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Effect of Temperature on the Function of a Proton Pump

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Summary. Vesicles with proton translocating activity were reconstituted with bacterial rhodopsin lipoprotein and synthetic phospholipids. Reconstitution with dimyristoyl phosphatidylcholine yielded vesicles which catalyzed light-driven proton uptake which was sensitive to nigericin, gramicidin or an uncoupler when tested at temperatures above 20 °C. At temperatures below 5 °C, the extent of proton uptake was actually greater than at 20 °C, but there was little effect of either nigericin or uncouplers even when these compounds had been added at 20 °C. Gramicidin inhibited at all assay temperatures provided it was added at 20 °C. With dipalmitoyl phosphatidylcholine similar results were obtained except that nigericin lost its effectiveness at higher temperatures. On illumination tetraphenylboron was taken up by the reconstituted vesicles. We propose that an electrogenic proton translocation takes place by a channel mechanism.

There are two basic mechanisms that have been implicated in ion transport – a mobile carrier and a transmembranous channel or pore. While the former requires fluidity of the membrane for mobility, the latter does not. The marked temperature-dependence of the action of valinomycin on lipid membranes suggests that this ionophore functions as a carrier, while the response of gramicidin conforms to the pore model (Mueller & Rudin, 1969; Krasne, Eisenman & Szabo, 1971). Studies of this type are difficult to conduct with natural membranes that remain relatively fluid even at 0 °C. Reconstituted systems of active transport offer broader possibilities for studies of the role of membrane fluidity, provided well-defined phospholipids with suitable phase transition temperatures can be used. In this communication we describe active proton transport vesicles of bacterial rhodopsin reconstituted with synthetic saturated phospholipids and their response to temperature variations.

Materials and Methods

Dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine and dilauryl phosphatidylethanolamine were obtained from Calbiochem; gramicidin and valinomycin from Sigma. Nigericin was a gift from Dr. R. J. Hosley of Eli Lilly. Purple membrane

preparations from *Halobacterium halobium* were generously supplied by Dr. W. Stoeckenius. A crude mixture of phospholipids from *Halobacterium cutirubrum* was a gift from Dr. J. K. Lanyi, Ames Research Center, California. Reconstitution of vesicles was performed by the sonication procedure (Racker, 1973) and measurement of proton uptake as described previously (Racker & Stoeckenius, 1974) except that an Esterline Angus recorder (Speed Servo) with adjustable chart speeds was used.

Results and Discussion

Effect of Temperature on Extent of Light-Driven Proton Uptake and Sensitivity to Ionophores

As can be seen from Fig. 1, the extent of proton uptake by vesicles reconstituted with dimyristoyl phosphatidylcholine (upper curve) is optimal between 5 and 15 °C. With crude soybean phospholipids the extent of proton uptake was also somewhat greater at lower temperatures, but variabilities in the optimal temperature were encountered with different sonication times. With crude phospholipids from *H. cutirubrum* the extent of proton uptake increased slightly at higher temperatures. The extent of proton uptake was far greater than the amount of rhodopsin used (5 to 12 ng atom H^+/nmole of rhodopsin). This indicates that rhodopsin acts as a catalyst rather than stoichiometrically.

When nigericin was added to the assay medium at 36 °C there was virtually no proton uptake detectable with either phospholipid vesicles. Nigericin catalyzes an exchange of K^+ for H^+ across the membrane and thus prevents the formation of a pH gradient. Other proton conductors had a similar effect. With dimyristoyl phosphatidylcholine vesicles, the light-driven proton uptake reappeared on lowering the temperature of the assay medium (Fig. 2). Between 12 and 14 °C the extent of proton uptake was about 50% of the control; at 1 °C it reached the value of the control without nigericin. The proton pump reconstituted with dipalmitoyl phosphatidylcholine responded similarly except that the curve was shifted to the right; i.e., nigericin had little effect at 18 °C but was strongly inhibitory at 40 °C. Multilayered liposomes made from dipalmitoyl phosphatidylcholine or dimyristoyl phosphatidylcholine have major transition temperatures at 42 and 24 °C, respectively (Hinz & Sturtevant, 1972). Sonication apparently broadens the phase transition (Papahadjopoulos, Jacobson, Nir & Isac, 1973) which may account for the rather broad temperature dependence of proton conduction by nigericin observed. Gramicidin inhibited proton uptake at all temperatures tested (Fig. 2) if it was added at the higher temperatures. When gramicidin was added to the assay medium at 1 °C there was little inhibition.

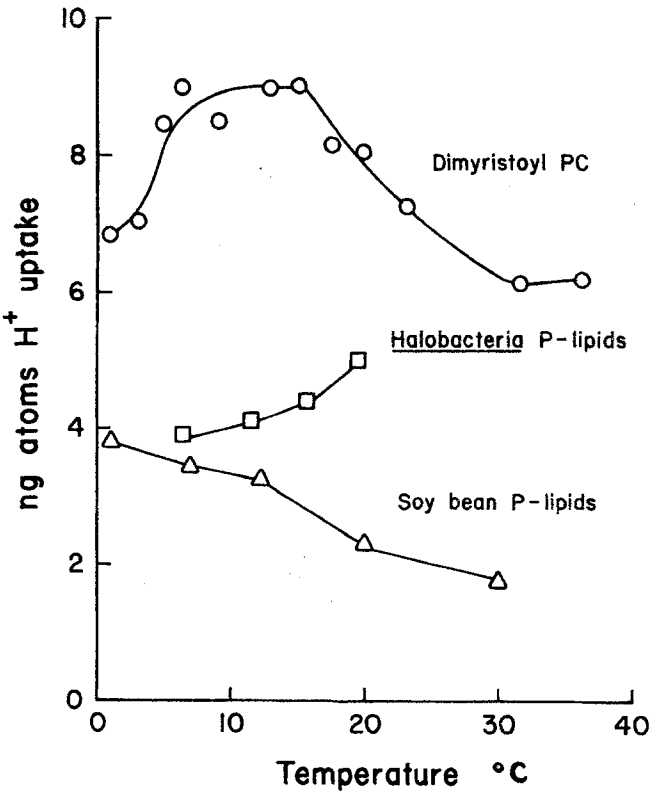

Fig. 1. Effect of temperature on proton uptake in reconstituted vesicles. Vesicles were reconstituted with 5 mg of dimyristoyl phosphatidylcholine or of phospholipids from *Halobacteria* suspended in 0.2 ml of 0.15 M KCl containing 100 μg of bacterial rhodopsin. Sonic oscillation was performed for 8 min at 34 °C as described previously (Racker, 1973). Samples containing 25 μg of rhodopsin were assayed in a final volume of 1 ml of 0.15 M KCl. Vesicles were reconstituted with soybean phospholipids by the same procedure except that 5 μmoles of phospholipids were used and sonication was performed at room temperature. The sample for assay contained 10 μg of rhodopsin. The temperature was controlled by a water jacketed cell (Racker & Stoeckenius, 1974) and was monitored by a thermistor (YSI Model 42SC) immersed in the assay mixture. PC = phosphatidylcholine

Kinetics of Proton Uptake

The extent of proton uptake is a function of the rate of proton uptake and of proton leakage from the vesicles. Somewhat variable results were encountered with valinomycin, which sometimes increased and sometimes decreased the extent of proton uptake, but invariably accelerated both the initial rate of uptake in the light and the decay in the dark (Table 1). In the presence of valinomycin the initial rate of proton uptake was accelerated

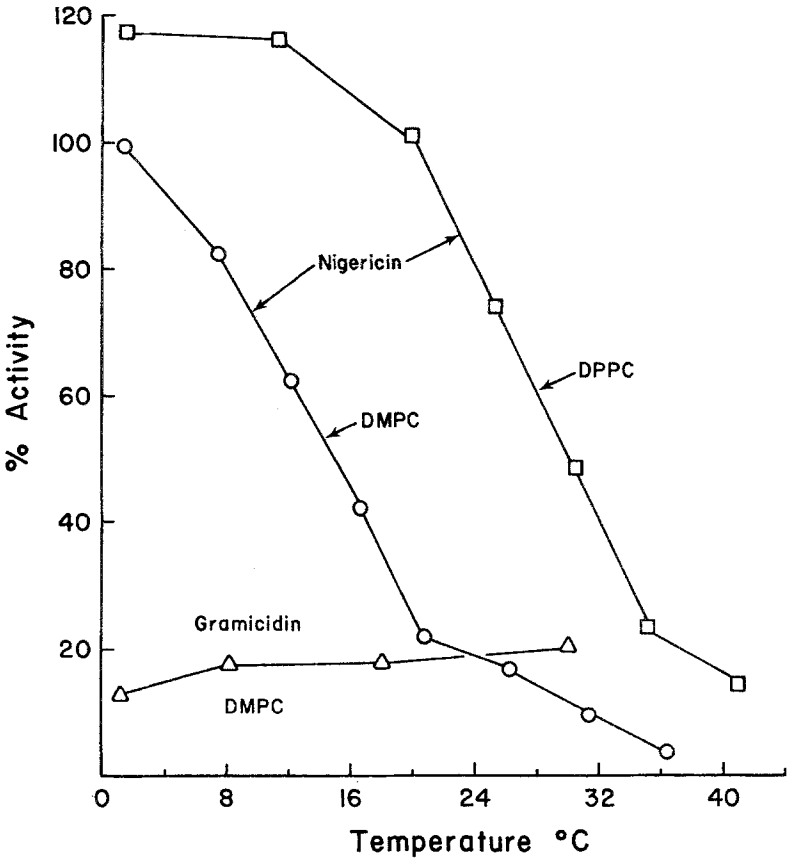

Fig. 2. Effect of temperature on the inhibition of proton translocation in reconstituted vesicles by nigericin and gramicidin. Reconstitution of vesicles with dimyristoyl phosphatidylcholine (DMPC) was performed as described in the legend of Fig. 1. Vesicles were reconstituted with 5 mg of dipalmitoyl phosphatidylcholine (DPPC) by the same procedure except that the lipids were sonicated prior to addition of rhodopsin at 44 °C for 12 min and for an additional 4 min at 41 °C in the presence of rhodopsin. Nigericin (0.2 μg) or gramicidin (2 μg) were added at the highest temperatures indicated in the figure. The % values were calculated with reference to the values obtained at the same temperature without nigericin

Table 1. Effect of valinomycin on initial rate of proton uptake in the light and dark and on proton efflux in the dark

Additions	Proton uptake		Proton efflux
	Initial rate (ng ions H^+/min)	Extent (ng ions H^+)	t 1/2 (sec)
Vesicles	12.4	2.7	35
Vesicles +0.2 μg valinomycin	20.2	2.4	12.6

Vesicles were reconstituted with 5 μmoles of soybean phospholipids suspended in 0.2 ml of 0.15 M KCl containing 100 μg of bacterial rhodopsin. Sonic oscillation was performed for 8 min at room temperature as described previously (Racker, 1973). A sample containing 10 μg of rhodopsin was assayed in a final volume of 1 ml of 0.15 M KCl.

but the extent was actually somewhat lower. This was readily explained by the marked difference in t 1/2 of the decay which was 35 sec without and 12.6 sec with valinomycin. The increase in the initial rate of proton uptake in the presence of valinomycin shows that the rate of the pump is partly limited by the permeability of co-ions. This problem could not be entirely eliminated because co-ion permeability was also temperature dependent, but was minimized by using 150 mM KCl.

A study of the initial rate of proton uptake and of the t 1/2 of decay of the pH gradient in reconstituted dimyristoyl phosphatidylcholine vesicles revealed why there was relatively little effect of temperature on the extent of proton uptake. As shown in Table 2 (Experiment 1) the initial rate of proton uptake at 5 °C was about 68% of that at 15 °C yet the extent was actually higher. This is readily explained by the decay time which rose from 8.6 sec at 15 °C to 23 sec at 5 °C. In the presence of nigericin both the initial rate as well as the decay time were much lower than the control, accounting for the pronounced fall in the extent of proton uptake at higher temperatures. At lower temperatures both the initial rate and extent of proton uptake in the presence of nigericin approached the values in the absence of the ionophore. Even more striking results were obtained with vesicles reconstituted with a mixture of dimyristoyl phosphatidylcholine and dilauryl phosphatidylethanolamine. The values at 10 °C were very similar with or without nigericin while at 5 °C the rate in the presence of nigericin exceeded those in its absence (Table 2, Experiment 2). Somewhat higher values were also observed at lower temperatures in the presence of nigericin with dipalmitoyl vesicles (Fig. 2). This paradoxical response may be caused by the increased uptake of K^+ prior to cooling of the sample.

Since the preparations of bacterial rhodopsin contained about 75% protein and 25% of phospholipids (Oesterhelt & Stoeckenius, 1971) the possibility was considered that the endogenous lipids may endow the protein with some mobility in an otherwise frozen membrane. However, with vesicles reconstituted with rhodopsin and phospholipids from *Halobacteria* (Fig. 1) the extent of proton uptake in the presence of nigericin (1 µg) was less than 10% that of the control at 20 °C and 39% that of the control at 10 °C. The initial rates of uptake were 25 ng ions H^+ and 14 ng ions H^+ per min per 25 µg rhodopsin at 20 and 10 °C, respectively. The corresponding values in the presence of nigericin were 3.2 ng ions H^+ at 20 °C and 7.2 ng ions at 10 °C. These data, though not as striking as those obtained with synthetic phospholipid vesicles, suggest that the phospholipids of the *Halobacteria* cannot endow the rhodopsin with mobility in a frozen bilayer of dimyristoyl phosphatidylcholine.

Table 2. Effect of temperature on kinetics of proton pump

Additions	Temperature (°C)	Proton uptake		Proton efflux
		Initial rate (ng ions H^+/min/25 μg)	Extent (ng ions H^+)	t 1/2 (sec)
Exp. 1				
Dimyristoyl	5	12.3	3.5	23
phosphatidylcholine	10	15.1	3.7	17
vesicles	15	17.9	2.8	8.6
	20	15.6	2.1	7.9
	25	13.4	1.6	6.3
Dimyristoyl	5	9.5	2.9 (83%)	17.4
phosphatidylcholine	10	11.7	2.4 (65%)	9.5
vesicles +0.1 μg nigericin	15	12.9	1.6 (57%)	5.5
	20	7.8	0.6 (28%)	3.1
	25	7.3	0.4 (25%)	2.9
Exp. 2				
Dimyristoyl	5	7.8	1.8	17.6
phosphatidylcholine +	10	10.6	2.3	16.8
Dilauryl	15	14.5	2.9	16.0
phosphatidylethanolamine	20	18.0	3.4	16.0
vesicles	25	17.0	2.8	11.0
	30	15.6	2.6	10.1
Dimyristoyl	5	9.5	2.6 (144%)	26
phosphatidylcholine +	10	10.5	2.3 (100%)	12.6
Dilauryl	15	13.1	1.72 (54%)	5.0
phosphatidylethanolamine	20	11.4	0.98 (29%)	4.2
vesicles +0.2 μg nigericin	25	5.5	0.57 (20%)	4.2
	30	5.2	0.33 (13%)	3.4

Experiment 1 was performed with 50 μliters of vesicles reconstituted as described (Racker, 1973) with 5 mg of dimyristoyl phosphatidylcholine suspended in 0.2 ml 0.15 M KCl containing 100 μg of bacterial rhodopsin membrane. The suspension was sonicated for 12 min at 34 °C. In experiment 2, 3 mg of dimyristoyl phosphatidylcholine and 2 mg of dilauryl phosphatidylethanolamine suspended in 0.2 ml of 0.15 M KCl were first sonicated without rhodopsin for 15 min at 38 °C then for an additional 6 min in the presence of 100 μg of rhodopsin. The % values given in parentheses were calculated with reference to the values obtained at the same temperature without nigericin.

We therefore propose that bacterial rhodopsin acts as a proton pump via a transmembranous channel that remains operative under conditions that do not permit the operation of a mobile carrier. That we are dealing with an electrogenic translocation of protons across the membrane is supported by the fact that rhodopsin acts as a catalyst (*see* Fig. 1, and Racker &

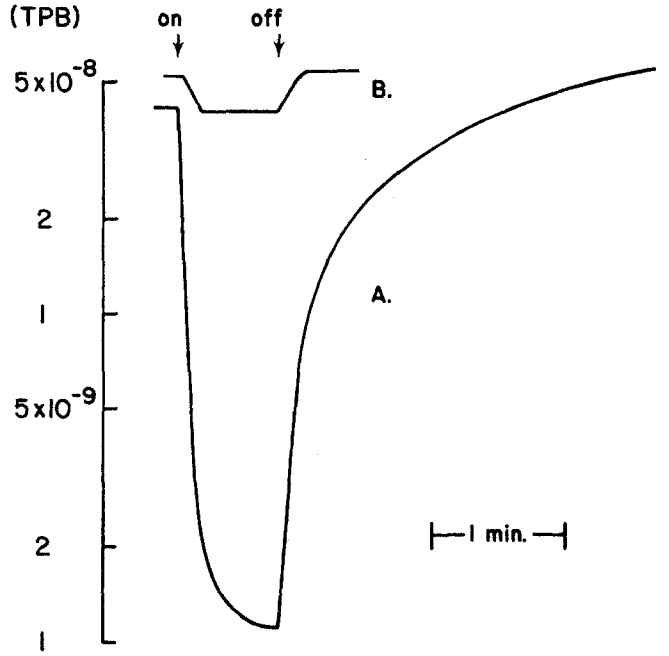

Fig. 3. Effect of light on uptake of tetraphenylboron by reconstituted proton pump. Reconstitution of vesicles with soybean phospholipids was performed as described in the legend of Table 1 except that 0.1 M Na_2SO_4 was used for the suspension of the phospholipids. A sample containing 10 μg of rhodopsin was placed in 3 ml of 50 mM Na_2SO_4, pH 6.0 in the inner compartment of an assembly (Grinius *et al.*, 1970) using a tetraphenylboron-sensitive phospholipid-electrode. The light source was the same as for proton uptake measurements. Gramicidin (2 μg) was used as inhibitor of proton translocation (Curve *B*). TPB = tetraphenylboron

Stoeckenius, 1974) and that the formation of a membrane potential could be demonstrated (Fig. 3).

To obtain further insight into the mode of action of this proton pump, the formation of a membrane potential was tested by the assay procedure of Grinius, Jasaitis, Kadziauskas, Liberman, Skulachev, Topali, Tsofina and Vladimirova (1970) with tetraphenylboron as penetrating anion. As shown in Fig. 3 tetraphenylboron uptake by the reconstituted vesicles was induced by light. In the dark, equilibration of tetraphenylboron to the original level was observed. The response to light was abolished in the presence of gramicidin. Although it is not possible at present to make a quantitative estimate of the membrane potential, these observations are consistent with the operation of an electrogenic pump.

Two challenging problems emerge from these studies. Why is the orientation of the proton pump in the reconstituted vesicles asymmetric and

opposite to that in intact bacteria (Oesterhelt & Stoeckenius, 1973) which translocate protons from the inside to the outside? How are protons translocated from one side of the membrane to the other? The answer to the first question may come from further studies of the organization of the purple membrane within the bacterial membrane. Alternatively, the conditions of reconstitution may be responsible for the vectorial assembly in one direction and variations in the method may yield vesicles which are right-side in. An answer to the second question will be more difficult to obtain. A most intriguing possibility is proton channelling through the polypeptide chain via the amino groups or via bound water on the protein surface.

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