Two-dimensional protonic percolation on lightly hydrated purple membrane

(dielectric/hydration/proton transfer)

JOHN A. RUPLEY\textsuperscript{1}, LINDA SIEMANKOWSKI\textsuperscript{1}, GIORGIO CARERI\textsuperscript{2}, AND FABIO BRUNI\textsuperscript{3,4}

\textsuperscript{1}University Department of Biochemistry, Biological Sciences West, University of Arizona, Tucson, AZ 85721; and \textsuperscript{2}Dipartimento di Fisica, Università di Roma 1, Rome 00185, Italy

Communicated by John T. Edsall, March 30, 1988 (received for review June 26, 1987)

ABSTRACT The capacitance and dielectric loss factor were measured for a sample of purple membrane of \textit{Halobacterium halobium} as a function of hydration level (0.017 to >0.2 g of water/g of membrane) and frequency (10 kHz to 10 MHz). The capacitance and the derived conductivity show explosive growth above a threshold hydration level, $h_c \approx 0.0456$. The conductivity shows a deuterium isotope effect, $H/\text{D} = 1.38$, in close agreement with expectation for a protonic process. The level $h_c$ is frequency independent and shows no deuterium isotope effect. These properties are analogous to those found for lysozyme in a related study. Protonic conduction for the purple membrane can be considered, as for lysozyome, within the framework of a percolation model. The critical exponent, $\nu$, which describes the conductivity of a percolative system near the threshold, has the value 1.23. This number is in close agreement with expectation from theory for a two-dimensional percolative process. The dielectric properties of the purple membrane are more complex than those of lysozyme, seen in the value of $h_c$ and in the frequency and hydration dependence of the loss factor. There appear to be preferred regions of proton conduction. The percolation model is based upon stochastic behavior of a system partially populated with conducting elements. This model suggests that ion transport in membranes and its control can be based on pathways formed of randomly connected conducting elements and that a fixed geometry (a proton wire) is not the only possible basis for a mechanism of conduction.

Two-dimensional movement of protons within the surface of membranes has been suggested as being important for the biophysical and biological properties of these systems (1, 2). Various authors have treated proton transfer along essentially linear paths within the membrane (3, 4). Proton conduction over the surface of lysozyme has been described by use of dielectric measurements made on partially hydrated powders (5, 6). Binding of substrate at the active site of lysozyme reduced the proton flux (5). This was taken to mean that preferred paths for proton movement pass through the active site and could be important for catalysis.

The results of the above experiments on lysozyme follow closely the percolation model. Percolative behavior typically is observed for systems that have random processes evolving on a topologically disorderous substrate (7). Percolative processes are characterized by a threshold above which they grow explosively. Long-range connectivity appears at the percolation threshold. Behavior near the threshold can be described at a set of critical exponents, analogous to those used to describe phase transitions of other kinds. A typical percolative process is electrical conduction through a mixture of conducting and nonconducting elements, a system that displays a transition and a specific power law dependence of the conductivity at the critical threshold value of the concentration of conducting elements. This behavior is fully explained by the general statistical arguments of percolation theory, which have wide applicability. Diffusion on a randomly filled lattice is also a percolative process, and it can be shown to be formally equivalent to conduction. Thus, one has reason to ask whether the percolation model can apply to movement of protons in a membrane. Because of the statistical character of the percolation model, for it to apply to a membrane, the trajectory followed by a particular proton would be along one of the many possible connected arrangements of conducting elements linking two distant sites or boundary regions. This view of a fluctuating set of pathways is in contrast to the fixed geometry assumed in a "proton wire" type of model.

We report here the application of the experimental techniques developed through study of lysozyme to measurement of the dielectric properties of the purple membrane of \textit{Halobacterium halobium}. The data demonstrate a cooperative transition for proton movement, with a threshold hydration level $h_c \approx 0.0456$ g of water per g of purple membrane. The critical exponent describing the hydration dependence of the protonic conductivity near the transition threshold is characteristic of a two-dimensional percolative system. These observations bear on our understanding of the pathways of proton movement within the membrane; they suggest that there may be a statistical component in the processes of proton and ion transport across membranes.

METHODS

Sample Preparation. Purple membrane fragments were prepared from a high-yield strain of \textit{H. halobium} by use of standard procedures (8). Preparations gave a single band in gel electrophoresis and showed the spectrum and extinction coefficient expected for the purple membrane. Samples were stored frozen as suspensions in sucrose solution. To remove all sucrose and supporting electrolyte before use, a thawed sample was subjected to cycles of centrifugation and resuspension in deionized water. The salt-free suspension then was lyophilized over P$_2$O$_5$. The lyophilization procedure did not irreversibly alter the membrane, judged by the spectrum and gel electrophoretic behavior of reconstituted material. This observation is consistent with other studies on dehydrated purple membrane fragments (9). During the dielectric measurements described below, the lyophilized membrane

Abbreviations: $h$, hydration level in g of water per g of protein; $h_c$, low hydration limit of the dielectric measurements; $h_t$, threshold for onset of the dielectric response; $h_f$, full hydration; $\theta$, fraction of surface covered at the percolation threshold; $\tau$, time constant of the dielectric relaxation; $C(f)$, frequency-dependent capacitance of the composite capacitor at fixed hydration level and temperature; $\sigma(h)$, hydration-dependent dc conductivity; $\epsilon$, dc dielectric constant.

\textsuperscript{1}Present address: Boyce-Thompson Institute, Cornell University, Ithaca, NY 14853-1801.
samples were subjected to cycles of isopiestic hydration followed by drying under a stream of H$_2$O$_2$-free air. Similar dielectric data were obtained for successive cycles.

**Dielectric Measurements.** Dielectric measurements were carried out as described for lysozyme powders (5). The membrane sample was maximally hydrated isopiestically versus pure water (H$_2$O or 2H$_2$O) at 5°C. The measurements were performed on a three-layer capacitor composed of the sample, a supporting glass dish above the lower electrode, and a vapor layer of negligible density below the upper electrode. The electrodes were not in contact with the sample. The capacitor was placed on a digital balance within a controlled environment. The hydration level was changed continuously, from maximal isopiestic hydration to the lowest level of hydration that could be reached through evaporation in an atmosphere purged of water vapor at the measurement temperature (28°C). The low hydration limit ($h_0$) was determined to be 0.017 g of water per g of purple membrane, by oven drying under conditions known to drive off all water (10). Data were acquired and reduced by on-line computer analysis of simultaneous measurements of the sample weight and of the capacitance and loss factor detected by a high-sensitivity digital ac bridge over the frequency range 10 kHz to 10 MHz. The experiments were designed to obtain many data at hydration levels near the transition. Sample weights were about 0.3 g and were determined to a precision of 10$^{-5}$ g. Capacitance was determined to a precision of 10$^{-4}$ pF.

**Data Reduction.** Percolation theory (7, 11) predicts the critical exponents of the dc conductivity ($\sigma$). To compare results obtained for the lightly hydrated powders with the theoretical models, values of the dc conductivity ($\sigma(h)$) were calculated from the recorded values of the capacitance as a function of frequency and hydration as follows. Inspection of the frequency dependence of the loss factor shows that it is correct to assume a single relaxation in these samples for the frequency range 10–100 kHz. The electrostatic analysis of the three-layer capacitor (glass (1)-hydrated sample (2)-dry air (3)) for a single Maxwell–Wagner relaxation process is straightforward. By assuming that the glass and the dry air layers display dielectric constants $\varepsilon_1 = 3$ and $\varepsilon_3 = 1$, respectively, and neglecting their dc conductivity, at fixed hydration level the frequency-dependent capacitance $C(f)$ of the composite capacitor can be expressed in terms of the dc dielectric constant $\varepsilon$ and conductivity $\sigma$ of the membrane sample.

$$C(f) = C^*[(1 + \varepsilon^*/\varepsilon)(f^2 \times \tau^2)/(1 + f^2 \times \tau^2)]^{-1},$$

where

$$\tau = \varepsilon_0 \times \varepsilon/\sigma$$

$$\varepsilon^* = (d_2 \times \varepsilon_1 \times \varepsilon_3)/(d_1 \times \varepsilon_3 + \varepsilon_3 \times \varepsilon_1)$$

$$C^* = \varepsilon_0 \times L \times \varepsilon^*/d_2,$$

and where $\varepsilon_0$ is the vacuum permittivity, $d$ is the thickness of a layer, and $L$ is the electrode area. From a set of $C(f)$ data obtained at constant hydration level $h$, the value of $\sigma$ can be derived by a standard least-squares best-fit procedure. The error in estimation of $\sigma$ is 0.1%.

From percolation theory (7), above and near the threshold $p_c$,

$$\sigma(h) \sim (p - p_c)^t,$$

where $p$ is the probability of site occupancy by the conducting species and $t$ is the critical exponent for the dc conductivity. The exponent $t$ depends on the dimensionality of the system. To obtain values of the critical exponent from dc conductivity data, we write for the experimental system

$$\sigma(h) - \sigma(h_c) = K \times (h - h_c)^t,$$

where $K$ is a constant. Values of $h$ are directly proportional to the site occupancy probability. The value of the dc conductivity at the percolation threshold, $\sigma(h_c)$, is subtracted from $\sigma(h)$ to remove the nearly negligible contribution of nonpercolative processes to the total conductivity. The values of the percolation threshold $h_c$ and the critical exponent $t$ were determined by minimizing the variance of the fit to Eq. 3 for conductivity data obtained at hydration levels close to $h_c$.

To compare the conductivities measured for purple membrane hydrated with H$_2$O or 2H$_2$O, the same membrane sample was used in consecutive runs, by repetition of the cycle of maximal isopiestic hydration and drying in the dielectric-gravimetric apparatus. Because the sample was small (0.3 g), slight differences in instrument geometry contributed to the total capacitance. To eliminate this systematic error, the limiting low dry-sample capacitance for all runs was set equal. Five runs were performed. Similar results were obtained for all. The data shown below are for the last pair of runs, with H$_2$O and 2H$_2$O, for which particular attention was paid to the low hydration range near $h_c$.

**RESULTS**

Fig. 1 shows the hydration dependence of the capacitance at several frequencies for purple membrane hydrated with H$_2$O. The data show the explosive increase of the capacitance above a critical hydration level, $h_c \approx 0.0456$, expected for a percolation transition. As was found for lysozyme (6), the hydration level of the transition is frequency independent, as is the capacitance below the transition (see legend of Fig. 1), in contrast to the frequency dependence of the capacitance above the transition.

Fig. 2 shows the loss factor measured contemporaneously with the capacitance data given in Fig. 1. Inspection of the results of Fig. 2 indicates that there is more than one

![Fig. 1. Reduced capacitance (C/C0) of the composite capacitor containing an H2O-hydrated sample of purple membrane as a function of hydration level at the three frequencies indicated. Values of the limiting low hydration capacitance C0 are 2.506 pF at 10 kHz, 2.493 pF at 100 kHz, and 2.481 pF at 1 MHz.](image-url)
relaxation process at high hydration and possibly also more than one process at low hydration. When determining values of the conductivity \( \sigma \), we have therefore restricted analysis of the data to the set of three frequencies, 10, 20, and 40 kHz, which in the range of low hydration \((h < 0.5)\) are within the tail of a single Maxwell–Wagner relaxation.

Reduction of the capacitance data of Fig. 1 according to Eq. 1 yields the dc conductivity displayed in Fig. 3. Fig. 4 gives the fit to Eq. 3 of the dc conductivity data in the neighborhood of \( h_c \) for the \( H_2O \) and \( ^2H_2O \)-hydrated samples.

Table 1 summarizes the values of \( h_c \) and the critical exponent \( t \), obtained in the least-squares fitting shown in Fig. 4 for \( H_2O \) and \( ^2H_2O \) hydration. Neither \( h_c \) nor \( t \) is affected by deuteration, in agreement with expectation for a percolative process (6). Also given in Table 1 is the proportionality constant \( K \) of Eq. 3. The ratio of the constants \( K \) gives the effect of replacement of hydrogen by deuterium on the rate process determining the conductivity. This ratio is 1.38, in close agreement with the square root of the mass ratio \( H/^2H \), expected for a classical kinetic process involving protons.

**DISCUSSION**

Dielectric experiments carried out on lysozyme, similar to these on purple membrane, gave evidence that protonic conduction on the protein surface exhibits a two-dimensional percolative transition (6, 12). The following quantitative arguments led to the above conclusion. (i) The critical fractional coverage for protonic conduction is \( h_c/h_m = 0.40 \), in close agreement with the theoretical prediction for surface percolation, \( \theta_c = 0.45 \) (7). (ii) The critical exponent for the conductivity, \( t = 1.25 \), is also in close agreement with theoretical and experimental studies on surface percolation, for which \( t = 1.1-1.3 \) (7, 11, 13).

The dielectric properties of the purple membrane are qualitatively similar to those reported for lysozyme and are characteristic of percolation: the conductivity shows the deuterium isotope effect expected for a protonic process; it shows explosive growth above a critical hydration level, \( h_c = 0.0456 \); \( h_c \) is frequency-independent; \( h_c \) and the critical exponent \( t \) show no deuteration isotope effect.

However, there are important quantitative differences between the membrane and protein systems. (i) The sorption isotherms for the same purple membrane preparations analyzed in this work show 65% of the water binding capacity of bovine serum albumin or lysozyme (14)—i.e., the point of full coverage for the membrane fragments, analogous to full coverage of a protein, is at \( h_m = 0.25 \). Thus, the critical fractional coverage for protonic conduction in the membrane is

<table>
<thead>
<tr>
<th>Hydration</th>
<th>( h_c )</th>
<th>( t )</th>
<th>( K )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( H_2O )</td>
<td>0.0456</td>
<td>1.231</td>
<td>1951.</td>
</tr>
<tr>
<td>( ^2H_2O )</td>
<td>0.0439</td>
<td>1.232</td>
<td>1418.</td>
</tr>
</tbody>
</table>

**Fig. 2.** The dielectric loss factor tan \( \delta \) of the composite capacitor containing the sample of Fig. 1 as a function of the logarithm of the frequency of measurement, \( f \). The various symbols represent different hydration levels, from top to bottom: 0.2504, 0.1297, 0.0675, 0.0448, 0.0308.

**Fig. 3.** Reduced dc conductivity \( (\sigma/\sigma_0) \) versus hydration level \( h \) of the purple membrane sample of Fig. 1.

**Fig. 4.** Hydration dependence of the conductivity for \( H_2O \) and \( ^2H_2O \) hydration, considered in Table 1, plotted according to Eq. 3. Only data near the percolation transition, with values of \( (h - h_c) < 0.01 \), were used.
is $h_r/h_m = 0.18$. This value is widely different from that for lysozyme and from that found and predicted for two-dimensional percolation. It is in close agreement with the value for three-dimensional percolation, $\theta = 0.15$ (7). (ii) The data of Fig. 4 give the value $t = 1.23$ (Table 1) for the critical exponent describing the growth of the conductivity near the percolation threshold. This number is closely similar to the value found for lysozyme and to the prediction from theory for two-dimensional percolation. It differs substantially from the value expected for three-dimensional percolation ($t = 1.65 - 2.00$) (7, 11, 13). (iii) The loss factor for purple membrane (Fig. 2) differs in its hydration and frequency dependence from the simpler behavior displayed by lysozyme (see figure 1 of ref. 5).

Clearly, the dielectric behavior of the membrane system is not amenable to the straightforward interpretation possible for the protein. This is not surprising, considering the compositional and structural complexity of the membrane.

There is an apparent conflict, in the case of the purple membrane, between the values of the critical fractional coverage (characteristic of three-dimensional percolation) and the critical exponent $t$ (characteristic of two-dimensional percolation). We suggest the following resolution. The long-range proton conduction paths, reflected in the growth of the conductivity at the percolation threshold, may be predominantly within one of the several regions of the membrane: the lipid surface, the lipid–protein interface, or entirely within the protein. If the region containing the principal conduction paths is preferentially hydrated relative to other regions of the membrane, then a two-dimensional percolative process would show the critical fractional coverage expected for a process of higher dimensionality. In this view, we place weight on the critical exponent as a reflection of the character of the percolative process.

There is evidence for preferred paths of proton conduction, described above for purple membrane, also in the case of lysozyme (5). Complexation of lysozyme with substrate reduced the surface proton flux by perhaps half, although the active site comprises only 10% of the protein surface. This suggested that the active site serves as a channel for proton movement.

The dc photoelectric response of the purple membrane has been studied as a function of hydration (15). The signals for light- and dark-adapted samples showed a sharp change at the hydration level of the percolation threshold found for the dielectric measurements. This observation suggests that the percolative behavior of the purple membrane, described above, may be part of the functional process and may reflect the movement of protons across the membrane. We emphasize that, in the absence of additional structural information, this conclusion remains tentative. The dielectric measurements do not give information on the direction of proton movement, specifically, whether it is in the direction of proton transport, orthogonal to the plane of the membrane, or along the surface of the membrane. However, the observation of percolation for a membrane is itself of importance. In our view, it brings forward the need to consider the possible involvement of topological disorder and statistical processes when constructing models of membrane transport.

The fundamental emphasis of the percolation model is on randomness of the arrangements—i.e., of the conducting elements—here presumed to be water molecules distributed about the protein surface or the membrane surface and interior. This picture is particularly important for membranes, for which conduction and transport are principal biological activities. It may not be necessary to have a relatively rigid geometry and a definite pathway for conduction. Conduction can follow from statistical assemblies of elements only partially filling a surface or pore. In principle, to reach the conduction threshold for a three-dimensional pore, only one-sixth of the conduction sites need be filled. Because a percolative process exhibits a true phase transition (it displays cooperativity), conduction might be controlled by subtraction or addition of a few elements, moving the fractional occupancy backward or forward through the transition region. The measurements described here show that long-range proton motions in membranes indeed can occur at partial occupancy and can respond sensitively to change in the occupancy.