Functional Analysis of Nodulin 26, an Aquaporin in Soybean Root Nodule Symbiosomes*

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Upon infection of soybean roots, nitrogen-fixing bacteria become enclosed in a specific organelle known as the symbiosome. The symbiosome membrane (SM) is a selectively permeable barrier that controls metabolite flux between the plant cytosol and the symbiotic bacterium inside. Nodulin 26 (NOD 26), a member of the aquaporin (AQP) water channel family, is a major protein component of the SM. Expression of NOD 26 in Xenopus oocytes gave a mercury-sensitive increase in osmotic water permeability ($P_w$). To define the biophysical properties of NOD 26 water channels in their native membranes, symbiosomes were isolated from soybean root nodules and the SM separated as vesicles from the bacteria. Permeabilities were measured using stopped-flow fluorimetry in SM vesicles with entrapped carboxyfluorescein. Osmotic water permeability ($P_w$) of SM was high, with a value of 0.05 ± 0.003 cm/s observed at 20 °C (mean ± S.E.; $n = 15$). Water flow exhibited a low activation energy, was inhibited by HgCl$_2$ (0.1 mM), and exhibited a unit conductance of $3.2 ± 1.3 \times 10^{-15}$ cm$^2$/s, a value 30-fold lower than that of AQP 1, the red blood cell water channel. Diffusive water permeability ($P_d$) was 0.0024 ± 0.0002 cm/s, and the resulting $P_f$ to $P_d$ ratio was 18.3, indicating that water crosses the SM in single file fashion via the NOD 26 water channel. In addition to high water permeability, SM vesicles also show high mercury-sensitive permeability to glycerol and formamide, but not urea, suggesting that NOD 26 also fluxes these solutes. Overall, we conclude that NOD 26 acts as a water channel with a single channel conductance that is 30-fold lower than AQP 1. Because the solutes that permeate NOD 26 are far larger than water, and water appears to cross the channel via a single file pathway, solute flux across NOD 26 appears to occur by a pathway that is distinct from that for water.

Soil bacteria of the Rhizobium and Bradyrhizobium genera invade the roots of specific leguminous plants and establish a nitrogen fixing symbiosis. During this process, a developmental program is triggered resulting in the formation of root nodules. The bacterium becomes enclosed in a specific organelle, the symbiosome (1), within the infected cells of the nodule. This organelle is delimited by a membrane of plant origin, known as the symbiosome membrane (SM).¹ This membrane regulates the efflux of fixed nitrogen from the endosymbiont to the plant cytosol as well as the influx of dicarboxylates (e.g. malate) from the plant, providing energy for nitrogen fixation (2–4).

During nodule development, the expression of a number of nodule-specific proteins, known as nodulins, is induced. One of these proteins, nodulin 26 (NOD 26), is a major protein component of the SM (5). NOD 26 is a member of an ancient family of membrane channel proteins that include bacterial glycerol transporters, aquaporin (AQP) water channels, and various other intrinsic membrane proteins (6, 7). Structural similarities shared by family members include the presence of six putative transmembrane α-helical domains with highly conserved NPA motifs found within the loop structures between these helices (7–9). Carboxyl- and amino-terminal regions are relatively hydrophilic and are exposed to the cytosolic compartment. It has previously been shown that NOD 26 is phosphorylated specifically and uniquely, in vitro and in vivo, at Ser-262 within the carboxyl-terminal domain by a calcium-dependent protein kinase of the CDPK family (5, 10).

The symbiosome represents a metabolically active compartment with several transport activities that are essential for support of the symbiosis (2–4). The observation that NOD 26 is expressed only during nodule formation and is a major membrane component of this organelle argues for its importance in SM function. However, the question of the role that NOD 26 plays in symbiosome function is an open one. Similar to the major intrinsic protein of the lens (MIP or AQP 0) (11–13), NOD 26 forms a high conductance ion channel upon reconstitution into planar lipid bilayers (14), and phosphorylation confers voltage-sensitive gating behavior on the channel (15). However, recent experiments involving expression of MIP in Xenopus oocytes have led to a reevaluation of the channel properties of this protein, and it is now clear that MIP possesses aquaporin activity in this system (16, 17, 41). In the present report we show that NOD 26 also forms an aquaporin channel upon expression in Xenopus. To address a potential aquaporin function for NOD 26 in rhizobial symbioses, we have also examined the water and solute transporting activities of NOD 26 in its native membrane, both to define its potential

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¹The abbreviations used are: SM, symbiosome membrane; NOD 26, nodulin 26; AQP, aquaporin; CF, carboxyfluorescein; MIP, major intrinsic protein; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid;ANTS, aminomethylpropanesulfonic acid.
role in symbiosome function and to compare its transport properties with other members of the AQP family.

MATERIALS AND METHODS
Expression of NOD 26 cRNA in Xenopus Oocytes and Permeability Determinations—For expression in Xenopus oocytes, a NOD 26 cDNA (15) was cloned into the BgII site of the pXGe-vplasmid (19) (a gift of Dr. Peter Agre). Capped cRNA encoding NOD 26 (flanked by 3′- and 5′-untranslated regions of the Xenopus β-globin mRNA) was produced by in vitro transcription of XhoI linearized plasmid by T7 RNA polymerase (Stratagene). Xenopus oocytes (stage VI) were prepared as described previously (17) and were injected with 50 nl of water (Control) or 50 nl of 1 mg/ml NOD 26 cRNA. Oocytes were incubated at 18 °C in ND96 media for 24 h prior to analysis.

Osmotic water permeability (P) of oocytes was measured at 10 °C by monitoring the change in cross-sectional area of the oocyte upon 70% dilution as described (16). The relative change in volume (V/Vo) was calculated as follows (Equation 1).

\[ \Delta(V/V_0) = V/V_0 \]  
\[ (Eq. 1) \]

A0 and V0 are the cross-sectional area and cell volume, respectively, at time 0, and A and V are the cross-sectional area and cell volume, respectively, at time t. P was calculated as shown in Equation 2.

\[ P = \frac{d(V/V_0)/dt}{A_0} \Delta(V/V_0) = \frac{(A_0 - V_0)}{V} \times \left( \frac{S_{\text{real}}/S_{\text{sphere}}}{V_0} \right) \]  
\[ (Eq. 2) \]

S is the geometric surface area of the oocyte at time 0, \( \Delta(V/V_0) \) is the osmotic gradient, and \( V_0 \) is the partial molar volume of water. \( S_{\text{real}} \) is the actual area of the oolemma, and \( S_{\text{sphere}} \) is the surface area of the oocyte assuming it is a sphere. An \( S_{\text{real}}/S_{\text{sphere}} \) value of 0.9 was used for all \( P \) calculations. This factor corrects for the increase in surface area due to the presence of folds and microvolta in the oolemma (16).

Measurements of reversible HgCl2 inhibition of water flux were done as follows. After \( P \) determination, oocytes were incubated in ND96 media containing 3 mM HgCl2 for 4 min. \( P \) was then remeasured in diluted ND96 in the presence of 3 mM HgCl2. Reversibility was shown by then incubating the same oocytes in ND96 containing 5 mM 2-mercaptoethanol and no HgCl2 for 15 min prior to a third \( P \) determination. Between each measurement, oocytes were rinsed and incubated in ND96 medium containing no agents for 20 min.

Symbiosome Vesicle Preparation— nodulated soybean plants (Glycine max cv Essex) were grown by a modification of the procedure described by Weaver et al. (5, 10). Soybeans were planted in vermiculite and were inoculated directly by watering with a dilute culture of rhizobium japonicum and were inoculated directly by watering with a dilute culture of R. meliloti (5, 10). Soybeans were planted in vermiculite and were inoculated directly by watering with a dilute culture of R. meliloti. Nodules (20 to 40 g) were harvested from 28- to 40-day-old soybean plants. Nodules were ground to a fine powder in liquid nitrogen. The powder was washed in 25 mM MES-bis-trispropane, 3 mM MgSO4, 350 mM mannitol, 1 mg/ml bovine serum albumin, 2 μg/ml leupeptin, 350 mM mannitol. The extract was filtered through Miracloth, and the remaining material was isolated by centrifugation (5,550 rpm in a Sorvall HS-4 rotor (4936 g) on a Percol step gradient (7 ml of extract on a 15 ml gradient consisting of 5 ml each of 30, 60, and 80% Percoll in 10 mM MOPS-NaOH, pH 7.0, 3 mM MgSO4, 350 mM mannitol). Pure symbiosomes were collected from the 60/80% Percoll interface. The symbiosomes were washed in 25 mM MES-bis-trispropane, 3 mM MgSO4, 350 mM mannitol, 1 mg/ml bovine serum albumin, 2 μg/ml leupeptin, pH 7.0 (wash buffer). The final suspension was centrifuged in 3–4 ml of wash buffer containing 20 mM carboxyfluorescein (CF) and was vortexed 2 min. Symbiosome membrane (SM) vesicles were obtained by differential centrifugation. Bacteroids were pelleted at 6000 rpm for 10 min at 4 °C in a Sorvall HS-4 rotor (5874 g). The supernatant, containing the SM vesicles, was centrifuged at 100,000 × g for 1 h at 4 °C. The final vesicle pellet was suspended in 25 mM MES-bis-trispropane, 3 mM MgSO4, 20 mM CF, 2 μg/ml leupeptin, pH 7.0.

Permeability Measurements in Symbiosome Vesicles—Permeability measurements were performed as described (20, 21) using a stopped-flow spectrofluorimeter (SF17MV, Applied Photophysics, Leatherhead, UK) with a measured dead time of 0.7 ms to monitor the fluorescence quenching of CF entrapped within the vesicles. Extravesicular fluorescence was quenched completely using anti-CF antibody (21, 22). The excitation wavelength was 490 nm. The emission wavelength was filtered with a 515-nm cutoff filter. Fluorescence data from the stopped-flow device from 5 to 10 individual determinations were averaged and fit to a single exponential curve using software supplied by Applied Photophysics. The software utilizes a nonlinear regression (Marquardt) algorithm calculated from the time course using the “Curfit” routine.

Osmotic Water Permeability (P) of all solutions were measured by freezing point depression (Precision Instruments Osmette A osmometer, Natick, MA). NOD 26 containing vesicles were washed three times with buffer solution (25 mM MES, 3 mM MgSO4, titrated to pH 7.0 with NaOH; osmolality 45 mOsm/kg). P was determined by abruptly doubling the osmolality of the extravesicular solution with additional sucrose and monitoring the time-dependent quenching of entrapped CF. Upon mixing, intravesicular CF concentration increased as water efflux occurred, leading to concentration-dependent CF quenching. P was calculated from the time course of relative fluorescence by comparing simple exponential time constants fitted to simulated curves in which P was varied. Simulated curves were calculated using a commercially available software package (Mathcad) from the osmotic permeability equation (Equation 3).

\[ d(V/V_0)/dt = (P) (SAV) (MVW) \times (C/V_0 - Cout) \]  
\[ (Eq. 3) \]

V(t) is the relative volume of the vesicles at time t, \( P \) is osmotic water permeability, SAV is the vesicle surface area-to-volume ratio, MVW is the molar volume of water (18 cm3/mol), and Cin and Cout are the initial concentrations of total solute inside and outside the vesicle, respectively. SAV was calculated from the vesicle diameters, which were measured by quasiliatc light scattering using a Nicomp Model 270 instrument.

Since the volume within the vesicle was small compared with the volume outside, it was assumed that Cout remained constant throughout the experiment. Parameters from the exponential fit (amplitude and end point) were used to relate relative fluorescence to relative volume using boundary assumptions that relative fluorescence and volume are 1.0 at time 0 and that relative volume reaches a known value (if at time 0 the osmolality outside is double that inside, the relative volume reaches 0.5) at the end of the experiment.

Unit Conductance of NOD 26—Unit conductance (P) for NOD 26 was determined by estimation of the NOD 26 content of the symbiosomes per unit surface area as described (23, 24). The number of vesicles per unit volume of preparation was determined by quantifying the total amount of entrapped CF per unit volume by lysing the vesicles with Triton X-100 and comparing the total fluorescence in the cuvette (SLM Aminco 500 C spectrofluorimeter) to standard curves obtained on the same instrument at identical settings using known quantities of CF. From the quench curve of the intact vesicles containing CF, the internal concentration was 20 μM. From this concentration, the total amount of CF per unit volume of the preparation, and the diameter of each vesicle, it was possible to calculate the total amount of vesicle surface area in the preparation. The density of NOD 26 per unit surface area of vesicle was determined from the concentration of NOD 26 in the SM preparations. Two separate procedures were used. First, the total protein concentration in the SM preparation was determined as described (5), and the proportion of protein which is NOD 26 was estimated by densitometry of a Coomasie Blue-stained SDS-polyacrylamide gel electrophoresis gel of vesicle preparation. The molar concentration was determined using a molecular weight of 28,887 calculated from the deduced amino acid composition of NOD 26. Second, a quantitative Western blot procedure with NOD 26 specific antibodies (18) was done on SM preparations, and the amount of nodulin 26 was determined by densitometry of the immunooreactive band using a purified nodulin 26 standard curve. Concentration values determined by the two methods showed excellent agreement (data not shown). Based on the density of NOD 26 per unit surface area of vesicle, the unit conductance of NOD 26 (pF) could then be calculated as shown in Equation 4.

\[ pF = P - SuD \]  
\[ (Eq. 4) \]
measurements were so rapid, they were performed at 12 °C instead of 20 °C to slow the rate and permit maximal definition of the time course of water diffusion.

Small Nonelectrolyte Solute Permeability—SM permeabilities to urea, glycerol, acetamide, and formamide were measured by loading the vesicles with the small nonelectrolytes and abruptly mixing them into isoosmotic medium containing half the intravesicular concentration of the permeant solute as described (20, 21). As the permeant solute escaped from the vesicles, water osmotically followed, and the rate of vesicular shrinkage was dependent upon permeant solute exit. As with osmotic water permeability, vesicle shrinkage was monitored by the self-quenching of CF. Calculations of small nonelectrolyte (urea or glycerol) permeabilities were performed using Equation 5.

\[
dV_{\text{rel}}/dt = P_{\text{relative}}(SAV_0)/(141)(188/V_{\text{rel}} - 235) \quad \text{(Eq. 5)}
\]

The equation has been derived and validated as described previously (20, 21). \( P_{\text{relative}} \) is the permeability coefficient (cm/s) for solute. \( V_0 \) is the relative volume of the vesicles at a given time, and \( V_{\text{rel}} \) is their relative volume at time 0. \( SA \) is the surface area of the vesicles. By use of parameters from the single exponential curve fit to the data, \( P_{\text{relative}} \) was solved using MathCad.

Statistical Analysis—The program SigmaStat (Jandel Corp., Corte Madera, CA) was used for the paired t test and for analysis of variance. Numerical results were considered statistically significant if \( p < 0.05 \).

RESULTS

Ability of NOD 26 cRNA to Induce Water Channel Activity in Xenopus Oocytes

Fig. 1 shows \( P_f \) values obtained in oocytes injected with water (control) or NOD 26 cRNA (NOD 26). Injection of NOD 26 cRNA markedly and significantly increased \( P_f \) above that of control oocytes (\( p < 0.001 \)). This increase was abolished by exposure to HgCl\(_2\), and the mercurial sensitivity was reversed in the presence of 2-mercaptoethanol (for both comparisons, \( p < 0.005 \)). Consistent with the water flux through a protein channel, the \( E_a \) calculated from Arrhenius plots was reduced 2-fold in NOD 26-injected oocytes compared with water injected controls (data not shown). Overall, these results are consistent with water flux through an aquaporin-like channel (reviewed in Ref. 7) and demonstrate that NOD 26 cRNA encodes a mercurial-sensitive water channel.

Permeability Properties of NOD 26 in the Symbiosome Membrane

Size Characteristics of the Symbiosome Vesicle Population—Since the symbiosome membrane represents a specialized plant membrane of which NOD 26 is a major (if not the major) protein component (5, 10), we set out to investigate further the water permeability of this protein in its native state in isolated SM vesicles. To perform permeability measurements, vesicles must behave as a single population in size measurements. Fig. 2 shows a representative quasielastic light scattering trace of SM vesicles. It is apparent that the vesicles exhibit a single population profile. The diameters obtained in two such measurements were 217 and 240 nm, so an average radius of 228 nm was used for all permeability calculations.

Osmotic Water Permeability of Symbiosomes—Fig. 3 shows representative time courses of osmotic vesicle shrinkage in the absence and presence of HgCl\(_2\). SM vesicles exhibit high water permeabilities with an average \( P_f \) of 0.05 ± 0.003 cm/s at 20 °C (mean ± S.E., \( n = 15 \)). Consistent with the mercury sensitivity of the NOD 26 aquaporin activity, treatment of vesicles with 0.1 mm HgCl\(_2\) drastically reduced \( P_f \) to an average value of 0.00380 ± 0.0006 cm/s (\( n = 7 \)). The effect of varying temperature (12–39 °C) on \( P_f \) is shown in the form of an Arrhenius plot in Fig. 4. From the slope, the activation energy, \( E_a \), was determined and from three such plots averaged 3.3 ± 0.4 kcal/mol. This low \( E_a \) is characteristic for water flow through aquaporins (26, 27). Based on immunoassay, the NOD 26 concentration in SM was estimated at 3 to 4 nmol/mg protein which represents 10% of the total SM protein. From this value and the calculated vesicle surface area, the conductance of each NOD 26 monomer was determined to be 3.2 ± 1.3 × 10\(^{-15}\) cm\(^3\)/s (\( n = 3 \), a value 30-fold lower than that of AQP 1, the red blood cell water channel (23)).

Diffusive Water Permeability (\( P_d \)—Measurements of \( P_f \) and \( P_d \) of the red cell water channel, AQP 1, have permitted calculation of the ratio \( P_f/P_d \). Values for this ratio of greater than 1 have been interpreted to indicate that water molecules line up single file within the lumen of the pore and provide an estimate of the number of water molecules lined up within the single file portion of the pore at any one time (26, 27). We therefore measured \( P_d \) in NOD 26 containing SM vesicles to determine whether we could find evidence for single file water transport and, if so, to estimate the number of water molecules lined up within the pore at any one time. Fig. 5 shows a representative
DISCUSSION

The present studies provide new insights in two areas: the transport functions of the symbiosome membrane between en-
NOD 26 awaits reconstitution of the pure protein into proteoliposomes, it appears highly likely that NOD 26 mediates small nonelectrolyte transport. The flux of small nonelectrolytes across NOD 26 may also help in osmoregulation, by balancing the movements of other solutes and ions across the membrane. Interestingly, in a previous study it was found that phosphorylation of Ser-262 of NOD 26 correlates with enhanced malate translocation across symbiosome membranes (29). Thus, whether this protein also participates in the transport of metabolically important compounds and the potential effects of phosphorylation of Ser-262 on NOD 26 water and solute transport activities require further investigation.

NOD 26 as an Aquaporin—The present study establishes that NOD 26 functions as an aquaporin, mediating water flow when expressed in Xenopus oocytes and in its native membrane, the symbiosome. Based on previous and present studies, NOD 26 and the major intrinsic lens protein, MIP or AQP 0, exhibit striking similarities of function. Both NOD 26 and AQP 0 (16, 41) have been shown to effect clear-cut increases in water permeability when expressed in oocytes. The water conductances of these proteins, although measured in different systems, are relatively similar (17). When each one is reconstituted and placed into planar lipid bilayers, they form high conductance nonselective ion channels which develop the ability to gate in response to voltage when phosphorylated in their carboxyl-terminal domains (11–15). These functional similarities suggest that AQP 0 and NOD 26 may form a functional subclass of AQPs which exhibit moderate water conductance (30-fold lower than AQP 1) which is roughly comparable to that of AQP 0. There is evidence from the present studies that NOD 26 also conducts some small nonelectrolytes. These results raise the possibility that AQP 0 may also function in a similar manner under appropriate conditions.

Using van der Waal’s volumes for comparison, water has a volume of 10.6 cm$^3$/mol, formamide a volume of 24.8 cm$^3$/mol, urea 32.8 cm$^3$/mol, acetamide 35.9 cm$^3$/mol, and glycerol a volume of 51.4 cm$^3$/mol (38). The relative permeability of the solutes (formamide $>$ glycerol $>$ acetamide $>$ urea) indicates that the apparent selectivity of NOD 26 for small nonelectrolytes is not based solely on size. It will now be of great interest to examine the transport properties of purified NOD 26 in proteoliposomes to determine whether reconstitution alters the biophysical behavior of the protein.

The $P_l/P_o$ ratio of NOD 26 of 18 provides additional information about its function and configuration in the membrane. The comparable value for AQP 1 is 13 (39), so that it appears that the single file water pore is longer for NOD 26 than for AQP 1. It is interesting, in this regard, that AQP 1 exhibits a higher conductance than NOD 26, raising the possibility that the length of the single file pore has an impact on conductance. Similar measurements in a number of other aquaporins will be needed to address this issue further.

Although the high $P_l/P_o$ ratio of NOD 26 indicates single file water flow through the water pore (26, 27), the apparent ability of NOD 26 to mediate transport of formamide, acetamide, and glycerol suggests a site that permits passage of molecules far...
larger than the diameter of two water molecules side-by-side. Therefore, if the water permeation occurs via a single file pathway, then the transport of small nonelectrolytes must be occurring via a pathway distinct from that of water flow. Such a conclusion appears reasonable given the clear-cut dissociation of water and small nonelectrolyte permeabilities exhibited by the aquaporin family. Thus, AQP 1 exhibits high water conductance and little or no solute permeability (23, 24), and AQP 3 exhibits low water conductance and mediates relatively rapid solute fluxes (40).

Although definitive answers to questions about NOD 26 biophysics await its reconstitution, the availability of its permeability profile in its native membrane will permit unambiguous interpretation of results obtained when the protein is reconstructed or expressed in heterologous systems. Moreover, detailed biophysical profiles of different aquaporins will permit structure-function relationships between different aquaporin sequences to emerge and will provide information critical to the development of a structural model of the water channels that these proteins form.

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