Plant Physiol. (1996) 112: 759–766

Mechanism of Proton Permeation through Chloroplast Lipid Membranes[1]

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Electrical measurements were carried out to investigate the contribution of chloroplast lipids to the passive proton permeability of both the thylakoid and inner-envelope membranes. Permeability coefficient and conductance to protons were measured for solvent-free bilayers made from monogalactosyldiglyceride:digalactosyldiglycerid:sulfoquinovosyldiglyceride:phosphatidylglycerol (2:1:0.5:0.5, w/w) in the presence of a pH gradient of 7.4/8.1. The permeability coefficient for protons in glycolipids was 5.5 ± 1.1 × 10^{-4} cm s^{-1} (n = 14). To determine whether this high H^+ permeability could be explained by the presence of lipid contaminants such as weak acids, we investigated the effects of (a) bovine serum albumin, which can remove some amphiphilic molecules such as free fatty acids, (b) 6-ketocholestanol, which increases the membrane dipole potential, (c) oleic acid, and (d) chlorodecane, which increases the dielectric constant of the lipid bilayer. Our results show that free fatty acids are inefficient protonophores, as compared with carbonylcyanide-*m*-chlorphenylhydrazone, and that the hypothesis of a weak acid mechanism is not valid with glycolipid bilayers. In the presence of deuterium oxide the H^+ conductance was reduced significantly, indicating that proton transport through the glycolipid matrix could occur directly by a hydrogen bond process. The passive transport of H^+ through the glycolipid matrix is discussed with regard to the activity of the thylakoid ATP synthase and the inner-envelope H^+-ATPase.

The chloroplast of plant cells is enclosed by a double-envelope membrane and contains photosynthetic membranes called thylakoids, which convert light into chemical energy. The proton pumping during the illumination of thylakoids builds up a pH gradient of about 3 units. Proton efflux down this gradient via the CF_o-CF_1 complex of an ATP synthase is linked to ATP synthesis (Junge and Jackson, 1982). The contribution of the electrical membrane potential difference to the H^+ electrochemical potential gradient is negligible because counterfluxes of K^+, Mg^{2+}, and Cl^- ions are linked to H^+ pumping (Hind et al., 1974; Vredenberg, 1976; Junge, 1977). The lipid matrix of thylakoid bilayers, which contains about 90% of glycolipids, is highly permeable to protons; however, it cannot be considered a pathway for passive fluxes of K^+, Mg^{2+}, and Cl^- (Fuks and Homblé, 1994). In addition to the proton flux through the CF_o-CF_1 complex that is linked to photophosphorylation, there appear to be two other parallel pathways for the efflux of protons across thylakoids (Cole et al., 1981): a passive diffusion through the bilayer and an efflux through the CF_o-CF_1 complex that is not linked to ATP synthesis. Monitoring the decay of the light-induced H^+ gradient, Schönfeld and Schickler (1987) demonstrated that the H^+ efflux can occur significantly through the pathway that is not associated with the CF_o-CF_1 channel. The proton permeability of this pathway through the bilayer was about 10^{-5} cm s^{-1}, which is at least 5 orders of magnitude higher than the alkali or halide ion permeability.

The outer-envelope membrane of chloroplasts is freely permeable to small molecules, in contrast to the inner-envelope membrane, which is a selective barrier for solutes (Douce and Joyard, 1990) and contains many transport proteins, e.g. a phosphate translocator (Flügge et al., 1992), ion channels (Mi et al., 1994; Fuks and Homblé, 1995) and H^+-ATPase (Berkowitz and Peters, 1993). On the other hand, experimental evidence suggests that chloroplast envelopes are freely permeable to protons (Heldt et al., 1973; Werdan et al., 1975; Bligny et al., 1990). Therefore, the inner-envelope H^+-ATPase is required to sustain a pH gradient of about 0.5 units across the membrane.

The molecular process involved in the passive proton transport through the lipids of both thylakoid and inner-envelope membranes is unresolved. Three hypotheses have been suggested to explain the high H^+ permeability of phospholipid bilayer membranes. First, chains of hydrogen-bonded water molecules extend across the hydrophobic core of the membrane, providing a pathway for H^+ to jump from one side of the bilayer to the other side (Deamer, 1987; Nagle, 1987; Deamer and Nichols, 1989; Deamer and Volkov, 1995). Second, lipid contaminants such as free fatty acids diffuse across the bilayer in their neutral form, carrying H^+-like weak acid uncouplers (Gutknecht, 1987a, 1987b, 1987c, 1988). Third, the proton permeability arises from hydronium ($H_9O_4^+$) diffusion, which has a larger permeability coefficient than the proton in the nonhydrated state (Deamer and Volkov, 1995). None of these proposed mechanisms has been demonstrated in chloroplast glycolipid bilayers.

In the present study we have carried out electrical measurements of H^+ permeability coefficients in planar lipid bilayers that were made from glycolipids found in thylakoid and inner-envelope membranes to characterize the

[1] B. Fuks is a research assistant and F. Homblé is a senior research associate of the Belgian National Fund for Scientific Research, which provided financial support.

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Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; CF_o-CF_1, H^+-ATP synthase; G_H, specific proton conductance.

molecular mechanism of passive H^+ transport through chloroplast lipid bilayers.

MATERIALS AND METHODS

Plant lipids were purchased from Lipid Products (Redhill, Surrey, UK). They were mixed to form monogalactosyldiglyceride:digalactosyldiglyceride:sulfoquinovosyldiglyceride:phosphotidylglycerol (2:1:0.5:0.5, w/w) and stored in chloroform at −20°C. This composition is similar to that of both thylakoid and inner-envelope membranes (Dorne et al., 1990). Unless otherwise stated, membrane-forming solutions were constructed to give a lipid concentration of either 0.5% in hexane or 1% in *n*-decane and chlorodecane. All of the solvents used (hexane, chloroform, *n*-decane, chlorodecane, and squalene) were more than 99% pure. Oleic acid, BSA (free of fatty acid), CCCP, and 6-ketocholestanol were purchased from Sigma. D_2O was more than 99% pure (Janssen Chimica, Beerse, Belgium), and all other chemicals were of analytical reagent grade. Solutions were made using triple-distilled water and were filtered through 0.2-μm filters (Acrodisc, Gelman Sciences, Ann Arbor, MI) before being used.

Planar Lipid Bilayers

Solvent-free lipid bilayers were formed from the quaternary mixture of chloroplast lipids using the technique of Montal and Mueller (1972) by bringing together two monolayers of lipids over a hole (200 μm in diameter) that was drilled in a thin sheet (25 μm thick) of Teflon treated with squalene. Hexane was evaporated at least 5 min before the folding of the monolayers.

In some cases we used solvent-containing planar bilayers that were formed by the technique of Mueller et al. (1962) by painting the membrane-forming solution over a 200-μm-diameter hole that was made in a plastic foam cup.

Electrical Recordings

Currents across the lipid bilayers and capacitance measurements were carried out using a current-voltage converter (RK-300, Bio-Logic, Claix, France) as described previously (Fuks and Homblé, 1994). The H^+ conductance and permeability were measured in an ionically balanced buffer mixture to avoid the current that was produced by the other ions. A pH gradient was produced by adding 20 mM Hepes to the *cis* side (pH 7.4) and 20 mM Tris to the *trans* side (pH 8.1) of a symmetrical concentration of buffers that was bathing the membrane (30 mM Hepes and 30 mM Tris, pH 7.75).

Since it is not possible to determine the individual contribution of H^+ and OH^- to the electrical current measured, H^+/OH^- is expressed as the single term H^+. The permeability for H^+ is calculated from the reverse potential difference and the ionic transference numbers measured in the pH gradient of 7.4 to 8.1 when there are no gradients of the buffer cation and anion. The zero-current voltage (V_m), referred to hereafter as the diffusion potential, can be expressed as follows:

$$V_m = T_H \times E_H, \quad (1)$$

where E_H is the equilibrium electrical potential of H^+ ions. The ionic transference number (T_H) is defined as:

$$T_H = G_H / G_m, \quad (2)$$

where G_H is the H^+ conductance and G_m is the total membrane conductance. The H^+ permeability coefficient is calculated from the following relation:

$$P_H = (G_H \times R \times T)/(F^2 \times C_H), \quad (3)$$

where R is the gas constant, T is temperature (25°C), F is Faraday, and C_H corresponds to an H^+ concentration of $10^{-7.75}$ M (Gutknecht, 1984; Deamer and Gutknecht, 1990).

Data are expressed as means ± SE (n = number of replicates). Unless otherwise stated, statistical significance refers to a Student's t test ($P < 0.05$), and the experiments were carried out at room temperature.

RESULTS

H^+ Selectivity and Permeability of Glycolipid Bilayers

The permeability coefficient for H^+ was calculated from the membrane conductance and the diffusion potential of solvent-free planar lipid bilayers made of monogalactosyldiglyceride:digalactosyldiglyceride:sulfoquinovosyldiglyceride:phosphotidylglycerol (2:1:0.5:0.5, w/w). The membrane conductance was measured from the ohmic part of the I-V relationship between +60 and −60 mV (Fig. 1). Its value of $6.6 \pm 0.5 \times 10^{-8}$ siemens cm^{-2} (n = 30) was similar when it was calculated either in a symmetrical pH of 7.75 or in a pH gradient of 7.4 to 8.1. The pH gradient applied across the lipid bilayer produced a diffusion potential of −23.1 ± 1.6 mV (n = 14). From the equilibrium potential for H^+ of −41.3 mV and the diffusion potential,

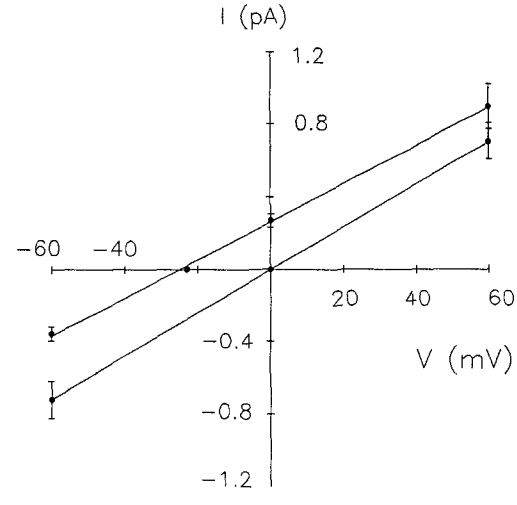

Figure 1. The I-V relationship of solvent-free bilayers made of monogalactosyldiglyceride:digalactosyldiglyceride:sulfoquinovosyldiglyceride:phosphotidylglycerol (2:1:0.5:0.5, w/w) under a pH gradient (*cis*, pH 7.4; *trans*, pH 8.1) and a symmetrical pH (7.75).

the transference number was calculated to be 0.55 ± 0.03 (n = 14) (see Eq. 1). This means that the chloroplast lipid bilayer was not perfectly proton-selective. The H^+ conductance and the H^+ permeability coefficient were calculated from Equations 2 and 3 to be $G_H = 3.7 \pm 0.7 \times 10^{-8}$ S cm^{-2} (n = 14) and $P_H = 5.5 \pm 1.1 \times 10^{-4}$ cm s^{-1} (n = 14), respectively.

Inhibition of G_H by BSA

The high H^+ permeability could be explained by the presence of weak acid contaminants in glycolipids. To test this hypothesis, we added BSA (free of fatty acids) to both sides of a solvent-free lipid bilayer. BSA is known to bind strongly to amphiphilic molecules and, in particular, to fatty acids (Hamilton and Cistola, 1986; Gutknecht, 1987a; Kamp and Hamilton, 1992); therefore, it is expected to extract and to remove contaminants from the bilayer. BSA was added from a stock solution (40 mg/mL) to give a final concentration of 0.2 mg/mL. We have verified that this concentration did not alter the pH gradient. Each compartment was stirred for 30 s, and both the diffusion potential and the membrane conductance were measured after the addition of BSA. We calculated that G_H was reduced 3-fold after the addition of BSA ($G_H = 1.3 \pm 0.1 \times 10^{-8}$ S cm^{-2}, n = 4), but in one case its value decreased 20-fold (Fig. 2).

The Effect of Dipole Potential Changes on G_H

Previously, it has been suggested that the proton transport process of free fatty acids is similar to that of weak acid uncouplers such as fluorocarbonyl-cyanmide-phenylhydrazone and CCCP (Gutknecht, 1987a). This process requires that the protonated form of the weak acid permeates easily through the lipid matrix, and the anionic recycling form is the rate-limiting step (Benz and Mc Laughlin, 1983).

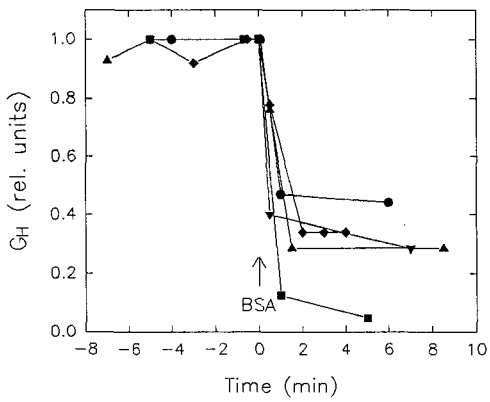

Figure 2. The effect of the addition of BSA on the G_H measured in solvent-free bilayers formed from monogalactosyldiglyceride: digalactosyldiglyceride:sulfoquinovosyldiglyceride:phosphotidylglycerol (pH 7.4/8.1). The arrow indicates the addition of BSA (0.2 mg/mL) in the *cis* compartment of the bilayer membrane. Each symbol corresponds to a separate experiment. The initial conductance was 5×10^{-8} (♦), 4.2×10^{-8} (▲), 3.4×10^{-8} (●), and 3.5×10^{-8} (■), and the final conductance was 1.7×10^{-8} (♦), 1.2×10^{-8} (▲), 1.5×10^{-8} (●), and 0.2×10^{-8} (■) (S cm^{-2}).

Lipid bilayers have a dipole potential of several hundred millivolts (positive inside the membrane), which originates from oriented dipoles located just below the water-hydrocarbon chain interface (Gawrisch et al., 1992; Simon et al., 1992). This dipole contributes significantly to the energy barrier of the rate-limiting step of weak acid diffusion through phospholipid liposomes (Perkins et Cafiso, 1987). Therefore, the weak acid contaminant hypothesis can be verified by investigating the effect of a change in the dipole potential on the proton permeability of the bilayer. 6-Ketocholestanol is a cholesterol analog with a large dipole moment associated with its keto group, and it is known to increase the dipole potential of liposomes and biological membranes. In contrast to cholesterol, it has been shown that this molecule does not modify the fluidity, packing, or thickness of the bilayer (Simon et al., 1992; Franklin and Cafiso, 1993; Gross et al., 1994). The change in dipole potential induced by 6-ketocholestanol is expected to enhance the translocation rate of the weak acid CCCP anion.

Figure 3A shows the result of a typical experiment in which the current carried by CCCP (1 μM in *cis*) was measured at different electrical potentials, either in the absence or in the presence of 6-ketocholestanol (17 mol% in chloroplast lipids). In the absence of 6-ketocholestanol CCCP increased the H^+ selectivity to a value of T_H = 0.90 ± 0.04 (n = 14), and the H^+ conductance was found to be $G_H = 8.6 \pm 1.4 \times 10^{-7}$ S cm^{-2} (n = 14). This effect was enhanced in the presence of 6-ketocholestanol (Fig. 3). This result agrees with previously published results on phospholipids, thus indicating that the effect of 6-ketocholestanol is affected neither by the lipid composition of the bilayer nor by the model membrane investigated.

To check whether a molecule with transport properties that are similar to those of a weak acid uncoupler exists in our lipid bilayers formed from glycolipids, we measured H^+ conductances at three concentrations of 6-ketocholestanol with no uncouplers added to the aqueous solution (Fig. 3B). The modification of the membrane dipole potential did not alter the intrinsic H^+ conductance of the glycolipid bilayers (Fig. 3B). Thus, this result does not agree with a weak acid hypothesis, in which a negatively charged species is expected to be the rate-limiting step carrying the current.

The Effect of Oleic Acid on G_H

The envelope contains the enzymes required for glycolipid synthesis. Oleic acid is the main fatty acid synthesized by the chloroplast and is exported to the cytosol through the double envelope for further desaturation (Douce and Joyard, 1990). To investigate the protonophore activity of oleic acid in glycolipid bilayers, we measured the H^+ conductance before and after the addition of the free fatty acid in the *cis* compartment. Figure 4A shows a typical experiment in which different concentrations of oleic acid were added to the solution in a pH gradient of 7.4 to 8.1. Oleic acid increased the

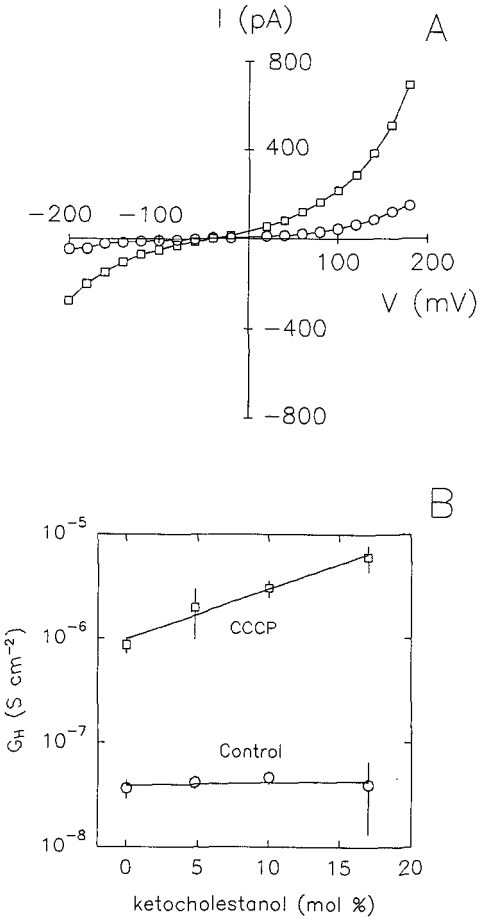

Figure 3. The effect of 6-ketocholestanol on the proton transport through solvent-free glycolipid bilayers. A, The I-V relationships of bilayer membrane without (○) and with (□) 6-ketocholestanol (17 mol%) measured after the addition of CCCP (1 μM). B, G_H as a function of the concentration of 6-ketocholestanol mixed with glycolipids in the presence (□) and in the absence (○) of CCCP.

selectivity of the chloroplast lipid bilayer for H^+. For example, with 500 μM fatty acid, T_H shifted from 0.56 in the unmodified lipid bilayer to 0.76 ± 0.08 (n = 4). However, G_H was increased only 5-fold by this high concentration of fatty acid (Fig. 4B).

When 30 mol% oleic acid was mixed directly with the lipid solution that was used to form the bilayer, the conductance of the bilayer was slightly higher than that measured in the absence of fatty acids (Fig. 5A). This level of fatty acid increased the selectivity for H^+ to T_H = 0.87 ± 0.06 (n = 6), whereas G_H was enhanced only 2-fold (Fig. 5B). Even at a level as high as 60 mol%, G_H did not increase more than 5-fold, and we also obtained similar results with linolenic acid (data not shown). These results indicate that oleic and linolenic acids are very weak uncouplers in chloroplast lipid bilayers.

H^+ Permeability Coefficient Measured in D_2O

We performed experiments in which the water solution that was bathing the solvent-free lipid bilayer was replaced with buffers dissolved in D_2O. Under these conditions the H^+ permeability was expected to be altered if the H^+ transport was mediated by jumping along the hydrogen-bonded chains of water. Proton permeability coefficients were measured from diffusion potentials in asymmetrical pH concentrations and conductances of the chloroplast lipid bilayer. In the presence of D_2O, the membrane was moderately H^+-selective, with T_H = 0.26 ± 0.04 (n = 6); its conductance for H^+ was 9.7 ± 2.8 × 10^{-9} S cm^{-2} (n = 6). This value is lower than that of G_H measured in water in the same pH gradient (Fig. 6) and yields an H^+ permeability coefficient of 1.45 ± 0.42 × 10^{-4} cm s^{-1} (n = 6), which is significantly lower (4-fold) than the H^+ permeability coefficient measured in water ($P < 0.1$).

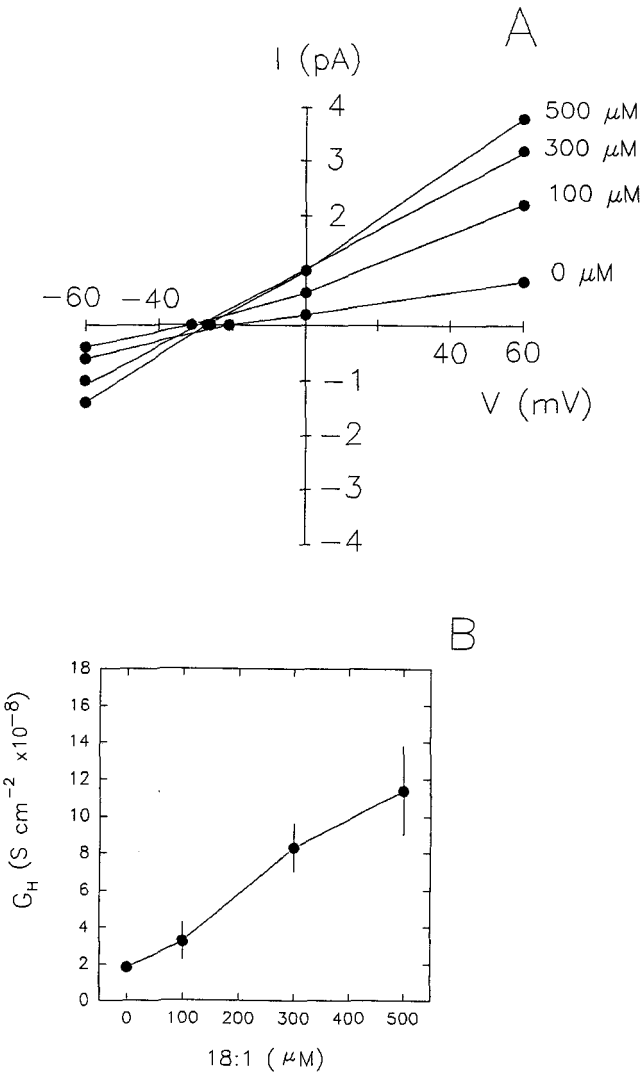

Figure 4. The effect of oleic acid added to the bathing solution on the G_H that was measured in solvent-free glycolipid bilayers. A, I-V relationship of a typical experiment measured at various concentrations of oleic acid. The free fatty acid was added to the *cis* compartment. B, Specific conductance of G_H as a function of the oleic acid (18:1) concentrations of the bathing medium.

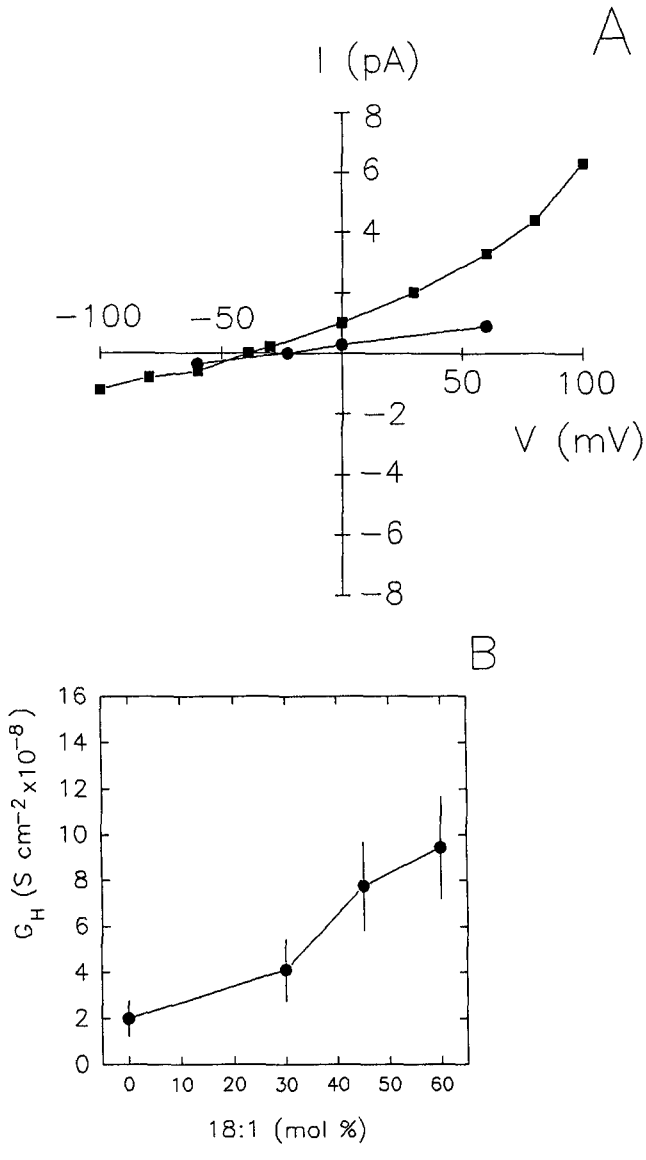

Figure 5. The effect of oleic acid mixed with glycolipids on the G_H that was measured in the solvent-free bilayers. A, I-V relationship of a typical experiment measured from a bilayer containing 30 mol% oleic acid (■) and free of oleic acid (●). B, Specific conductance G_H, as a function of the concentration of oleic acid (18:1) mixed with the lipid solution that was used to form the bilayer.

The Effect of the Bilayer Dielectric Constant on H^+ Current

To investigate the effect of the hydrophobic matrix on the H^+ conduction through the chloroplast lipids, we increased the dielectric constant of the nonpolar region of the bilayer by substituting *n*-decane (dielectric constant = 2.1) for chlorodecane (dielectric constant = 4.5). The substitution of *n*-decane by chlorodecane does not significantly change the bilayer thickness (specific membrane capacitance = 0.79 ± 0.02 μF cm^{-2} [n = 3] and membrane thickness = 5 nm) or other lipid properties (Dilger et al., 1979). Figure 7 shows H^+ conductances measured in

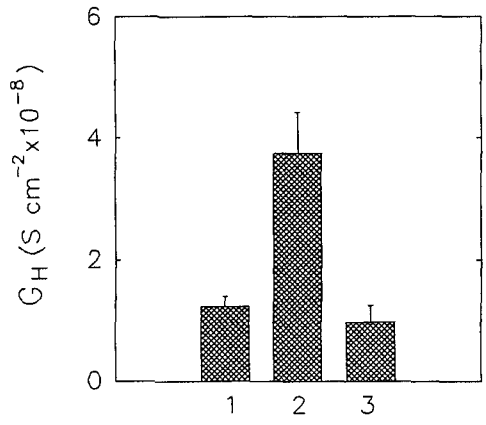

Figure 6. G_H of solvent-free glycolipid bilayers after the replacement of water (2) by D_2O (3). These G_H values were compared with those measured in decane-glycolipid bilayer membrane (1). The decrease of G_H that was measured in D_2O was statistically significant ($P < 0.1$).

solvent-containing chloroplast lipid bilayers. The addition of *n*-decane to the bilayer decreased the value of G_H that was obtained with solvent-free lipid bilayers to G_H = 1.22 ± 0.17 × 10^{-8} S cm^{-2} (n = 12). When chlorodecane was mixed with *n*-decane (50%, v/v) or used without *n*-decane, the H^+ conductances were enhanced 3- and 5-fold, respectively.

DISCUSSION

Are Weak Acid Contaminants Responsible for the High H^+ Permeability of Glycolipid Bilayers?

The H^+ conductance of the glycolipid bilayer is significantly decreased in the presence of BSA (Fig. 2). This effect was also observed in planar bilayers and liposomes that were made of phospholipids (Gutknecht, 1987a, 1987b; Kamp and Hamilton, 1992), and according to these authors, it arises from fatty acid contaminants. To check this hypothesis, we measured the effects of oleic acid on H^+ currents across chloroplast lipid bilayers (Figs. 4 and 5). A 5-fold increase in G_H can be observed only at very high

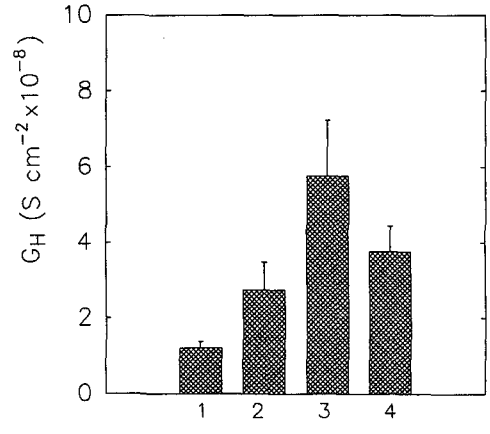

Figure 7. G_H of solvent-containing glycolipid bilayers. The lipids were solubilized in *n*-decane (1), chlorodecane/decane (1:1, v/v) (2), chlorodecane (3), or were solvent-free (4).

levels of oleic acid, either in the membrane (50 mol%) or in the solution (300 μM). This change in G_H occurs in parallel with an increase in H^+ selectivity. In contrast to our results obtained with solvent-free glycolipids, Gutknecht (1988) and Shinohara et al. (1995) observed that the conductivity of solvent-containing phospholipid bilayers increased 5-fold with 5 μM or 30 mol% oleic acid and 15-fold with 10 μM palmitic acid. The difference between our results and those obtained with phospholipids cannot be due to the presence of solvent in the bilayers, since we observed the same response when oleic acid was added to both solvent-free and decane-containing glycolipid bilayers (results not shown).

From the present results, it is clear that the 3-fold decrease in G_H after BSA addition cannot be attributed to free fatty acid contaminants in chloroplast lipids. Indeed, a membrane level of fatty acid higher than 10 mol% would be required to explain this change in G_H (Fig. 5B), whereas neither the total free fatty acid content of thylakoids nor the content of envelope membranes of chloroplasts exceeds 1 mol% of lipids (Siebertz et al., 1979). Alternatively, free fatty acids could be produced by hydrolysis of the fatty acid glycerol esters during the purification processes and the storage of lipids. However, we have verified that the chloroplast lipids used in our study were only slightly oxidized: about 0.4% of the double bonds per lipid molecule were detected according to the method of Klein (1970). Therefore, our lipid preparation was not hydrolyzed to a large extent and could not release enough amounts of free fatty acids to induce a significant change in proton permeability. Moreover, linolenic acid, the main fatty acid constituent of chloroplast lipids susceptible to being hydrolyzed, slightly enhances the G_H of chloroplast lipid bilayers, even at levels higher than 30 mol% (not shown). Finally, the basal H^+ conductance of $1.3 \pm 0.2 \times 10^{-8}$ S cm^{-2}, which remains in the presence of BSA, corresponds to an H^+ permeability coefficient of 1.9×10^{-4} cm s^{-1}. This value is lower than that of the unmodified bilayer but still is 6 orders of magnitude higher than permeability coefficients for K^+ and Cl^- (Fuks and Homblé, 1994). This indicates that the intrinsically high proton permeability of glycolipid bilayers does not arise from a fatty acid contaminant.

The hypothesis that a weak acid contaminant could act as a protonophore in chloroplast lipid bilayers is not valid, since modification of the magnitude of the membrane dipole potential does not alter the H^+ conductance, in contrast to what is observed with CCCP (Fig. 3). This result is consistent with those of Perkins and Cafiso (1986), who showed that H^+ currents measured across phospholipid liposomes were not modified by the dipole potential. However, they do not agree with those of Gutknecht, who found that the H^+ conductance of decane-phosphatidylcholine bilayers was sensitive to the membrane dipole potential (Gutknecht, 1987b, 1988).

Water-Wire Mechanism in the Lipid Matrix

Proton transport via transient hydrogen-bonded water chains is another mechanism proposed to explain the high H^+ permeability of phospholipids (Deamer, 1987; Nagle, 1987; Deamer and Nichols, 1989). Our results (Fig. 6) show that the H^+ permeability coefficient in D_2O is 4-fold lower than in water. This effect is similar to that observed in water and ice, and, therefore, our results provide experimental evidence for the transient hydrogen-bonded chain hypothesis.

The mechanism of H^+ permeation is also sensitive to the dielectric constant of the lipid matrix, since the increase of dielectric constant by chlorodecane enhances the H^+ currents (Fig. 7). Similar results have been observed in planar bilayers (Gutknecht, 1987a, 1988) and liposomes (Perkins and Cafiso, 1987) made of phospholipids. The increase in G_H when *n*-decane is replaced by chlorodecane is attributed to the change in the dielectric constant of the hydrophobic region of the membrane, because it has been shown that the thickness and other bilayer properties are not affected (Dilger et al., 1979). According to the Nagle model, the transport of protons along a transient hydrogen-bonded chain requires both proton hopping along the hydrogen-bonded chain and propagation of an L-type Bjerrum fault involving the rotation of successive hydroxyl groups (Nagle and Morowitz, 1978; Nagle et al., 1980). The rate-limiting step of the H^+ transport across the lipid bilayer will be either the rate of propagation of the turning defect or the assembly of a continuous hydrogen-bonded chain through the bilayer lipid membrane (Nagle, 1987). In a high dielectric constant medium it should be easier for water to partition into the membrane and form more hydrogen-bonded chains. Therefore, this could explain the increase in the membrane proton conductance in the presence of chlorodecane.

The high G_H of the solvent-free bilayer compared with that of the decane-containing bilayer cannot be explained by a dielectric effect, because both bilayers have the same dielectric constant. The thickness of solvent-free bilayers is about one-half that of decane-containing bilayers. Thus, the length of the transient hydrogen-bonded chain will be shortest in the solvent-free bilayer, which will increase the magnitude of the proton flux; our results are consistent with this explanation (Fig. 6). 6-Ketocholestanol increased the magnitude of the dipole moment at the water-membrane interface (Franklin and Cafiso, 1993). Our results (Fig. 3B) showing the lack of a 6-ketocholestanol effect on G_H agree with Nagle's hypothesis, according to which the rate of H^+ transport is not limited by the proton partition across the water-membrane interface. In addition, this would indicate that the Bjerrum fault migration is not the rate-limiting step, because Bjerrum faults also carry a charge.

Finally, Deamer and Volkov (1995) have claimed that $H_9O_4^+$ could be the permeant species. Their hypothesis rests on the fact that this species has a sufficiently large radius, so that the Born energy barrier for its translocation is significantly reduced. Consequently, the permeability coefficient of $H_9O_4^+$ is relatively high (10^{-5} cm s^{-1}). If we disregard the fact that the presence of $H_9O_4^+$ has not been proven, it is difficult to believe that this species could account for the proton flux, either by an electrodiffusion process or by a partition effect (which would be the rate-

limiting step for the proton flux) because in those cases G_H is expected to be pH-dependent, which it is not (Gutknecht, 1984).

Physiological Significance of the Proton Permeability of the Glycolipid Bilayer

The H^+ leakage due to the intrinsic H^+ permeability of glycolipids corresponds to the proton flux $J_H = RTG_H/F^2$ of 1×10^{-14} mol H^+ cm^{-2} s^{-1} when $G_H = 4 \times 10^{-8}$ S cm^{-2} (Sten-Knudsen, 1978). During ATP synthesis this passive H^+ flux is not significant, since it corresponds to 0.1% of the maximum H^+ flux through the CF_o-CF_1 complex of the thylakoid membrane of a sun plant under normal light conditions (Vredenberg, 1976). However, it is well known that a basal electron transfer persists in thylakoids, even when ATP synthesis is inhibited because of passive H^+ efflux via the ATP synthase CF_o channel and an unspecific pathway (Schönfeld and Neumann, 1977; Schönfeld and Schickler, 1984). This H^+ leakage still occurs after the inhibition of the CF_o channel, and its magnitude is similar to that measured in the present work (Schönfeld et al., 1987). Therefore, we can conclude that the glycolipid matrix could be the pathway for this unspecific H^+ leak measured in native membranes.

The inner-envelope membrane is known to be permeable to protons, but the molecular mechanism of H^+ permeation has not yet been investigated (Heber et al., 1973; Werdan et al., 1975; Bligny et al., 1990). In the light, proton pumping into the intrathylakoid space increases the stromal pH to approximately 8.0 and builds up a pH gradient across the chloroplast envelope (Heldt et al., 1973; Werdan et al., 1975). It has been shown that the inner-envelope H^+-ATPase is involved in the maintenance of the pH gradient in the light (Berkowitz and Peters, 1993). Because the specific activity of the inner-envelope H^+-ATPase is not known, it is unwise to speculate about the contribution of this H^+ pump in terms of bioenergetics. However, from our measurements we can conclude that the leakage of H^+ through the glycolipid matrix of the membrane would not be able to short-circuit the active H^+ transport. Indeed, the inner envelope of one chloroplast has an area of about 10 μm^2, which can account for a proton leak through the glycolipid matrix of 10^2 H^+ s^{-1} $chloroplast^{-1}$. Assuming a turnover rate of 10^3 H^+ s^{-1}, which is commonly found for proton pumps of plant and animal cells, this current could be carried by an unexpected low number (0.1) of H^+-ATPase.

In conclusion, our results demonstrate that the weak acid contaminant hypothesis is not valid to explain the high proton permeability of glycolipid bilayers. Moreover, free fatty acids cannot facilitate significantly the passive proton transport through the lipid membrane. Our results can be explained assuming a proton diffusion via transient hydrogen-bonded chains of water through the glycolipid bilayer. Since the magnitude of the proton flux reported in this study is close to that measured in thylakoids when the CF_o-CF_1 complex is blocked, we suggest that the diffusion through glycolipids can account for the unspecific H^+ leakage in native membranes.

ACKNOWLEDGMENTS

We thank Dr. John F. Nagle (Pittsburgh, PA) and Dr. Jean-Marie Ruysschaert (Brussels, Belgium) for a critical reading of the manuscript.

Received March 26, 1996; accepted July 8, 1996.
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