune its ion selectivity by about 50%, whereas current block remained unchanged.

Plastid organelles perform vital biosynthetic functions in every plant organ. They are surrounded by double membranes, the inner and the outer envelope, which delimit spatially and temporally the plastid compartment from the cytoplasm. Both envelope membranes are distinguishable by their structure, function, and biochemical properties but also cooperate, for example, in the synthesis of lipids or in protein translocation. Chloroplasts are the site of carbon dioxide reduction and its assimilation into carbohydrates, amino acids, fatty acids, and terpenoid compounds. The manifold biosynthetic functions of chloroplasts require the existence of different selective transport mechanisms across the envelope membranes to provide the cell with carbohydrates, organic nitrogen, and sulfur compounds (1, 2). On the other hand, chloroplasts take up inorganic cations ($K^+$, $Na^+$, $Mg^{2+}$, $Ca^{2+}$, $Fe^{2+}$, $Mn^{2+}$, and $Zn^{2+}$), anions ($NO_3^-$, $SO_4^{2-}$, $PO_4^{3-}$), and a variety of organic biosynthetic pathway intermediates, such as phosphoenolpyruvate, dicarboxylic acids, acetate, amino acids, and ATP, to fulfill their biosynthetic tasks.

Until recently, the outer envelope membrane was considered to be freely permeable for most small molecular weight solutes up to 10 kDa (1). Correspondingly, it was believed that the osmotic barrier against the cytosol is formed exclusively by the inner envelope membrane, containing specific carrier proteins, some of which have been identified on the functional and also on the molecular level (2). However, our recent reports reveal the presence of several specific solute pores in the outer envelope, indicating that the intermembrane space is not freely accessible to low molecular weight solutes (3–6).

Whereas the inner envelope carrier proteins (e.g. the triose phosphate-phosphate translocator, the dicarboxylic acid translocator, and the hexose phosphate carrier) show a distinct substrate selectivity and specificity, it remains elusive to what extent the transport through these outer membrane channels is regulated (7). The ancestral relation between plastids and Gram-negative bacteria suggests the presence of different solute channel proteins in the organellar outer membrane. In pea chloroplasts, four channel proteins have been identified and functionally characterized so far. Toc75 (translocase of the outer chloroplastic membrane of 75 kDa), probably consisting of 16 amphipathic transmembrane strands, forms the preprotein-conducting channel (8–10). Oep16 (outer envelope protein of 16 kDa) forms a four-helical (11, 12) cation-selective channel of about 1-nm width with a high specificity for amino acids and amines (4, 6). Oep16 allows the passage of molecules containing the amino acid backbone but excludes $C_4$ and $C_5$ sugars and other compounds, although the pore size of the channel would be large enough to allow the passage of these molecules. The current fluxes through the Oep16 channel were also regulated by the redox state of two spatial close cysteine residues. The third identified channel protein, Oep24, also constitutes a high conductance solute channel with a diameter of about 2.5 nm. The slightly cation-selective Oep24 channel allows the passage of triose phosphates, ATP, $P_i$, dicarboxylic acid, and positively or negatively charged amino acids (5) and has been shown to functionally replace the voltage-dependent anion channel in yeast mitochondria (14). Oep21 displays an anion-selective channel with asymmetric transport properties that are modulated by nucleotides and phosphorylated metabolic intermediates (3). Oep21 and Oep24 proteins reveal mainly β-sheet topology. In agreement with this, the voltage-dependent gating of both solute channels resembles closely the gating behavior observed for β-barrel membrane channels (15). In summary, evidence is accumulating that transport of different solute classes across the outer chloroplast membrane is facilitated through distinct pores in a selective, regulated fashion.

Here we describe the refined molecular characterization of the Oep21 channel and in particular the regulation of its transport properties by nucleotides and phosphorylated intermediates. Our results show that the currents through the Oep21 channel are regulated through tuning of its ion selectivity and by a modulated current block from the intermembrane space. The Oep21 channel has an asymmetric topology.
with a wider vestibule of about \( \sim 2.4 \text{ nm} \) diameter at the intermembrane space, whereas the restriction zone revealed at the lower limit a diameter of \( \sim 1.0 \text{ nm} \). The Oep21 contains two binding sites modulating the selectivity of the channel. One of the binding sites is responsible for the block of the channel currents.

**EXPERIMENTAL PROCEDURES**

### Materials

- CHAPS, Deoxy-Big CHAP, digitonin, and thermolysin were purchased from Calbiochem. Purified phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine) were from Avanti Polar Lipids, egg yolk phospholipids were from Sigma, and L-\( \alpha \)-phosphatidylcholine (egg) was from Larodan Fine Chemicals.

- Isolation of Organelles and Membrane Vesicles—Pea plants (Pisum sativum, var. Golf) were grown in a growth chamber and used for the isolation of intact, silica sol-purified chloroplasts as described before (16). Envelope membranes were separated and purified from hypertonically treated chloroplasts (17).

- Chloroplast Isolation and Proteolysis—Chloroplasts were isolated as described (18) and treated with either 0.5 \( \mu \text{g} \) of thermolysin/\( \mu \text{g} \) of chlorophyll for 5 min at 25 °C or with 8 \( \mu \text{g} \) of endopeptidase GluC (Roche Applied Science)/\( \mu \text{g} \) of chlorophyll for 30 min at 25 °C. The digestion was stopped by the addition of EDTA to a 10 \( \text{mM} \) final concentration or by the addition of a 2-fold excess of \( \alpha \)-macroglobulin, respectively. Subsequently, the chloroplasts were reisolated over a 40% Percoll cushion (330 mM sorbit, 50 mM Hepes/KOH, pH 7.6) and analyzed by SDS-PAGE and subsequent immunoblotting. For import reactions, psOep21 in pET21b (3) was translated in wheat germ lysate according to the protocol.

- Overexpression and Purification of the Recombinant Protein—cDNA encoding for Oep21 was cloned into the pET system (Novagen, Madison, WI), and proteins were expressed in Escherichia coli BL21 (Novagen). After lyses of E. coli by French press, proteins were recovered from insoluble inclusion bodies and purified in the absence of detergent as described before (3). The C-terminal deletion of pea Oep21 as well as the mutation of the FX4K-binding motif was performed by standard polymerase chain reaction and controlled by DNA sequencing. The final protein sequences are shown in Table 1.

### Table 1

| Protein | FX4K motif |
|---------|------------|
| psOep21C | \( ^{155} \text{QIREN}^\text{WTFNADYKGWRVNLRYDL} \) |
| psOep21D | \( ^{144} \text{QIREN}^\text{WTLNAMKGGKWKNLRLYDL} \) |
| psOep21E | \( ^{155} \text{QIREN}^\text{WTLNADYKGWRVNLRYDL} \) |
| psOep21F | \( ^{155} \text{QIREN}^\text{WTLHHTHHHH} \) |
| psOep21G | \( ^{155} \text{QIREN}^\text{WTFNATNLGKWKVRVL} \) |

#### Footnotes

3 The abbreviations used are: CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; OEV, outer envelope membrane; PGA, 3-phosphoglycerate; GAP, glycerinaldehyde 3-phosphate; MOPS, 3-(N-morpholino)propanesulfonic acid; WT, wild type.

4 0.5 microfarads/cm² and a resistance of >100 gigaohms. The noise was 3 pA (r.m.s.) at 5-kHz bandwidth. An osmotic gradient was used for vesicle fusion, whereby a channel-permeant solute is (in addition with the absolute necessity of the channel in the vesicle being in the open state (22)) a prerequisite for fusion of membrane vesicles with the bilayer.

Membrane potentials are in reference to the trans compartment. Recording and analysis of the data were performed as described (8). Voltage ramps were conducted with a rate of 10 mV/s. Ramps started from –50 to 0 mV and from 0 to +50 mV.
FIGURE 2. Rectifying current-voltage relationship of the Oep21 channel. Current-voltage relationship of the psOep21WT (A), psOep21WT-bc (B), atOep21-1 (D), psOEp21WT-OEV (E), and psOep21/CH9004 (G) deduced from the fully open channel state in symmetrical 1 M KCl, 10 mM Mops/Tris, pH 7. The inset shows the linear approximations of the current-voltage relationship in the ranges -100 mV < V < -75 mV, -25 mV < V < +25 mV, and +75 mV < V < +100 mV. Data points were averages of n ≥ 5 independent bilayers. In B and G, the current-voltage relationship of the most frequent subconductant state with the indicated conductance of 130 and 270 picosiemens, respectively are shown as well. C, the current-voltage relationship of the psOep21ATC under conditions described for A. In F and H, the current recording in the presence of an 8-fold transmembrane ion gradient (250/20 mM KCl) from a bilayer containing as single active psOep21WT-OEV (F) or psOep21ΔC channel (H) in response to a voltage (Vh) sweep (increment 5 mV/s) from −50 mV to +50 mV is presented.
Calculation of the pore size according to Ref. 23 was performed as described (22). The resistivity of the standard buffer solution ρ for a 1 M KCl, 10 mM Mops/Tris (pH 7) solution was determined by a standard conductometer (Knick, GmbH) to be 13.8 ohms cm. Taking the correction factor of 5 for the effective channel resistivity (24) into account, the effective value is ρ = 69 ohms cm.

**Data Analysis**—Changes in the reversal potential were fitted according to the following equation for a two-site binding model by Equation 1,

\[ \Delta V_{\text{rev}} = \frac{b_1 \cdot [\text{ATP}]}{k_1 + [\text{ATP}]} + \frac{b_2 \cdot [\text{ATP}]}{k_2 + [\text{ATP}]} \]  
**(Eq. 1)**

and fitted for the one-site binding model by Equation 2,

\[ \Delta V_{\text{rev}} = \frac{b_1 \cdot [\text{ATP}]}{k_1 + [\text{ATP}]} \]  
**(Eq. 2)**

where \( \Delta V_{\text{rev}} \) represents the change in the reversal potential, \( b_i \) is the maximal change in \( \Delta V_{\text{rev}} \) in the \( i \)th binding site, and \( k_i \) is the concentration where \( \Delta V_{\text{rev}} \) of the \( i \)th component is half-maximal. Confidence analysis was performed as described (25).

**RESULTS**

**Refined Evaluation of the Oep21 Membrane Topology**—To determine the topology of psOep21, we treated isolated chloroplasts with either thermolysin or endopeptidase GluC under conditions where the protease does not penetrate the outer envelope membrane (Fig. 1A, lanes 1–3). A few fragments of psOep21 were resistant to thermolysin as well as endopeptidase GluC, indicating the presence of membrane-protected regions (Fig. 1A, lanes 2 and 3). The treatment with thermolysin revealed a 14-kDa and a 10–12-kDa fragment, as previously described (Fig. 1A, lane 2 (3)). After digestion with GluC, we observed again two proteolytic fragments. Here, the fragments have a size of 18 and 14 kDa (Fig. 1A, lane 3). To determine the localization of the N terminus, we took advantage of the presence of a single methionine at the N terminus of psOep21. Thus, we imported in vitro translated [35S]methionine-labeled psOep21 into isolated chloroplasts (Fig. 1B, lane 1). The insertion of the protein in the outer envelope membrane was controlled by carbonate extraction at pH 11.5 (data not shown). Subsequently, chloroplasts were reisolated and treated with either thermolysin or endopeptidase GluC (Fig. 1B, lanes 5 and 6), and the appearance of proteolytic fragments was controlled by immunoblotting (data not shown). Neither one of the proteolytic products detected by immunoblotting was radioactively labeled (Fig. 1A, lanes 2 and 3, versus Fig. 1B, lanes 2 and 3). We used these data to model the topological structure of psOep21 (Fig. 1C). psOep21 was proposed to form a transmembrane β-barrel structure (3). The position of the transmembrane regions is based on the calculation of the exact β-sheet score as well as the alternating hydrophobicity of psOep21 (19). The structural model implies the presence of eight transmembrane β-sheets (Fig. 1C). According to the model, psOep21 provides two cleavage sites in transmembrane β-sheets for the endopeptidase GluC. Five cleavage sites for the endopeptidase GluC are present in loop regions exposed to the cytosolic side of the membrane, where one cleavage site seems to be buried in the structure as discussed below. Since the fragment of 18 kDa was not radioactively labeled, the C and N termini have to be exposed toward the cytosol (Fig. 1C), because penetration of the membrane by GluC under the conditions used is not observed (see also Ref. 26). We propose that GluC digested psOep21 at position 20, yielding an 18-kDa fragment and subsequently in loop VI, leading to a 14-kDa fragment (Fig. 1A, lane 3, and Fig. 1C). Therefore, loop IV seems to be shielded against both proteases, since no proteolytic product was observed, which could explain a cleavage event in this region (Fig. 1A). The cytosolic exposure of the N terminus is further supported by the appearance of a cleavage intermediate of 18 kDa by using thermolysin (Fig. 1A, lane 2) and by the production of exclusively nonradioactively labeled fragments by both proteases (Fig. 1B, lanes 4 and 5). Moreover, according to the model, we suggest that both thermolysin and GluC cleave psOep21 in loop VI, resulting in the 14-kDa fragment. A subsequent cleavage in loop II not exposing any GluC cleavage site (Fig. 1C) explains the occurrence of the second breakdown product of 10 kDa (Fig. 1A, lane 2). Again, as for the GluC cleavage, loop IV seems to be protected against processing.

**Conductance Properties of Oep21 from Different Preparations**—*Arabidopsis thaliana* contains two orthologs of psOep21, which are encoded for by the gene At1g20816 (atOep21-1) and At1g76405 (atOep21-2), respectively. Here, atOep21-1 contains the FXK motif, whereas in atOep21-2 the phenylalanine is exchanged for a leucine (see Table 1). Expression profiling data reveal that both orthologs are expressed in leaves (Fig. 1D). We therefore heterologously expressed atOep21-1 and compared key electrophysiological properties of the purified reconstituted protein with the previous identified protein from pea (psOep21WT) and two mutant forms (psOep21m and psOep21ΔC; Fig. 2 and Tables 1 and 2). In addition, the pea protein was biochemically purified from plastid membranes (psOep21WT-bp), or outer membrane vesicles were used directly (OVE; see Table 2).

One basic feature of channels is the rectifying current-voltage relationship, as shown for the recombinant psOep21WT, atOep21-1, psOep21WT-bp, psOep21WT-OEV, and psOep21ΔC (see Tables 1 and 2) (Fig. 2A, E–G). The degree of rectification was obtained from the linear tangent extrapolations of I/V relations at the high conductance site as compared with the low conductance site (e.g. Fig. 2A and B and Table 2). The rectification was observed at both lower and higher symmetrical KCl concentrations (250 mM and 1 M, Fig. 2) (25). The data in Fig. 2, B and G, were collected from bilayers where the cis compartment corresponded to the intermembrane space. This is the reason why rectification in these graphs is opposite to Fig. 2, A, D, and E, where the trans compartment corresponded to the intermembrane space. Fig. 2F shows the current-voltage relation of a single

| Protein                  | Δmain | Rec. ratio | Selectivity (Pf/Pt) |
|--------------------------|-------|------------|---------------------|
| psOep21WT-r              | 720   | 4.3        | 4:1                 |
| psOep21WT-bp             | 695   | 3.2        | 3:1                 |
| psOep21m                 | 660   | 1.5        | ND                  |
| atOep21-1                | 670   | 2.4        | 3:1                 |
| psOep21WT-OEV            | 630   | 3.7        | 3:1                 |
| psOep21ΔC               | 1000  | 2.0        | 4:1                 |

| Ion        | Pf/Pt-intermembrane space | Pf/Pt-cytosol |
|------------|---------------------------|---------------|
| K⁺ (mean conductance) | 0.33 ± 0.01 | 0.33 ± 0.01 |
| K⁺ (subconductance)  | 0.21 ± 0.01 | 0.21 ± 0.01 |
| NO₂⁻        | 1.3 ± 0.2 | 1.1 ± 0.2 |
| NO₃⁻        | 2.4 ± 0.4 | 1.9 ± 0.4 |
| SO₄²⁻       | 3.6 ± 1.5 | 0.4 ± 0.2 |
| H₂PO₄⁻      | 6 ± 1.7  | 0.8 ± 0.3 |
| Malat²⁺     | 2.8 ± 1  | 0.7 ± 0.3 |
FIGURE 3. Change of the psOep21 selectivity in response to metabolites added from the intermembrane space. A, current voltage relation of single active psOep21WT, in the presence of an 8-fold transmembrane ion gradient (control) and in the presence of 3 mM ATP added from the site corresponding to the intermembrane space (3). Values present means of n = 3 bilayers. Inset, current voltage relation of a single active psOep21WT, in the presence of an 8-fold transmembrane ion gradient (control) and in the presence of 3 mM ATP. B, the changes of the reversal potential ($V_{rev}$) of psOep21WT, in the presence of different ATP concentrations at the trans compartment are given. Measurements were performed in 2 M/250 mM NaCl (cis/trans) 10 mM Mops/Tris, pH 7, with the indicated ATP concentrations in the trans compartment. The line shows the best fit of the data using a two-side binding model with a confidence of 97% according to Equation 1 (see “Experimental Procedures”). Data points are averages from n = 8 independent bilayers. The inset shows a logarithmic plot of the data for analysis according to a modified GHK approach (23) (confidence of 83%). C, E, and F, changes of the reversal potential ($V_{rev}$) of psOep21WT, in the presence of different concentrations of ATP, ADP, and AMP (C), GAP and 3PGA (E), or Pi and glucose 6-phosphate (G6P) (F) at the trans compartment were determined as in B. The lines show the best fit of the data using a two-side binding model (Equation 1). Data points were averages from n = 5 independent bilayers. The parameters of the fit are summarized in Table 3. D, the same measurements as in B were performed using psOep21WT-OEV. Data points were averages from n = 5 independent bilayers. The parameters of the fit are summarized in Table 3.
psOep21\textsubscript{WT-OEV} channel, and Fig. 2H shows the one of psOep21\textsubscript{OMV} in the presence of a transmembrane ion gradient. The channel formed by the C-terminal deletion revealed the same selectivity as the full-length recombinant protein ($P_C/P_K = 3.1$, $n = 5$), whereas its conductance was significantly higher, and the rectification ratio was significantly lowered. For the psOep21\textsubscript{OMV}, the conductance of the open channel was close to the one observed for the recombinant protein, whereas its selectivity was slightly decreased (Table 2). Both psOep21\textsubscript{OMV} and psOep21\textsubscript{WT}, revealed a rectifying current-voltage relation (Fig. 2, A and B) as well. Although the conductance (Fig. 2, C and G) and selectivity (Fig. 2E, data not shown) of psOep21\textsubscript{OMV} were similar to that of atOep21-1 (Fig. 2D) (3) the rectification of these pore proteins was less pronounced than in the recombinant and the wild type channel of the pea. However, the reversal potential of the atOep21-1 channel was for ions with $z > -1$ dependent on the access side of the ions (Table 3).

### Refined Analysis of the Interaction between the Oep21 Channel with ATP

To further characterize the regulation of Oep21 by metabolites, a refined analysis of the interactions with nucleotides and phosphorylated metabolic intermediates was performed. In order to mimic physiological conditions, we analyzed the psOep21\textsubscript{WT-r} channel characteristics at zero electrical driving force ($V_h$ (holding potential) = 0 mV) but in the presence of a transmembrane ion gradient. We further investigated the effect of ATP on $V_{\text{rev}}$ and on the current block at $V_h = 0$ mV for psOep21\textsubscript{WT-bp}. Unfortunately, it turned out that psOep21\textsubscript{WT-bp} was randomly incorporated into the liposomes, and subsequently, only liposomes containing a single active channel could be used in these experiments. Fig. 3A (inset) shows the current voltage relation of a single active Oep21 channel in the presence of an 8-fold transmembrane ion gradient (control), whereas in Fig. 3A, averaged data from $n = 3$ bilayers are shown. When 3 mM ATP were added from the site corresponding to the intermembrane space (3), the reversal potential ($V_{\text{rev}}$) was drastically changed ($V_{\text{rev}} = +22$ mV to $V_{\text{rev}} = +8$ mV). At zero electrical driving force ($V_h = 0$ mV), the positive current, which is equivalent to an anion current from the cytosol into the intermembrane space (3), changes to a small negative current, which is equivalent to an cation current from the cytosol into the intermembrane space. In the following, we used this current block at $V_h = 0$ mV to analyze the effect of different charged metabolites on the regulation of the currents through the Oep21 channels. As a second parameter, we used the changes in the reversal potential, which were observable when nucleotides and other metabolites were added from the site corresponding to the intermembrane space (3).

Previously, we observed that the dependence of the reversal potential ($V_{\text{rev}}$) on different concentrations of nucleotides (ATP, ADP, AMP, GTP, and GDP (not shown)) at the intermembrane side could be described by a two-site equilibrium binding model (3). Here we extend this approach by analyzing the data by a modified constant field diffusion (the Goldman, Hodgkin, and Katz, or GHK) approach (23) and tested the validity of the parameters by a rigorous confidence analysis (for details, see “Experimental Procedures”) (25). Fig. 3B shows the fit of the data by the two-site binding model (confidence 97%), and the inset shows the fit of the data using the modified GHK approach (confidence 87%). In both approaches, binding of ATP is well described by the two-site binding model. The addition of ADP revealed a similar effect as ATP, whereas AMP did not significantly reduce the reversal potential (Fig. 3C). GTP and GDP also changed $V_{\text{rev}}$; however, the maximal change was about 70% of that obtained with ATP (data not shown). For the psOep21\textsubscript{OMV} channel, we already observed the reduction of $V_{\text{rev}}$ at lower ATP concentrations (Fig. 3D). The parameters of the fits in Fig. 3, B–D, are summarized in Table 4.

The phosphorylated intermediates GAP, 3PGA, glucose 6-phosphate, and PP (details not shown) also decreased the anion selectivity of the psOep21 channel when added from the intermembrane space (Fig. 3, E and F). Whereas the extent of $\Delta V_{\text{rev}}$ was similar to that of the nucleotides (Table 4), the binding affinities were different. Confidence analysis of the binding revealed that only PP binding occurred at two binding sites, whereas the binding of the other metabolites could be best described by a single class of binding sites with lower affinity (see Table 4).

**Metabolite-dependent Current Block at $V_h = 0$ mV**—The dependence of the current block in the psOep21 channel on different concentrations of ATP, ADP, and AMP when added to the intermembrane space side in the absence of an electrical driving force is shown in Fig. 4. The parameters for the fits of the experimental data (Fig. 4) are listed in Table 5.

The confidence analysis of the data revealed that one high affinity binding site in the Oep21 channel is responsible for the block of the currents at $V_h = 0$ mV. ATP with the highest affinity was about 6 times more effective than ADP, whereas AMP did not block the psOep21 channel. It is worth noting that GTP and GDP had a similar effect on the psOep21 channel currents as described above for ATP and ADP (data not shown). The phosphorylated metabolites also blocked the psOep21 channel currents at $V_h = 0$ mV (Fig. 4, B and C, and Table 5). The fit of the data and the confidence analysis showed only one class of binding sites with almost identical affinity (Table 5).

**Transmembrane Dielectric Distance of the Current-blocking Binding Sites in psOep21**—The relative dielectric distance of the binding sites responsible for the current block can be determined from the voltage dependence of the current block using the classical Woodhull approach (28). For this, the $K_p$ values have to be determined for the particular membrane voltage (28). The data can be analyzed according to Equation 3.

$$\ln(K_p V) = \ln(K_p V = 0 \text{ mV}) + \frac{z F \delta}{RT} . V$$

**Table 4**

| Metabolite      | Model | $K_p$ | $\Delta V_{\text{rev}}$ | Confidence |
|-----------------|-------|-------|------------------------|------------|
| ATP             | 2-site| 0.12 ± 0.02, 15.4 ± 3.37 | 20.8 ± 1.1, 22.5 ± 1.2 | 97         |
| ATP (OEV)       | 2-site| 0.1 ± 0.04, 4.57 ± 1.7 | 13.6 ± 0.6, 33.1 ± 7.6 | 99         |
| ADP             | 2-site| 0.4 ± 0.06, 4.9 ± 0.9 | 8.7 ± 3, 13.6 ± 4 | 95         |
| AMP             | 1-site| 0.2 ± 0.1 | 2.4 ± 0.6 | 91         |
| PP              | 2-site| 0.18 ± 0.1, 9.50 ± 6 | 10.3 ± 2.7, 22.7 ± 3 | 92         |
| P               | 1-site| 0.17 ± 0.5 | 26.3 ± 1.9 | 42         |
| GAP             | 1-site| 0.17 ± 0.5 | 32.5 ± 4 | 74         |
| 3PGA            | 1-site| 2.39 ± 0.6 | 16.7 ± 1.3 | 87         |
| Glucose 6-phosphate | 1-site | 3.5 ± 1.5 | 8.3 ± 1.8 | 62         |
The Chloroplast Channel Oep21

where the relative dielectric distance $\delta$ represents $0 < \delta < 1$, $z_i$ is the number of charges of the ion ($i$), $K_i$ is the voltage-dependent binding constant of the ion ($i$), and $R$, $T$, and $F$ are defined as usual.

A logarithmic plot of the data for ATP ($z_i = -4$) and 3PGA ($z_i = -3$), GAP ($z_i = -1.8$) is shown in Fig. 4C. The slope of the straight line revealed a value of $\delta = 0.53$ for ATP and values of $\delta = 0.33$ (3PGA), $\delta = 0.23$ (GAP). Therefore, the ATP binding site responsible for the channel block is located significantly deeper inside the psOep21 channel pore than the one of the metabolites.

**TABLE 5**

| Metabolite        | Model | $K_a$ (mM) | $\Delta f$ (pA) | Confidence |
|-------------------|-------|------------|-----------------|------------|
| ATP               | 1-site | 0.21 ± 0.01 | -20.8 ± 1.1 | 92 |
| ADP               | 1-site | 1.2 ± 0.1 | -8.7 ± 3 | 97 |
| AMP               | No block | | | |
| 3PGA              | 1-site | 0.5 ± 0.14 | -17.5 ± 0.8 | 74 |
| GAP               | 1-site | 0.9 ± 0.1 | -11.5 ± 0.4 | 92 |
| Glucose 6-phosphate | 1-site | 0.7 ± 0.15 | -7.8 ± 0.2 | 92 |

The analysis revealed that the effects of metabolites with one binding site in psOep21 counteracted the effect of ATP on the current block at $V_h = 0$ mV remained the same as described above for the full-length psOep21WT-r channel ($V_h = 0$ mV) by metabolites added from the intermembrane site.

**FIGURE 4.** The Chloroplast Channel Oep21

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**Competition between ATP, ADP, and Phosphorylated Metabolites in psOep21**—Next, we performed competition experiments where the changes of the psOep21 selectivity and the current block at $V_h = 0$ mV were measured in the presence of 2 mM ATP and varied concentrations of metabolites at the intermembrane space. At 2 mM ATP, only one of the ATP binding sites is occupied (see Table 4). Hence, we observed that the effects of ATP and ADP on psOep21 were additive (Fig. 5, first panel). However, the presence of GAP or 3PGA decreased the changes $\Delta V_{rev}$ with GAP being more effective than 3PGA (Fig. 5, second panel). Concomitantly with increasing concentrations of the metabolites, the ATP-induced current block at $V_h = 0$ mV is partially relieved and returns to the equilibrium values of the corresponding metabolites (Fig. 5, third panel). The obtained data (Fig. 5, first three panels) were fitted using the dose-response relation and subjected to confidence analysis (25).

\[
\Delta V_{rev} = A_1 + \frac{A_2 - A_1}{1 + 10^{\frac{x - x_{50}}{p}}} \quad \text{(Eq. 4)}
\]

\[
\Delta I(V_h = 0 \text{mV}) = A_1 + \frac{A_2 - A_1}{1 + 10^{\frac{x - x_{50}}{p}}} \quad \text{(Eq. 5)}
\]

where $A_1$ and $A_2$ represent the asymptotic values of $\Delta V_{rev}$ and $\Delta I(V_h = 0 \text{mV})$, respectively, and $p$ is the Hill coefficient.

Interestingly, for psOep21WT-r, a decrease of the reverse potential of $\Delta V_{rev} = 23 ± 2$ mV in the presence of 2 mM ATP was observed ($n = 3$; Fig. 5, first panel), whereas for psOep21ΔC, only values of $\Delta V_{rev} = 10 ± 2.1$ mV could be conducted ($n = 5$; details not shown). Notably, in both types of Oep21 channel proteins, the ATP-induced channel block at $V_h = 0$ mV remained the same as described above for the full-length using a two-side binding model. Data points are averages from $n \geq 5$ independent bilayers.
The Chloroplast Channel Oep21

The putative FX_K binding site in Oep21 is clearly related to the low affinity ATP binding site that is only involved in the tuning of the Oep21 channel selectivity.

To confirm our notion of a phosphor-metabolite interaction with Oep21, we investigated the effect of various concentrations of NADH as an example of noncharged physiological relevant metabolites on $\Delta V_{\text{rev}}$ and $\Delta V_{\text{restr}}$. The data for NADH are shown in Fig. 5, fourth panel. Obviously, NADH did not change the selectivity of the Oep21 channel; however, it blocked the channel current at $V_n = 0$ mV with $K_D = 1.0 \pm 0.3$ mM and $\Delta I_{\text{max}} = -15.2 \pm 2$ pA.

Estimation of the Oep21 Channel Pore Size—The topological properties of the Oep21 pore may be assessed from the conductance of the channel and its asymmetric current-voltage relation. At a first approach the model of Hille (23) can be used to determine the pore radius of a comparable channel pores has been shown to be 5-fold lower than in the corresponding bulk medium (24). In addition, the crystal structures revealed that the length of the restriction zones within these channels was typically 1 nm < $l_{\text{restr}}$ < 3 nm. The asymmetric current voltage relationship of the Oep21 channel indicates a ~4-fold lower resistance in the channel from the intermembrane space as compared with the cytosolic side (see Fig. 2, A–D). Therefore, we may calculate the lower limit for the diameter of the channel restriction zone ($d_{\text{restr}}$), corresponding to the low restriction side, and the widths of the vestibule, corresponding to the high conductance side, from Equation 6,

$$d_{\text{restr}} = \frac{\rho G}{\pi \left( \frac{\pi}{2} + \sqrt{\frac{\pi}{2} + \frac{4\pi I}{\rho G}} \right)}$$

where $G$ represents the conductance (in 1 M KCl symmetrical solution at $V_n = 0$ mV ($G_{\text{cyt}} = 340$ picosiemens, $G_{\text{max}} = 1.46$ nS)) $l_{\text{restr}} = l_{\text{vest}} = 2.5$ nm is the length of the constriction and vestibule zone, and $\rho = 67$ ohms cm is the effective resistivity of the solution (see “Experimental Procedures”). Following the model of (23), we obtain a value of $d_{\text{restr}} = 0.99$ nm and a value of $d_{\text{vest}} = 2.36$ nm.

**DISCUSSION**

**Topology of the Oep21 Channel**—The different prediction methods applied (see supplemental material) yielded two possible arrangements of eight transmembrane $\beta$-sheets. However, using the data from the proteolytic digests of Oep21 in the outer chloroplast membrane, one model could be ruled out. Subsequently, the model presented is favored (see Fig. 1C). According to this model, the FX_K binding motif is located between $\beta7$ and $\beta8$ and should be easily accessible from the intermembrane space. This proposal is in good agreement with our data and with the proteolytic digestion results.

Measurements at the trans compartment were obtained. Measurements were performed in 2 w/250 mM NaCl + 2 mM ATP (cis/trans) 10 mM Mops/Tris, pH 7, with the indicated nucleotide concentrations in the trans compartment. The line shows the best fit of the data using a two-side binding model with a confidence of 97% according to Equation 2 (see “Experimental Procedures”). Data points are averages from at least three independent bilayers. Second panel, changes of the reversal potential ($\Delta V_{\text{restr}}$) of psOep21WT-r in the presence of different ATP and ADP concentrations at the trans compartment were obtained. Measurements were performed in 2 w/250 mM NaCl + 2 mM ATP (cis/trans) 10 mM Mops/Tris, pH 7, with the indicated nucleotide concentrations in the trans compartment. The line shows the best fit of the data using a two-side binding model with a confidence of 97% according to Equation 2 (see “Experimental Procedures”). Data points are averages from at least three independent bilayers. Second panel, changes of the reversal potential ($\Delta V_{\text{restr}}$) of psOep21WT-r in the presence of different ATP and ADP concentrations at the trans compartment were obtained. Measurements were performed in 2 w/250 mM NaCl + 2 mM ATP (cis/trans) 10 mM Mops/Tris, pH 7, with the indicated nucleotide concentrations in the trans compartment. The line shows the best fit of the data using a two-side binding model with a confidence of 97% according to Equation 2 (see “Experimental Procedures”). Data points are averages from at least three independent bilayers.
with our electrophysiological results, which show that this binding site is located near to the membrane surface and easily accessible from only one side of the bulk medium. Using model one and the electrophysiological data, we have to conclude that β8 (which is not present in the psOep21ΔC) is not part of the Oep21 pore region. Moreover, since a pore with the estimated pore size of Oep21, \( d_{\text{pore}} > 0.96 \text{ nm} \), cannot be formed adequately by the remaining seven \( \beta \)-sheets, we have to postulate that the functional unit of the Oep21 pore is composed of at least two monomeric units.

**Conductance Properties of the Oep21 Channel**—Our comparative studies on Oep21 with the psOep21 either recombinant expressed, biochemically purified, or present in outer envelope vesicles, the recombinant atOep21-1 and two mutants (psOep21\(_m\) and psOep21ΔC) show that Oep21 from the different preparations revealed rectifying current-voltage relationships at symmetrical ionic conditions. As previously shown (3), the experimentally observed higher conductance at one site of the channel corresponds to a rectification of the currents from the intermembrane space into the cytosol. Rectifying current-voltage relation at symmetric ionic concentrations on both sides of the channel is an intrinsic property of channels with a net dipole moment along the channel axis (29). Asymmetric \( I/V \) relations have been also observed for porins (13, 30), but only at low ionic strength (<30 mM). In contrast, Oep21 displayed an asymmetric current-voltage relationship even in symmetrical 1 M KCl solutions. This shows that the channel contained a strong permanent dipole moment along the channel axis, which cannot be “saturated” even at 1 M ionic strength. It is remarkable that psOep21ΔC, psOep21\(_m\), and atOep21-1 (lacking the putative FX4K ATP binding motif) reveal a significantly reduced degree of rectification, which shows that the putative FX4K ATP binding motif contributes significantly to the generation of the permanent dipole moment in the channel.

**Properties of the Effector Binding Sites in the Oep21 Channel Pore**—As outlined above, the Oep21 channel carries an intrinsic permanent dipole moment along the channel axis, which is formed by anisotro-
pic distribution of charges along the channel axis. In particular, the Oep21 channel contains two classes of positively charged binding sites that strongly interact with negatively charged metabolic intermediates of the charge $z \approx -3$. The requirement of multiple negative charges and, in particular, the high affinity of the channel binding sites for di- and triphosphates shows that the charged “filter” binding sites in Oep21 are spatially in a close and defined geometrical arrangement.

Refined analysis of the dependence of the Oep21 channel currents and its site-dependent selectivity on the concentrations of phosphorylated intermediates, at zero electrical driving force, showed that the ATP binding site with the highest affinity (see Tables 3 and 4) is located at a relative electrical distance of $\delta = 0.5$ along the channel axis. This site is responsible for the current block and in part for the modulation of the channel selectivity. The second binding site revealed a $\sim 100$-fold lower affinity for ATP and was only responsible for the modulation of the channel selectivity. Binding of monophosphates to the Oep21 channel could be accounted for by a single class of binding sites with intermediate affinity (see Tables 4 and 5). Binding of monophosphates to these sites also partially blocked the Oep21 channel currents at a relative dielectric distance of $\delta$ and decreased the anion channel selectivity. The second low affinity binding site for ATP is related to the $c$-terminally located putative $F_{\chi}K$ binding site, since after deletion of this binding site in the Oep21$\Delta C$ mutant, the absolute extent of the decrease in the reversal potential was halved, and the binding isotherm revealed only a single remaining ATP binding site where ATP binding still blocked the channel currents. In summary, di- and triphosphates ($z \leq -3$) can discriminate between two binding sites in the Oep21 channel, whereas monophosphates and other anions with $z \geq -3$ can only recognize a single class of binding site in the Oep21 channel (see Fig. 6).

Although binding of phosphorylated metabolites with $z < -3$ can be described by a single class of binding sites, it is likely that these compounds bind to the same sites as ATP, but their affinity for both sites is indistinguishable. This conclusion is supported by the observation that the asymptotic values of $\Delta V_{\text{rev}}$ for ATP and the phosphorylated metabolites are almost identical (i.e. both binding sites were saturated to yield a unique value for $\Delta V_{\text{rev}}$ independent from the ligand) (see Fig. 6). Finally, it should be pointed out that the Oep21 channel asymmetry was observed even at a very high ionic strength about 3–4 times above the estimated pore size of the Oep21 channel, it is likely that ATP can permeate the channel with a tight fit. NADH will be excluded through its size and block the channel when forced to enter from the intermembrane vestibule, whereas triose phosphates are easily permeable. The permeation properties of the Oep21 channel will thus be regulated by the ratio of the concentrations of anions with charges of $z > -3$ and $z < -3$.

Beside this, the Oep21 channel may also be regulated through its voltage-dependent open probability, which is maximal at a membrane potential of $V_{m} = 0\, \text{mV}$ and decreases steeply with increasing potentials (3).

In summary, our results provide a topology model of the Oep21 channel in the open conformation, which, in combination with the detailed electrophysiological characterization of the Oep21 channel, will be useful to design new experiments in order to emphasize the supposed roles of the metabolite channels in the chloroplast outer membrane.

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