Thylakoid membranes contain a non-selective channel permeable to small organic molecules

The thylakoid lumen is a membrane-enclosed aqueous compartment. Growing evidence indicates that the thylakoid lumen is not only a sink for protons and inorganic ions translocated during photosynthetic reactions but also a place for metabolic activities, e.g. proteolysis of photodamaged proteins, to sustain efficient photosynthesis. However, the mechanism whereby organic molecules move across the thylakoid membranes to sustain these luminal activities is not well understood. In a recent study of *Cyanophora paradoxa* chloroplasts (muroplasts), we fortuitously detected a conspicuous diffusion channel activity in the thylakoid membranes. Here, using proteoliposomes reconstituted with the thylakoid membranes from muroplasts and from two other phylogenetically distinct organisms, cyanobacterium *Synechocystis* sp. PCC 6803 and spinach, we demonstrated the existence of nonselective channels large enough for enabling permeation of small organic compounds (e.g. carbohydrates and amino acids with $M_r < 1500$) in the thylakoid membranes. Moreover, we purified, identified, and characterized a muroplast channel named here CpTPOR. Osmotic swelling branes. Moreover, we purified, identified, and characterized a muroplast channel named here CpTPOR. Osmotic swelling branes. Moreover, we purified, identified, and characterized a muroplast channel named here CpTPOR. Osmotic swelling branes. Moreover, we purified, identified, and characterized a muroplast channel named here CpTPOR. Osmotic swelling branes. Moreover, we purified, identified, and characterized a muroplast channel named here CpTPOR. Osmotic swelling branes. Moreover, we purified, identified, and characterized a muroplast channel named here CpTPOR. Osmotic swelling branes. 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dye Lucifer yellow (M_r of 444.24), supporting the possible existence of pore-like channel(s). However, the molecular identity of the channel remained unknown, and the permeability of organic molecules across the thylakoid membranes has not been explored further.

In this study, using proteoliposomes reconstituted with the thylakoid membrane proteins of muroplasts, cyanobacterium Synechocystis sp. PCC 6803 (referred to as PCC 6803) and spinach (Spinacia oleracea) chloroplasts, we demonstrated the presence of nonselective channels with a pore size large enough to allow permeation of organic molecules. Further, we purified, identified, and characterized the muroplast channel by employing biochemical and electrophysiological approaches.

Results

The existence of non-selective channels in the thylakoid membranes of muroplasts, PCC 6803, and S. oleracea chloroplasts

To clarify whether the presence of organic molecule-permeable channels is a common characteristic of the thylakoid membrane, we separated the thylakoid membranes from the muroplasts, cyanobacterium Synechocystis sp. PCC 6803 (referred to as PCC 6803) and spinach (Spinacia oleracea) chloroplasts, we demonstrated the presence of nonselective channels with a pore size large enough to allow permeation of organic molecules. Further, we purified, identified, and characterized the muroplast channel by employing biochemical and electrophysiological approaches.
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respective. Because the charges of the substrates frequently affect the influx rates through pore-forming diffusion channels, e.g. porin channels (23-25), we examined the effect of charge using glucose and its anionic and di-anionic derivatives glucosinate (M, = 196) and glucarate (saccharate, M, = 210) (Fig. 2D).

No facilitation or retardation of the influx rates of these derivatives was observed. This suggested that TPORs, at least in principle, functioned as diffusion pores for substrates irrespective of the charges of these substrates.

Purification and identification of CpTPOR

Because the muroplast TPOR (CpTPOR) showed the highest activity per unit amount of thylakoid membrane proteins among the three species tested, we chose CpTPOR as a target for purification and identification. The muroplast thylakoid membranes were first solubilized in the presence of 1% dodecyl maltoside (DDM) and resolved by gel filtration chromatography (Fig. 3A). The channel activity was examined in a liposome-swalling assay using proteoliposomes reconstituted with each fraction and arabinose as a substrate. The channel activity was solely observed with the 23- to 25-min fractions, which contained proteins with molecular masses of approximately 40 – 50 kDa. The proteins in the 23- to 25-min elution fraction were further separated by anion exchange chromatography (Fig. 3B). When the channel activity was examined as above, a single peak at the very beginning of the LiCl concentration gradient (approximately 7-min elution time) was observed. SDS-PAGE analysis of this fraction revealed a single band of approximately 30 kDa (Fig. 3C). Because solubilized membrane proteins bind a substantial amount of detergent molecules and are eluted faster than their predicted molecular mass in gel filtration LC experiments (26, 27), we assumed that the channel activity peak observed at 40 – 50 kDa during gel filtration chromatography corresponded to the CpTPOR monomer. The channel property of the purified CpTPOR was almost identical to that of the muroplast thylakoid membranes. The estimated pore radius size was 1.3 nm (Fig. 3D). The relative channel activity was about 8.2-fold higher than that of the thylakoid membrane preparation (data not shown).

The CpTPOR protein was next identified by MALDI-TOF MS (Fig. S1), and a full-length cDNA sequence encoding CpTPOR was determined by the rapid amplification of cDNA ends method. The CpTPOR gene is nucleus-coded and possesses six introns. The protein consists of 275 amino acid resid-
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The activity of a recombinant CpTPOR protein

The purified CpTPOR preparation migrated as a single band on the SDS-PAGE gel; nevertheless, we went on to confirm whether this 30-kDa protein was indeed CpTPOR because SDS-PAGE analysis itself cannot rule out the possibility of contamination with other minor protein(s), which might have been responsible for the channel activity. Consequently, we constructed an N-terminally His-tagged CpTPOR protein and expressed it in E. coli. The produced recombinant CpTPOR (rCpTPOR) formed inclusion bodies. The inclusion bodies were solubilized using 8 M urea, and rCpTPOR was purified (Fig. S2A) and then refolded in the presence of 0.1% octylglucoside and 10% glycerol. The channel property of the refolded rCpTPOR was next examined by liposome-swelling assay. The channel property of rCpTPOR was almost identical to that observed with the muroplast thylakoid membrane preparation and purified CpTPOR (Fig. S2B). Accordingly, we concluded that the 30-kDa protein was the CpTPOR protein.

CpTPOR sequence characteristics

CpTPOR homologs were identified by BLAST via the UniProt website. The top-ten matching hits are listed in Table S1. Proteins that shared a moderate (25–35%) identity with CpTPOR are widespread among eukaryotes, but no obvious homologs were found within prokaryotes. This suggested that TPOR of PCC 6803 was not evolutionarily related to CpTPOR or S. oleracea chloroplasts. The finding that the CpTPOR sequence appeared to be related to a voltage-dependent anion-selective channel (VDAC) of the mitochondrial outer membrane (31, 32) was unexpected, although the degree of similarity was not particularly high, and CpTPOR did not facilitate the influx of an anionic substrate (Fig. 2D). Putative VDAC motifs (Table S2) described by Young et al. (33), Homblé et al. (32), and Imai et al. (34) were indeed identified in CpTPOR (Fig. 5). In addition, the predicted secondary structure of CpTPOR contained a single N-terminal α-helix and following β-strand-rich region, similarly to VDACs (31).

Figure 5. The evaluation of mitochondrial contamination in the muroplast preparation. A, muroplasts were isolated from C. paradoxa cells and purified by Percoll gradient centrifugation (i), and the cytochrome c oxidase activity in each fraction was determined (ii). The data are presented as the means ± S.D. from three independent experiments (n = 9). ND, not detected. B, diffusion channel activities of the thylakoid membranes of muroplasts collected from fractions B and C after Percoll gradient centrifugation. The proteins of each thylakoid membrane (5 μg) were reconstituted into liposomes, and the diffusion channel activity was determined by liposome-swelling assay with arabinose as a substrate. The data are presented as the means ± S.D. from triplicate determinations (n = 3). C, detection of CpTPOR by immunoblotting. Fifteen micrograms of proteins of the total membranes in fraction A (lane I) and the thylakoid membranes isolated from the muroplasts in fractions B and C (lanes II and III, respectively) were separated by SDS-PAGE (i). Proteins were then blotted onto a polyvinylidene fluoride membrane, and CpTPOR was detected using anti-CpTPOR antibody and alkaline phosphatase-conjugated anti-rabbit IgG antibody (ii). M, molecular mass standards; CBB, Coomassie Brilliant Blue.

Figure 4. The CpTPOR sequence characteristics. The predicted secondary structure is shown underneath the amino acid sequence. The putative VDAC motifs, listed in Table S2, are boxed.
CpTPOR is not a mitochondrial protein contaminant

Because the CpTPOR sequence was related to VDAC, it was essential to evaluate the possibility of mitochondrial contamination of the muroplast preparation. The cytochrome c oxidase activity was used as an indication of the presence of mitochondria. In all experiments, the muroplasts were purified by a discontinuous Percoll gradient centrifugation and were collected from the interface between 40% and 80% Percoll (Fig. 5). The cytochrome c oxidase activity was examined in fraction A, the interface between 0% and 40% Percoll, which was expected to contain other cellular organelles and cell debris; fraction B, the interface between 40% and 80% Percoll; and fraction C, the fraction from the bottom of the tube, which contained aggregated muroplasts generated during the preparation. The cytochrome c oxidase activity was predominantly detected in fraction A (Fig. 5). Very low activity was also detected in fraction B, but it was less than 3% of the activity in fraction A. Fraction C contained no detectable activity. Further, when the thylakoid membranes were isolated from the muroplasts in fraction C and analyzed, the CpTPOR activity therein was similar to that of the muroplasts in fraction B (Fig. 5B). Finally, we prepared the anti-CpTPOR antibody and confirmed the existence of CpTPOR protein by immunoblotting (Fig. 5C). An intense band of CpTPOR was detected in the thylakoid membranes isolated from the muroplasts in fractions B and C, indicating that CpTPOR is indeed localized to the thylakoid membrane. An about 4-fold less intense band was also detected in the total membrane proteins in fraction A, but this was not unexpected because fraction A contained mitochondria, and the anti-CpTPOR antibody might cross-react to the VDAC protein therein. Based on these observations, we concluded that the CpTPOR protein was not a mitochondrial protein contaminant. Other lines of evidence supporting this conclusion are found in the literature. Facchinelli et al. (36) detected CpTPOR in the thylakoid membranes of muroplasts using proteomics (see Table S2 in Ref. 36). The mitochondrial envelopes, if any, are expected to be localized to the 15–35% sucrose fraction after isolation by sucrose density gradient centrifugation (37), whereas CpTPOR in our study was isolated from the 45–55% sucrose fraction, which predominantly contained the thylakoid membranes.

Electrophysiological characterization of CpTPOR

To characterize the channel activity of CpTPOR, single-channel current recordings were made using the contact bubble bilayer method (38, 39). Reconstituted channels in the liposomes were included in one of the bubbles, and the channels were spontaneously incorporated into the bilayer. The channel current was first measured in 1 M KCl. Channels with several varying conductance levels were observed. Typical current traces at various membrane voltages and the current–voltage curve are shown in Fig. 6, A and B. The single-channel conductance calculated based on these data was 1.8 nS. Multiple subconductance levels were occasionally detected (Fig. 6C). The channel stayed open most of the time, but infrequent closings to the zero current level were observed (e.g., the current traces at +50 mV or +100 mV in Fig. 6A). The channel gating appeared to be voltage-independent. Finally, the channel current was examined at low KCl concentration (50 mM) to clarify the sensitivity of the channel activity to changes in ionic strength, as reported earlier for a plant VDAC channel (40). No particular change in channel activity was observed, and, as shown by the representative current trace (Fig. 6D), the single-channel current amplitude was 4 pA at +50 mV, which was within the range anticipated from the current levels in 1 M KCl.

Discussion

CpTPOR is the first example of an identified and characterized organic molecule–permeable diffusion channel in the thylakoid membrane. We demonstrated that CpTPOR functions as a nonselective diffusion pore with an estimated pore radius of approximately 1.3 nm. Considering its amino acid sequence, CpTPOR appears to be related to VDAC (Fig. 4). However, biochemical and electrophysiological characterization revealed major differences with VDAC, as follows. The negative charge of the substrate did not appreciably affect its CpTPOR influx rate (Fig. 2), in contrast with the commonly observed selectivity of VDAC for anionic substrates (31). The single-channel conductance of CpTPOR (1.8 nS in 1 M KCl) was almost half that of the generally observed conductance of VDAC (~4 nS in 1 M KCl) (Fig. 6). No voltage-dependent gating was observed for CpTPOR (Fig. 6), in contrast with the well-known bell-shaped voltage dependence of VDAC that generally exhibits a decreasing conductance when the membrane potential is V ≥ 30 mV (31, 32). These observations suggest that the function of CpTPOR is noticeably diverged from that of VDAC, even though their amino acid sequences suggest a close evolutionary relationship. On the other hand, CpTPOR exhibited characteristics of the high-conductance channel described by Hinnah and Wagner (20) in the thylakoid membranes of pea chloro-

Figure 6. The electrophysiological characterization of CpTPOR. A, typical current traces of CpTPOR. The single-channel conductance of purified CpTPOR was determined in 1 M KCl at various membrane voltages using the contact bubble bilayer method. B, the current–voltage relationship of CpTPOR determined in 1 M KCl. The data are presented as the current amplitude of the predominant conductance level for each membrane potential. C, representative subconductance current traces of CpTPOR, observed in 1 M KCl with +100 mV membrane potential. D, typical current trace of CpTPOR observed in 50 mM KCl with +50 mV membrane potential.
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Plants; the estimated pore radius of 1.3 nm for that protein was the same as that of CpTPOR, and the channel showed no apparent selectivity for anions and no obvious voltage dependence.

Since the report by Hinnah and Wagner (20), no significant progress has been made regarding the permeability of organic molecules across the thylakoid membranes. This is in clear contrast to the rapid progress made in the last decade in the understanding of the molecular basis of ion flux across the thylakoid membranes (41, 42). This seems to be ascribed to the fact that the permeability of thylakoid membranes has been mainly explored by electrophysiological measurements; consequently, the permeability of organic molecules was not well discussed. Nonetheless, the requirement for membrane transport of organic molecules across the thylakoid membrane is obvious, as growing evidence points to several metabolic activities in the thylakoid lumen, e.g. the proteolysis of photodamaged proteins. The existence of TPORs indicates that various organic molecules are potentially able to move across the thylakoid membrane via simple diffusion. We propose that the thylakoid lumen is more readily connected to the cyanobacterial cytosol or the chloroplast stroma than commonly appreciated, presumably to enable rapid exchange of various metabolites (e.g. degradation products of photodamaged proteins) between the lumen and the stroma to sustain luminal activities.

It is obvious that TPOR activity should be regulated to not disturb photosynthetic light reactions. This could be achieved by either of the following: direct control of the channel opening and closing or spatial regulation to separate the site of photosynthetic electron transfer from the site of TPOR function. Lipid bilayer experiments with purified CpTPOR revealed multiple subconductance levels and infrequent channel closing (Fig. 6), suggesting that the channel permeability may vary in vivo. As represented by the stromal and granum thylakoid, the membranes spatially separate thylakoid functions (43). In addition, recent studies revealed a functional and compositional heterogeneity within the cyanobacterial thylakoid membranes even though they do not differentiate into grana (44, 45). Clarifying the precise location of TPORs in the thylakoid membrane is essential for understanding its physiological role.

Although TPOR activity is conserved in the cyanobacterium (PCC 6803), the primitive chloroplast (muroplast), and the chloroplast of higher plants (spinach), the evolutionary relationship of TPORs is not straightforward. CptTPOR homologs are absent from cyanobacteria; in accordance, the estimated pore radius of PCC 6803 TPOR (3.0 nm) was roughly 2-fold larger than that of CpTPOR (1.3 nm) and S. oleracea TPOR (1.7 nm) (Fig. 2), suggesting a fundamental difference in their channel structures. Furthermore, it remains unclear whether CptTPOR-like proteins are widespread in the thylakoid membranes of higher plants because no CptTPOR homolog was detected in the currently available proteomics dataset for the thylakoid membranes of Arabidopsis thaliana (46). The immunoblot analysis of the thylakoid membrane proteins of spinach chloroplasts using anti-CpTPOR antibody detected multiple bands, but further experiments are obviously required to eliminate the possibility of a nonspecific reaction (data not shown). Identification of TPORs in other species constitutes one of the essential research topics for further study.

**Experimental procedures**

**Strains and culture conditions**

*C. paradoxa* NIES-547 was grown at 24 °C under 14 h/10 h light/dark conditions, as described elsewhere (47). PCC 6803 was cultured in BG11 medium with continuous light at 30 °C.

**Isolation of the muroplasts and S. oleracea chloroplasts**

The muroplasts were isolated from *C. paradoxa* cells as described by Koike et al. (48) with the following modification. The muroplasts were purified by centrifugation (10,000 × g for 10 min) in a step gradient of 0%, 40%, and 80% Percoll and collected from the interface between 40% and 80% Percoll. The *S. oleracea* chloroplasts were isolated from the leaves of market spinach as described by Nakatani and Barber (49).

**Isolation of the thylakoid membranes**

The muroplast membranes were isolated and separated as described by Yusa et al. (50) and Koike et al. (51) with minor modifications as follows. The step gradient of sucrose concentrations was prepared as follows, from the bottom of the tube: 2 ml of 60% (w/v) sucrose, 3 ml of the membrane suspension in 55% sucrose, 2 ml of 45% sucrose, 2 ml of 35% sucrose, 1 ml of 15% sucrose, and 1 ml of HEM buffer (50 mM HEPES (pH 7.5), 2 mM EGTA, and 2 mM MgCl2). The centrifugation was performed at 100,000 × g for 18 h at 4 °C. The thylakoid membranes were found mainly in the 45–55% sucrose fractions. The absorbance spectrum of the thylakoid membrane suspension showed typical peaks of chlorophyll (approximately 440 nm and approximately 670 nm) and phycobilisomes (approximately 630 nm), whereas the major peaks of the carotenoids (440 – 480 nm) were absent (51) (Fig. S5A). The respective fractions were collected, diluted 10-fold in the HEM buffer, and then centrifuged at 100,000 × g for 30 min to pellet the membranes. The isolated membranes were washed once and suspended in HEM buffer.

The PCC 6803 membranes were isolated and separated as described by Omata and Murata (52) with the same modifications as above. The thylakoid membranes were found mainly in the 45–55% sucrose fractions. The absorbance spectrum of the thylakoid membranes was similarly confirmed (Fig. S5B), and the membranes from the respective fractions were collected as above. The *S. oleracea* chloroplast membranes were isolated and separated following the same protocol as for the muroplast membranes, except that the lysozyme treatment was omitted. The thylakoid membranes were mainly found in the 35–55% sucrose fractions, and the inner and outer membranes (the envelopes) were found in the 15% sucrose fraction. The identities of the membranes were confirmed by immunoblotting using anti-Lhcb2 (a thylakoid marker), anti-Tic40 (an inner membrane marker), and anti-Toc75 (an outer membrane marker) antibodies purchased from Agrisera (Vännäs, Sweden) (Fig. S5C). The membranes were collected from each fraction as above.

**The liposome-swelling assay**

To determine the permeability of the nonelectrolytes, proteoliposomes were prepared using 2.4 μmol of phosphatidyl-
choline, 0.15 μmol of dicetylphosphate, and the thylakoid membranes or purified protein samples, according to the protocol described by Nikaido et al. (53). The proteoliposomes were mixed with the substrates to be tested under isotonic conditions, and the swelling of the proteoliposomes caused by the influx of substrates was monitored by measuring the reduction of $A_{560}$.

To determine the permeability of negatively charged substrates, the proteoliposomes were prepared using 6.2 μmol of phosphatidylycholine and 0.2 μmol of dicetylphosphate, and suspended in 1 mM NAD-imidazole buffer (pH 6.1) containing 4 mM sodium NAD and 12 mM stachyose, as described by Nikaido et al. (24). The influx rates were determined as described above under isotonic conditions (1 mM sodium NAD and 1 mM NAD-imidazole buffer (pH 6.1)) and in the presence of the substrates to be tested.

Estimation of the pore radius size

The pore size was estimated based on the relative influx rates of the substrates determined by the liposome-swelling assay, as described by Nikaido and Rosenberg (22). Briefly, the theoretical relative influx rate was first calculated based on the Renkin equation, which describes the effect of substrate size on the influx rate through a pore (54). The expected influx rate, $J$, is calculated as follows: $J = D \times \frac{[1 - (r/R)^2]}{[1 - 2.104 \times (r/R) + 2.09 \times (r/R)^3 - 0.95 \times (r/R)^4]}$, where $D$, $r$, and $R$ are the diffusion coefficient of a substrate in water, radius of the substrate, and radius of the pore, respectively. The values of substrate radii determined by Schulz and Solomon (21) were used. The molecular radius of dextran was roughly estimated based on its molecular weight, as described by Venturoli and Rippe (55). The relative diffusion coefficient was calculated using the Stokes–Einstein equation, $D = kT/6πηr$, where $k$, $T$, and $η$ are the Boltzmann constant, the absolute temperature, and the coefficient of viscosity, respectively. Finally, the pore radius size was estimated by fitting the theoretical influx rates to the experimentally observed influx rates.

Purification of CTPOR

The muroplast thylakoid membrane preparation was solubilized by incubation in 1% DDM in 20 mM Tris-HCl (pH 7.5) for 15 min at 37 °C. The supernatant was collected by centrifugation at 20,000 × g for 20 min at room temperature. The supernatant was then resolved by gel filtration HPLC using a Superdex 200 Increase 10/300 GL column (GE Healthcare, Chicago, IL). The HPLC parameters were as follows: eluent, 0.1% DDM in 20 mM Tris-HCl (pH 7.5); flow rate, 0.5 ml/min. The 23- to 25-min elution fractions were collected and concentrated 10-fold using centrifugal filters (Amicon Ultra; Merck, Darmstadt, Germany). They were then subjected to anion exchange HPLC using a TSKgel DEAE-5PW column (Tosoh, Tokyo, Japan). The HPLC parameters were as follows: eluent A, 0.1% DDM in 20 mM Tris-HCl (pH 7.5); eluent B, 0.1% DDM and 0.5 M LiCl in 20 mM Tris-HCl (pH 7.5); flow rate, 0.5 ml/min. The timetable of the eluent A/B gradient is shown in Fig. 3B. The purified CTPOR was collected from the 6- to 7-min elution fractions and concentrated 10-fold as above.

Identification of CTPOR

A single 30-kDa band appeared after SDS-PAGE of the purified CTPOR preparation and was subjected to peptide mass fingerprinting as described by Kojima et al. (19). The CTPOR protein was identified after searching a database of predicted protein sequences of C. paradoxa (30) using the MS-Fit program. The detected peptide fragments covered 42.9% of the CTPOR sequence. The full-length CTPOR-coding cDNA sequence was determined by rapid amplification of cDNA ends method as described by Kojima et al. (19).

Expression and purification of rCTPOR

The CTPOR-coding DNA fragment was amplified by PCR from cDNAs of a 10-day culture of C. paradoxa cells and inserted into the pET-15b vector. The resulting plasmid, pCTPOR, was used to transform E. coli BL21(DE3). For expression of the rCTPOR gene and protein production, BL21(DE3) cells harboring pCTPOR were cultured in LB (1% Tryptone, 0.5% yeast extract, 0.5% NaCl) medium containing 100 μg/ml ampicillin at 37 °C with shaking. When $A_{600}$ reached approximately 0.5, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. The cells were further cultured for 2 h, and harvested by centrifugation at 3000 × g for 10 min at 4 °C. The cells were washed once with 50 mM potassium phosphate buffer (pH 7.5) containing 5 mM MgCl$_2$ (K-P buffer), suspended in the same buffer, and disrupted by sonication (eight cycles of 30-s pulses at 30-s intervals) in an ice water bath. The disrupted cells were removed by centrifugation at 3000 × g for 5 min at 4 °C, and the supernatant was centrifuged at 20,000 × g for 20 min at 4 °C to pellet the inclusion bodies. Next, the inclusion bodies were solubilized by incubation for 15 min at 37 °C in a denaturing buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 8 M urea, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride). The rCTPOR protein was purified on a His SpinTrap column (GE Healthcare) according to the manufacturer’s instructions, with the denaturing buffer supplemented with 500 mM imidazole as an elution buffer. The purified rCTPOR protein was refolded by dialyzing at 4 °C against a refolding buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.1% w-octyl-β-D-glucoside) containing the following successively decreasing concentrations of urea: 4, 2, 1, 0.5, and 0 M. The dialysis was conducted for 3 h at each urea concentration.

Antibody production

A rabbit polyclonal antiserum was raised against 2.5 mg of the purified rCTPOR protein, and the antibody was purified by using a protein A column (Cosmo Bio, Tokyo, Japan).

Immunoblotting

Protein samples were separated by SDS-PAGE and then electroblotted onto a polyvinylidene fluoride membrane. The membrane was soaked in TNT solution (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.02% (v/v) Tween 20) containing 1% skim milk for 60 min at room temperature on an orbital shaker. The membrane was washed with TNT solution and incubated in TNT solution containing primary antibody of the appropriate
Diffusion pores in thylakoid membranes
dilution (i.e. anti-CpTPOR antibody, 4000-fold; anti-Lhcb2 antibody, 5000-fold; anti-Tic40 antibody, 2500-fold; and anti-ToC75 antibody, 2500-fold) for 60 min at room temperature. It was then washed with TnT solution and incubated for 60 min at room temperature in TnT solution containing 3000-fold diluted alkaline phosphatase–conjugated anti-rabbit IgG antibody (Bio-Rad). Detection of alkaline phosphatase activity was performed with SIGMAFAST 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich, St. Louis, MO).

CpTPOR sequence analysis

The secondary structure prediction of CpTPOR was performed with PSIPRED (56). The VDAC motifs were identified using the putative sequences listed by Homblé et al. (32) (Table S2) as references. For the phylogenetic analysis, CpTPOR homologs were identified by BLAST, and protein sequences of the homologs from animals, fungi, stramenopiles, red algae, green algae, bryophytes, Pteridophyta, Charophyta, and seed plants were retrieved from the NCBI or UniProt websites. The phylogenetic tree was created using the RAXML program (35) and visualized using the FigTree software. The multiple sequence alignment was created by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).3

Determination of cytochrome c oxidase activity

C. paradoxa cells were disrupted as described by Koike et al. (48) and subjected to Percoll gradient centrifugation as described above. Fractions from the interface between 0% and 40% Percoll, the interface between 40% and 80% Percoll, and the pellet from the bottom of the tube were collected and diluted 10-fold in the HEM buffer. These fractions were centrifuged at 2500 × g for 10 min at 4 °C, and the pellets were suspended in HEM buffer. The cytochrome c oxidase activity in these suspensions was examined using a cytochrome c oxidase assay kit (Merck).

Electrophysiological measurements

The single-channel current of CpTPOR was determined as described by Iwamoto and Oiki (38). Briefly, two glass pipettes (1.50 mm outer diameter and 1.05 mm inner diameter) were filled with a suspension of liposomes (2 mg/ml in 1 M KCl or 50 mM KCl) made of azolecin (P3644, Sigma-Aldrich); one of the lipettes additionally contained 45 mM of CpTPOR. The suspensions were swollen at the tip of the glass pipettes (30 µm in diameter) in hexadecane to generate small water-in-oil bubbles lined by a lipid monolayer. Two bubbles were placed in contact with each other by pipette manipulation, forming a contact bubble bilayer at the interface. Ag/AgCl electrodes were placed inside the glass pipettes, and the pipette with the CpTPOR-containing bubble was set as a reference. CpTPOR was spontaneously reconstituted into the contact bubble bilayer, and the ionic current was measured under voltage-clamped conditions using a patch clamp amplifier (EPC800USB, HEKA, Lambrecht, Pfalz, Germany). The signal from the current was low pass–filtered (1-KHz cutoff frequency) and sampled at 5 kHz using an A/D converter (Digidata 1550A, Molecular Devices, Sunnyvale, CA).

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