Na^+ TRANSPORT VIA Na^+/H^+ ANTIPORT IN *HALOBACTERIUM HALOBIUM* ENVELOPE VESICLES

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1. Introduction

The role of protons in chemiosmotic energy transduction has been widely recognized. The difference in pH and electrical potential between the bulk phases across membranes, generated by substrate oxidation, ATP hydrolysis, or light-energy transducing components, constitutes a form in which energy is conserved. In many microbial systems the extrusion of intracellular Na^+ against its electrochemical gradient is energized by the protonmotive force, and evidence suggests that in *Streptococcus faecalis* [1] and *Escherichia coli* [2,3] this occurs by exchange of Na^+ for H^+ via a proposed antiporter [4]. In *Halobacterium halobium* envelope vesicles protonmotive force can be established by light-activated proton translocation through bacteriorhodopsin (purple membrane), a protein–retinal complex. Studies of Na^+-dependent amino acid transport and light-induced pH effects led to the proposal of a Na^+/H^+ antiporter in this organism as well [5,6]. Light-activated uncoupler-insensitive Na^+ extrusion has been found in vesicles prepared from the *H. halobium* strain R-1 [7,8], as well as from bacteriorhodopsin-deficient strains ([8,9], H. J. W., I. Probst, unpublished). This primary Na^+ translocation has been attributed to a protein–retinal complex with many spectroscopic properties distinguishing it from bacteriorhodopsin [10]. With the discovery of this Na^+ translocating pigment questions arise as to the importance or even existence of the Na^+/H^+ antiporter, since many of the results which gave rise to the concept of the antiporter in *H. halobium* can be explained by the operation of a primary Na^+ translocation mechanism and passive H^+ fluxes.

Investigative tools to distinguish phenomena related to a Na^+ pump from those having to do with bacteriorhodopsin have been provided with the isolation of *H. halobium* mutants which lack either bacteriorhodopsin alone or both the primary Na^+ pump and bacteriorhodopsin (H. J. W. et al., unpublished). In the latter case the purple pigment can be obtained by adding *trans*-retinal to isolated membrane vesicles, yielding a preparation containing functional bacteriorhodopsin but little or no Na^+ translocating pigment. These strains were used in this investigation to re-evaluate the evidence for a Na^+/H^+ antiport mechanism.

2. Materials and methods

2.1. *Strains and culture conditions; vesicle preparation*

Isolation and characterization of the 'colorless' *H. halobium* strains ET-15 and W5002-7 will be described elsewhere, strain R-1 was from W. Stoeckenius. They were grown in a peptone medium and used to prepare membrane envelope vesicles by the sonication technique [11,12]. Vesicle membrane orientation was ascertained by assaying for NADH-menadione oxidoreductase [13], and all preparations showed 85–95% inside-in direction. Vesicles were stored at 5–10 mg protein/ml in 4 M NaCl and kept refrigerated. Protein was determined by the method in [14]. Vesicles used in transport experiments were loaded by osmotic shocking, i.e., dilution into a 3 M salt solution of the composition desired for the vesicle interior, and pelleting by centrifugation. This process was repeated twice and the final concentration was adjusted to 5 mg protein/ml.

The strain W5002-7 lacks spectroscopically detectable levels of either bacteriorhodopsin or Na^+ pump pigment, and shows no light-dependent H^+ uptake or

extrusion. Bacteriorhodopsin could be assembled in this strain by addition of 1 nmol *trans*-retinal/mg protein to W5002-7 vesicles in 4 M NaCl. The treated vesicles were protected from light and stored in the refrigerator overnight before osmotic loading.

2.2. *Transport assay*

Uptake of glutamate and triphenylphosphonium ion ($TPMP^+$) was determined by the Millipore filter method in [11–13]. Transport was assayed at 31°C. Actinic illumination was provided by a GE ELH quartz-halogen lamp through a Corning 3-68 cutoff filter, a heat reflecting mirror and 7.5 cm circulating water. Light intensity in the sample chamber was $\sim 8 \times 10^5$ ergs . cm^{-2} . s^{-1}, as measured with a Kettering Model 68 radiant energy meter. Assay mixtures contained 10 mM Hepes in 3 M NaCl, adjusted to pH 7.0, 2 μCi/ml of [^{3}H]glutamate or 5 μCi/ml of [^{3}H]$TPMP^+$, and 50 μg vesicle protein/ml. When the energy source was diphenylmethylene diamine (DPD), this agent was added to 1.2 mM.

Transmembrane electrical potential was determined from the following equation:

$$\Delta\psi = -\frac{RT}{F} \ln C_{out}/C_{in} \quad (1)$$

where R, T and F have their usual meaning, and C_{out} and C_{in} are the external and internal concentrations of $TPMP^+$. The concentration gradient of the accumulated [^{3}H]$TPMP^+$ during illumination was calculated from the specific intravesicle and extravesicle radioactivities and the internal volume of the vesicles, i.e., 3 μl/mg protein [11,15].

2.3. *pH traces*

Continuous pH measurements were performed in a magnetically stirred chamber, thermostatted at 30°C, with a glass electrod (Beckman Instr. no. 39003) and a Corning Model 12 pH meter [16]. About 3 ml of 1 mg vesicle protein/ml in 4 M NaCl was used for each trace. The electrode and cable were wrapped in aluminium foil to within 1 in. of the electrode tip to reduce noise. The signal was recorded at 0.5 in./min with full scale deflection set to 0.1 pH units. Actinic illumination was provided as described for the transport assay. Light intensities were $\sim 2 \times 10^6$ ergs . cm^{-2} . s^{-1}.

2.4. *Difference spectroscopy*

Difference spectra were determined in a Cary 14 spectrophotometer equipped with a scattered transmission accessory and connected to a Nicolet LAB 80 computer as in [10]. Bacteriorhodopsin concentration was determined in an Aminco SP-2A spectrophotometer connected to a Nicolet 1080 computer. In these determinations spectra from dark-adapted vesicles, prepared by incubation at 37°C for 2 h in the dark, were subtracted from spectra of vesicles light-adapted by illumination with yellow light. The resulting difference peak at 590 nm and its known extinction coefficient [17] were used to calculate bacteriorhodopsin contents.

3. Results and discussion

3.1. *Assembly of bacteriorhodopsin in W5002-7 membranes*

H. halobium strain W5002-7 does not respond to illumination and lacks spectroscopically detectable levels of pigments absorbing above 500 nm (H. J. W. et al., unpublished), indicating that this strain has neither bacteriorhodopsin nor Na^+ pump in appreciable quantities. Apparently, this mutant synthesizes bacterio-opsin but not retinal, since bacteriorhodopsin can be assembled in isolated membranes by adding *trans*-retinal (fig.1). The decrease of the retinal A_{385} peak, the increase in A_{568}, and the existence of an isosbestic point at 443 nm are consistent with the incorporation and binding of retinal by bacterio-opsin [18,19]. Pigment formation proceeded until ~1.1 nmol bacteriorhodopsin/mg vesicle protein was prod-

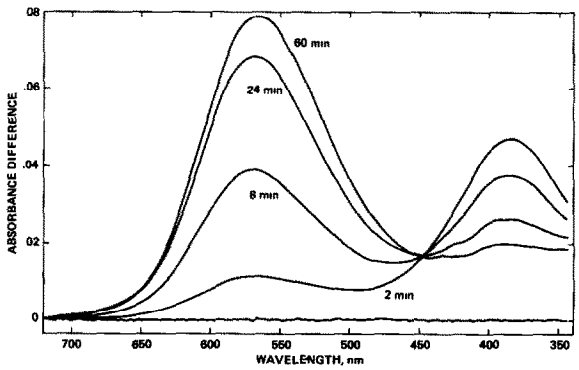

Fig.1. Retinal reconstitution of W5002-7 vesicles in 4 M NaCl at pH 6.0. Protein was 2 mg/ml. Difference spectra measured between sample with added *trans*-retinal (1.5 nM final conc., at $t = 0$) and sample with added methanol (6 μl/3 ml). Times indicated are after retinal addition.

uced, as expected since 1 nmol retinal/mg protein was added. Formation of the Na^+ pump pigment cannot be detected by this method, since this pigment is present in smaller quantities [10] and in spite of its red-shifted absorption maximum at 588 nm, its spectral contribution would be obscured by the larger amount of bacteriorhodopsin.

3.2. *pH responses to illumination*

To ascertain whether the Na^+ pump is also formed in W5002-7 membranes with retinal, pH changes during illumination in the presence of the H^+ conductor, *p*-trifluoromethoxycarbonylcyanide phenylhydrazone (FCCP), were determined (fig.2). The pH changes in the medium are interpreted as reflecting intravesicle pH changes of the opposite sign, as shown for R-1 vesicles [7,20]. Addition of 70 μM FCCP caused a reversal in pH response to illumination in R-1 vesicles, from H^+ extrusion to H^+ uptake, but only decreased the magnitude of H^+ extrusion in retinal-treated W5002-7 (W5002-7 + ret) vesicles. The H^+ uptake in R-1 vesicles in the presence of FCCP has been shown to occur as a result of primary Na^+ extrusion, which creates a membrane potential (inside negative) and induces passive H^+ entry until the pH gradient is balanced by the electrical potential [7,8]. Adding FCCP to 270 μM did not cause such H^+ uptake in W5002-7 + ret vesicles, but H^+ extrusion was progressively inhibited, as expected for active H^+ transport. The accumulation of $TPMP^+$ indicated that R-1 vesicles developed a membrane potential of −147 mV, while W5002-7 + ret vesicles produced −117 mV. The reversal of the light-induced pH change in the R-1 vesicles in the presence of FCCP, and the total absence of this effect in W5002-7 + ret vesicles indicates that R-1 vesicles show primary Na^+ transport but this activity is virtually absent in the W5002-7 + ret vesicles. This conclusion is strengthened by the fact that the R-1 vesicles contained ~4-times more bacteriorhodopsin than the W5002-7 + ret vesicles in these experiments, which would favor H^+ extrusion in R-1 vesicles, an obscuring effect to observing H^+ uptake. The inability of the W5002-7 vesicles to regain Na^+ pump activity implies that in this strain the absence of retinal during growth prevents the synthesis of the apoprotein for this pump. Experiments to be reported elsewhere (H. J. W., I. Probst) have indicated that if retinal is added to this strain during growth, Na^+ pump activity is regained.

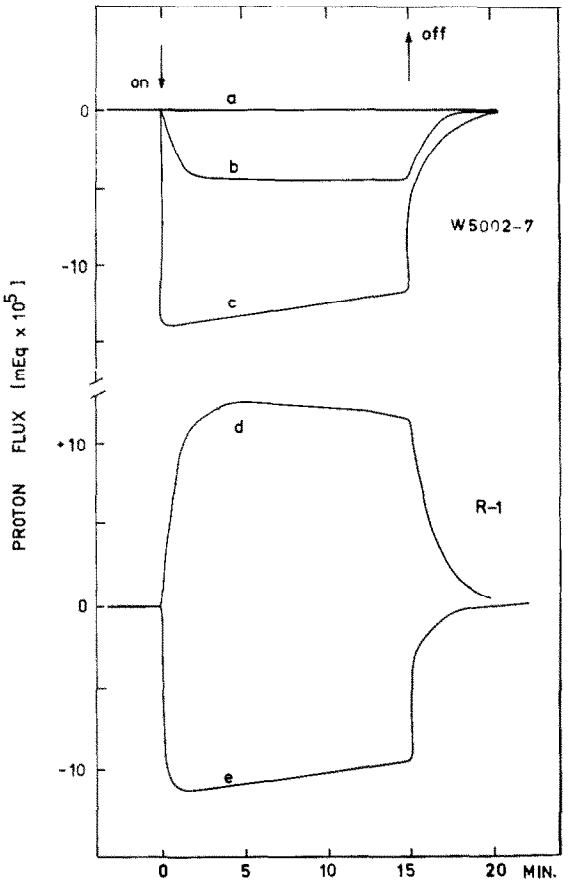

Fig.2. pH traces in the presence and absence of FCCP. W5002-7 curve: (a) W5002-7 without retinal; (b) W5002-7 + ret and 66.7 M FCCP in ethanol, added ~5–10 min prior to illumination; (c) W5002-7 + ret, no FCCP added. R_1 traces: (d) 66.7 M FCCP; (e) no FCCP added. Light on and off as indicated. All traces with 1.0 mg protein/ml in 4 M NaCl. Total volume in pH chamber, ~3.0 ml.

3.3. *Na^+ transport in R-1 and W5002-7 + ret vesicles*

In the presence of several molar NaCl the determination of intravesicle Na^+ is difficult and subject to many errors. Furthermore, extensive binding of Na^+ inside the vesicles, proposed [21], would make a considerable difference in Na^+ activity at low internal concentrations. For these reasons Na^+ extrusion was determined indirectly, by following the transport of [^{3}H]glutamate. The transport of this amino acid can be energized both by illumination and oxidation of artificial electron donors. In either case, transport proceeds after lag periods proportional to the initial intravesicle Na^+ content [5,22,23], as it has been established that glutamate transport is energized only

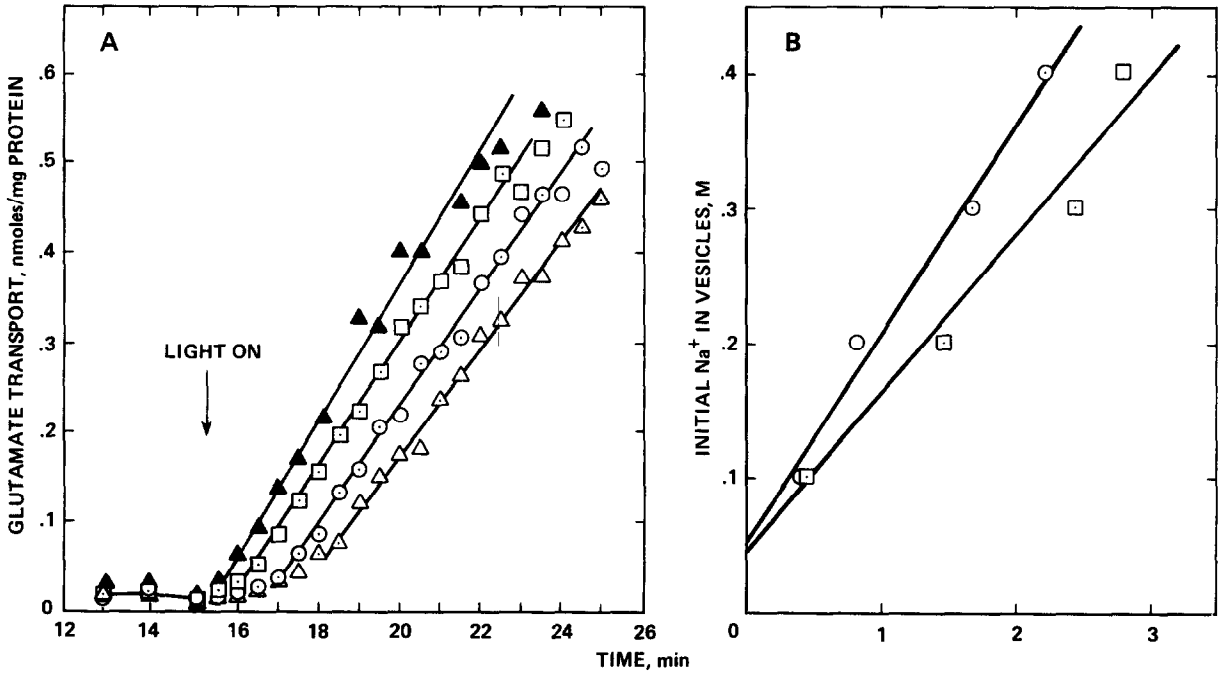

Fig.3. Light-dependent glutamate transport. Vesicles, loaded with NaCl and KCl, were suspended in 3 M NaCl containing [^{3}H]-glutamate and 10 mM Hepes (pH 7). (A) Internal [Na^+] (with KCl, so that total [salt] was 3 M): (▲) 0.1 M; (□) 0.2 M; (○) 0.3 M; and (△) 0.4 M. (B) A plot of lag time, obtained by extrapolating to baseline, as a function of the initial internal [Na^+] (○) R-1, (□) W5002-7 + ret.

by a concentration gradient of Na^+ and does not respond to electrical potential difference [23]. The observation is repeated in fig.3, which shows that the lag times for glutamate uptake are strictly proportional to loading NaCl at 0.1–0.4 M. Hence, measurement of glutamate transport is an excellent method to investigate Na^+ transport in *H. halobium* vesicles.

Table 1

Strain	$\Delta Na_i/\Delta t$, M . min^{-1}	
	hν	DPD, 1.2 mM
R_1	0.16	0.08
ET-15	n.d.	0.07
W5002-7	n.d.	0.08
W5002-7 + ret	0.12	0.07

n.d., not detectable; h*ν*, illumination as described in section 2; DPD for diphenylmethylene diamine

$\Delta Na_i/\Delta t$ was calculated from graphs of internal Na^+ versus lag times of glutamate accumulation. Glutamate transport assays were started at the onset of illumination or at the time of DPD addition. The stock solution of DPD was prepared in 3 M NaCl. No corrections were made for the small amount of KCl introduced with the vesicle suspension

Since observable glutamate transport responds to high concentration gradients of Na^+ [23] where the internal Na^+ content is very low, a plot of initial [Na^+] vs. lag time (fig.3B) should correspond directly to the time-course of Na^+ extrusion. The slope of the line therefore, gives the rate of Na^+ transport, and the intercept on the ordinate, the amount of Na^+ bound inside the vesicles. The latter is 0.05 M or below and does not greatly influence the calculations. Rates of glutamate transport and lag times generally correspond, and although the latter is preferred, rates can be used also for determining Na^+ transport.

A plot of initial Na^+ content as a function of lag time is shown in fig.3B for W5002-7 + ret as well as for R-1 vesicles. Non-retinal-treated W5002-7 vesicles did not transport glutamate when illuminated (not shown). The results in fig.3B and table 1 demonstrate that both vesicle preparations transport Na^+, and at roughly comparable rates. Addition of 5 μM FCCP inhibited glutamate transport by 94% in R-1 and 100% in W5002-7 + ret, and 10 μM FCCP inhibited transport in these strains by 98% and 100%, respectively. Assuming that the W5002-7 + ret vesicles

lack the Na^+ pump, the results are consistent with the suggestion in [5,15,23] that it is the protonmotive force generated by bacteriorhodopsin which drives Na^+ transport, via Na^+/H^+ antiport. The antiport mechanism must be by far the major Na^+ translocating pathway in these membranes.

3.4. *Na^+ transport in ET-15 vesicles*

H. halobium strain ET-15 lacks spectroscopically detectable quantities of red carotenoids and bacteriorhodopsin (H. J. W. et al. unpublished). After bleaching isolated membranes from this strain addition of retinal caused the appearance of an A_{588} band while absorbance due to free retinal decreased [10]. The 588 nm band corresponded to action spectra for light-mediated but passive H^+ uptake as measured in this strain (H. J. W., I. Probst, unpublished), and was related to the transport activity. Vesicles from ET-15 did not transport detectable amounts of glutamate during illumination, although a membrane potential of −50 mV developed in NaCl-loaded vesicles and uncoupler-sensitive glutamate uptake could be measured in the dark when reducing substrate (DPD) was present (see table 1). Indeed, light-dependent Na^+ extrusion in bacteriorhodopsin-deficient vesicles is much slower than in vesicles from the R-1 strain [8].

4. Summary

Using *H. halobium* cell envelope vesicles containing either bacteriorhodopsin plus Na^+ pump, bacteriorhodopsin alone, Na^+ pump alone, or no light-responsive pigment altogether, it could be shown that the large majority of light energized Na^+ extrusion in these mutants is linked to bacteriorhodopsin and to protonmotive force, and therefore must be facilitated by a Na^+/H^+ antiporter. Thus, the recently discovered primary Na^+ pump makes only a minor contribution to light-mediated Na^+ flux. The activity of the Na^+/H^+ antiporter appears to be independent of the presence of any photoreactive pigments, since an artificial electron donor will drive rapid Na^+ extrusion in all of the vesicle preparations tested.

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