*Biochimica et Biophysica Acta*, 514 (1978) 178—184


**BBA Report**

BBA 71363

# LIGHT-INDUCED pH CHANGES INSIDE BACTERIORHODOPSIN VESICLES AS MEASURED BY $^{31}P$ NMR

M.C. BLOK [a], K.J. HELLINGWERF [a], R. KAPTEIN [b] and B. DE KRUIJFF [c]

[a] *Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam,* [b] *Department of Physical Chemistry, University of Groningen, Nijenborgh 16, 9747 AG Groningen and* [c] *Institute of Molecular Biology, State University of Utrecht, Transitorium III, Padualaan 8, Utrecht (The Netherlands)*

(Received September 4th, 1978)

## Summary

$^{31}P$ NMR has been used to measure light-induced pH changes inside bacteriorhodopsin vesicles containing entrapped sodium glucose-6-phosphate. Reversible light-induced pH changes were observed at various pH values. The results indicate that our vesicle preparations were not homogeneous with respect to the generation of pH gradients.

It is generally accepted that bacteriorhodopsin, a chromoprotein present in the plasma membrane of *Halobacterium halobium* [1], functions as a light-driven, electrogenic proton pump [2—5]. Several methods have been described to incorporate bacteriorhodopsin into lipid vesicles in such a way that net proton movement is observed upon illumination [2,6—8]. In most studies the number of protons taken up from the extravesicular medium has been used as a measure of the activity of bacteriorhodopsin. Only in a few studies have the generation of a light-dependent pH gradient [5,9,10] and a membrane potential [3,5,9,10] been investigated. Either fluorescence techniques [5] or the accumulation of probe molecules [3,9,10] were used. To calculate the respective components of the proton motive force, however, certain assumptions have been made concerning the homogeneity of the vesicle preparations.

Recently, $^{31}P$ NMR has been shown to be a useful technique for measuring pH inside cells and organelles [11—13]. This method employs the change in chemical shift of the $^{31}P$ resonance of phosphate groups with their degree of ionization. We have used $^{31}P$ NMR to investigate the light-dependent generation of pH gradients in bacteriorhodopsin vesicles. $^{31}P$ NMR has the advantage that it is a non-perturbing technique. Moreover, one can obtain information about the heterogeneity of vesicle preparations with respect to the generation

of pH gradients. Glucose-6-phosphate was used, since its $^{31}P$ resonance is well separated from the resonance of the phospholipids. In addition its outward leak from vesicles could be measured continuously by enzymatic coupling to $NADP^+$ reduction [14].

Bacteriorhodopsin, in the form of purple membranes, was isolated [15] and incorporated into egg phosphatidylcholine vesicles as described previously [16], except for the following: sonication was carried out at approx. 18°C, and the medium contained 500 mM sodium glucose-6-phosphate, 5 mM Na-EDTA, pH 7.3 (EDTA binds paramagnetic cations, contaminating the glucose-6-phosphate). The final concentration of egg lecithin was 50 mM. The extravesicular medium was manipulated by a column-centrifugation method [17], except that we used disposable 5 ml syringes (Plastipak, Becton-Dickenson and Co., Rutherford, N.J.), filled with pre-swollen Sephadex G-50 coarse (Pharmacia). 0.20—0.25 ml vesicle suspension was applied to each syringe. 50—70% of the effluent glucose-6-phosphate was found to be enclosed in the vesicles. For NMR experiments 0.80 ml of vesicle suspension was centrifuged through these columns. The effluent was pipetted into an NMR tube, and $D_2O$ (approximately 15%) added. The final phospholipid concentration in the NMR tube was approximately 30—40 mM.

Egg phosphatidylcholine (Type V-E) was obtained from Sigma (St. Louis, Mo., USA). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were from Boehringer (Mannheim, F.R.G.).

$^{31}P$ NMR measurements were performed at 22°C at 145.7 MHz on a Bruker HX-360 spectrometer, operating in the Fourier-transform mode and equipped with quadrature detection and broad-band proton decoupling (2 W). Flat-bottomed 10-mm tubes were used (inner diameter 9 mm). During the experiments the NMR tube was spinning at a rate of 30 cycles/s providing efficient mixing of the vesicle solution, thus ensuring homogeneous illumination of the sample. Typically, 300—400 scans were recorded, using 90° resonance frequency pulses, a 2 s interpulse time and a spectral width of 10 kHz. Triphenyl phosphine in chloroform was used as an external reference. A calibration curve was made by measuring the chemical shift position of glucose-6-phosphate as a function of pH.

The experimental arrangement for light irradiation in the NMR probe has been described elsewhere [18]. A Spectra Physics model 171 argon laser with power mainly at 488 and 512 nm was used. The diameter of the light beam was 8 mm. The beam was carefully centered onto the NMR tube. Illumination caused an increase in the temperature of the sample of about 4°C.

Fig. 1 shows $^{31}P$ resonances of glucose-6-phosphate, partially entrapped and partially outside the bacteriorhodopsin vesicles, under various conditions. In a dark control, taken before illumination, only one glucose-6-phosphate peak is observed (upper spectrum), indicating an average pH of 7.11. The middle spectrum shows the steady-state situation during illumination with light of saturating intensity. Two peaks are observed, one indicating a pH of 7.07, almost the same as in the dark control, the other indicating a pH of 7.64. This latter peak originates from external glucose-6-phosphate, since its intensity increased upon addition of extra glucose-6-phosphate. A spectrum taken in the dark, 3 h after illumination was terminated (lower trace in Fig. 1),

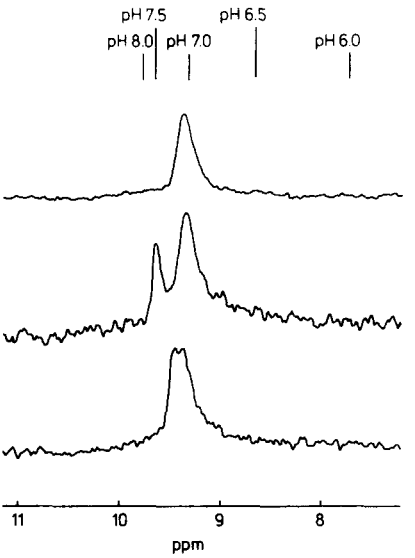


Fig. 1. $^{31}$P-NMR spectra of bacteriorhodopsin vesicles (protein/egg phosphatidylcholine ratio, 1/20, w/w) in 500 mM $Na_2SO_4$, 10 mM Na-EDTA, pH 7.0. Experimental details are given in the text. Upper spectrum, before illumination; middle spectrum, during steady-state illumination with saturating light (320 mW); lower spectrum, in the dark, 3 h after finishing illumination. Chemical shifts are expressed in ppm downfield from the peak of external triphenylphosphine at 22°C.

shows that most of the light-induced pH gradient had decayed in this time. From this it can be inferred that it is a reversible phenomenon. No pH changes could be detected upon illumination if the outside medium contained 700 mM KCl, 25 mM Tris-acetate, 10 mM Na-EDTA, pH 7.0 (data not shown). It is likely that also under this condition bacteriorhodopsin is actively pumping but that no pH gradients are formed due to a rapid diffusion of acetic acid and hydrochloric acid.

From the experiment of Fig. 1 it seems that only small pH gradients were generated by the vesicles. Careful inspection of the spectra, however, revealed the following. In experiments with bacteriorhodopsin vesicles the total intensity of the glucose-6-phosphate peaks at 9.4—9.7 ppm relative to that of egg phosphatidylcholine decreased upon illumination. This is not the case with pure egg phosphatidylcholine vesicles (see also Table I). This indicates that part of the glucose-6-phosphate resonances is shifted to another position. In spectra of illuminated samples, but not in 'dark' spectra, the integral of the $^{31}$P-NMR spectrum increased significantly between the glucose-6-phosphate peaks and the phospholipid peak, indicating a very broad peak in the chemical shift region around 7 ppm. This shows that upon illumination part of the glucose-6-phosphate that is enclosed in the vesicles is in a more acidic environment than in the dark. That this peak is very broad and can only be observed in the integral most likely is due to the very strong pH dependence of the chemical shift of the glucose-6-phosphate resonance in this region such that very small variations in pH already cause a strong resonance broadening.

From a determination of the buffer capacity of the outside medium in the experiment of Fig. 1 and a titration of a 500 mM sodium glucose-6-

TABLE I

THE EFFECT OF BACTERIORHODOPSIN TO LIPID RATIO ON THE PROPERTIES OF BACTERIORHODOPSIN VESICLES

| Parameter | Bacteriorhodopsin/egg lecithin ratio (w/w) | | | | |
|---|---|---|---|---|---|
| | 0/1 | 1/50 | 1/20 | 1/10 | 1/5 |
| pH of glucose-6-phosphate-* containing compartment: | | | | | |
| Dark: inside + outside | 7.14 | 7.14 | 7.11 | 7.11 | 7.03 |
| Light: inside | 7.14 | 7.10 | 7.07 | 7.14 | 7.00 |
| outside | 7.14 | 7.57 | 7.64 | 7.45 | 7.00 |
| Intensity of the narrow glucose-6-phosphate peaks relative to egg lecithin peak: | | | | | |
| Dark | 0.207 | 0.298 | 0.316 | 0.519 | — |
| Light | 0.218 | 0.275 | 0.215 | 0.427 | — |
| $\Delta\nu$ of phospholipid peak (Hz)** | 56 | 86 | 93 | 108 | 133 |
| Calculated vesicle diameter (Å)** | 250 | 288 | 296 | 312 | 334 |
| Internal volume ($\mu$l/$\mu$mol lecithin)*** | 0.40 | 0.47 | 0.53 | 0.71 | 0.49 |
| Leakage of glucose-6-phosphate*** (% per 3 h) | 0 | 10 | 13 | 20 | 31 |

*Illumination (light power 600 mW) and calculation of the pH is described in the text.

**Vesicle diameter was calculated from $\Delta\nu$, the full peak width at half height of 145.7 MHz $^{31}$P NMR spectra, using the formula $\Delta\nu = ca^3 + d$ (see ref. 19), where $a$ is the vesicle radius and $c$ and $d$ are constants. Constant $d$ was neglected in our calculations. A value for $c$ at 145.7 MHz of $c = 28.67 \cdot 10^{-6}$ $s^{-1} \cdot Å^{-3}$ was obtained from the $\Delta\nu$ value for pure egg phosphatidylcholine vesicles and an assumed vesicle diameter of 250 Å for these vesicles (ref. 20).

***The leakage of glucose-6-phosphate was measured continuously as described in ref. 14, using vesicles that were treated according to the column centrifugation method. During the first 5 min of the enzyme assay no discrimination could be made between leakage and contamination of the outside medium with glucose-6-phosphate, due to incomplete removal during separation. The total amount of glucose-6-phosphate was determined in a duplicate assay where the vesicles had been lysed with Triton X-100. The internal volume was calculated from the amount of glucose-6-phosphate after incubation with enzyme and $NADP^+$ for 5 min.

phosphate solution it was estimated that an increase in the outside pH with 0.5 units should be accompanied by a decrease of the internal pH from 7.1 to 6.6—6.5. In this calculation it is assumed that the vesicle suspension is homogeneous and that glucose-6-phosphate is the only buffering substance in this pH region. Further, it was calculated that in this experiment 500—750 nmol $H^+$/mg bacteriorhodopsin are translocated upon illumination. Considering this number, it is very unlikely that the apparent absence of a change of the inside pH is due to the buffer capacity of bacteriorhodopsin. From the above considerations we conclude that part of the vesicle population does not generate a $\Delta$pH and that another part pumps to a range of more acidic pH values inside the vesicles.

In another experiment the effect of the bacteriorhodopsin content of the vesicles on the light-dependent generation of pH gradients was studied. The results are shown in Table I, where the influence of the protein/lipid ratio on various properties of the vesicles is summarized. Illumination of pure lipid vesicles did not cause a pH gradient across the vesicle bilayer. The change in outside pH upon illumination was highest at moderate bacteriorhodopsin to phospholipid ratios and decreased at higher ratios (Table I). The most likely explanation is an increased proton permeability of the bilayer, concomitantly with the increase in glucose-6-phosphate permeability discussed below. Table I shows that the average size of the vesicles, calculated from the width of the phospholipid resonance peak, increases with increasing bacteriorhodopsin content. In agreement with this, the internal volume determined from the

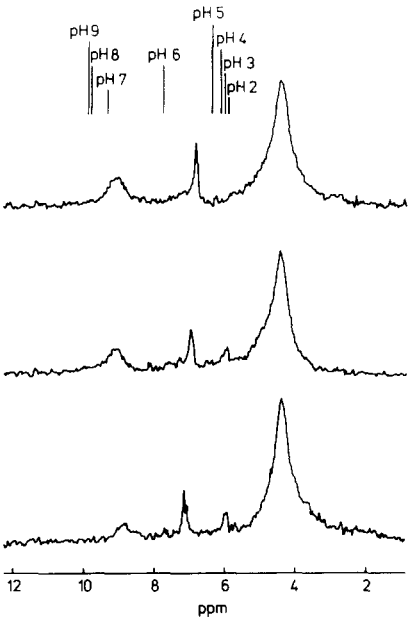


Fig. 2. $^{31}$P NMR spectra of bacteriorhodopsin vesicles without and with illumination at an outside pH of 5.5. An identical vesicle sample as in Fig. 1 was used, but the outside pH was brought to 5.5 with HCl. Upper spectrum, before illumination; middle spectrum, approximately 10 min after starting illumination (power 320 mW); lower spectrum, after illumination for more than 1 h. Chemical shifts are expressed in ppm downfield from the peak of external triphenylphosphine at 22° C.

amount of entrapped glucose-6-phosphate was found to be increased by increasing bacteriorhodopsin content. Above a bacteriorhodopsin/egg lecithin ratio of 0.10 the internal volume seemed to decrease. This apparent decrease is due, however, to a large increase in the rate of leakage of glucose-6-phosphate with increasing protein content, which is also shown in Table I. In none of the cases in Table I did the 9.4 ppm peak originating from intravesicular glucose-6-phosphate move to a position indicating a more acidic pH upon illumination. On the other hand, the spectra of bacteriorhodopsin vesicles (e.g. the relative decrease of the intensity of the narrow glucose-6-phosphate peaks; Table I) indicate the presence of a fraction of vesicles which generates a lower but relatively heterogeneous pH inside upon illumination.

This finding was further substantiated in the experiment shown in Fig. 2, in which the same vesicle preparation was used as in Fig. 1, but the outside pH was brought to 5.5 with HCl. The large peak at 4.40 ppm originates from the phospholipids. In the upper spectrum, taken in the dark 1.5 h after adjusting the pH, the narrow peak at 6.79 ppm is from glucose-6-phosphate in the outside medium and in vesicles that are relatively permeable to protons. The peak at 9.00 ppm has been assigned to the $^{31}$P resonance of glucose-6-phosphate in vesicles which are poorly permeable to protons, since its chemical shift agrees with an average pH of 6.76, which is close to the pH at which the vesicles were originally prepared. This peak moved very slowly with time to positions indicating a lower pH, due to a light-independent proton movement as a result of the applied pH gradient. A small but significant peak at 5.93 ppm appeared

upon illumination of the sample (Fig. 2, middle spectrum), which did not change in intensity nor chemical shift during prolonged illumination (lower spectrum). Incubation of the vesicles in the dark following illumination resulted in the disappearance of this peak. We conclude that this peak is from glucose-6-phosphate present in part of the vesicles which have an internal pH of 2.6 ± 0.4, indicating that these vesicles can generate a pH gradient of about 3 units. Furthermore, this experiment unambiguously shows the heterogeneity of the preparation with respect to the formation of a light-induced pH gradient since both vesicles with an internal pH of 2.6 and 6.7 coexist during illumination. A similar result was obtained in an experiment with bacteriorhodopsin vesicles, which were prepared in 500 mM sodium glucose-6-phosphate, 5 mM EDTA, pH 7.3, but resonicated after adjustment of the pH of the extravesicular suspension to 5.5 with HCl. After removal of the extravesicular glucose-6-phosphate the $^{31}$P NMR spectrum showed one glucose-6-phosphate resonance with a chemical shift corresponding to pH 5.5, demonstrating that the chemical shift of the enclosed glucose-6-phosphate is pH sensitive similar as for glucose-6-phosphate free in solution. Illumination of the sample causes the appearance of a new resonance with a chemical shift corresponding to a pH of 2.4. Since the intensity of this new resonance was only 20—25% of the initial intensity this demonstrates that only in a fraction of the bacteriorhodopsin vesicles a large pH gradient is formed upon illumination.

We conclude that $^{31}$P NMR can be used to study in a direct way light-induced pH changes inside bacteriorhodopsin vesicles. The vesicles were found to be heterogeneous with respect to the capacity to generate pH gradients. On account of the intensity of the $^{31}$P resonance of glucose-6-phosphate approximately 75% of the vesicles is inactive and 25% is actively pumping. One of the aspects that has to be studied in more detail is the question of whether this heterogeneity has to be ascribed to the high concentration of glucose-6-phosphate, which had to be used to ensure that sufficient was trapped inside the small vesicles.

The present study was supported in part by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.). The $^{31}$P NMR measurements were carried out at the SON-NMR facility in Groningen. We wish to thank Dr. K. van Dam and Dr. R.P. Casey for their continuous interest and their critical reading of the manuscript.

## References

1 Henderson, R. (1977) Annu. Rev. Biophys. Bioeng. 6, 87—109
2 Racker, E. (1973) Biochem. Biophys. Res. Commun. 55, 224—230
3 Kayushin, L.P. and Skulachev, V.P. (1974) FEBS Lett. 39, 39—42
4 Drachev, L.A., Jasaitis, A.A., Kaulen, A.D., Kondrashin, A.A., Liberman, E.A., Nemecek, I.B., Ostroumov, S.A., Semenov, A.Yu. and Skulachev, V.P. (1974) Nature 249, 321—324
5 Kagawa, Y., Ohno, K., Yoshida, M., Takeuchi, Y. and Sone, N. (1977) Fed. Proc. 36, 1815—1818
6 Racker, E. and Stoeckenius, W. (1974) J. Biol. Chem. 249, 662—663
7 Happe, M., Teather, R.M., Overath, P., Knobling, A. and Oesterhelt, D. (1977) Biochim. Biophys. Acta 465, 415—420
8 Hwang, S.-B. and Stoeckenius, W. (1977) J. Membrane Biol. 33, 325—350
9 Hellingwerf, K.J., Arents, J.C., Scholte, B.J. and Westerhoff, H.V. (1978) Biochim. Biophys. Acta, submitted

10 Hellingwerf, K.J., Tegelaers, F.P.W., Westerhoff, H.V., Arents, J.C. and van Dam, K. (1978) in Halophilism (S.R. Caplan, ed.), Elsevier/North-Holland, Amsterdam, in press
11 Moon, R.B. and Richards, J.H. (1973) J. Biol. Chem. 248, 7276—7278
12 Salhany, J.M., Yamane, T., Shulman, R.G. and Ogawa, S. (1975) Proc. Natl. Acad. Sci. U.S. 72, 4966—4970
13 Casey, R.P., Njus, D., Radda, G.K. and Sehr, P.A. (1977) Biochemistry 16, 972—976
14 Bergmeyer, H.-U. (1970) Methoden der Enzymatischen Analyse, pp. 1200—1204, Verlag Chemie, Weinheim
15 Oesterhelt, D. and Stoeckenius, W. (1974) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. XXXI, pp. 667—678, Academic Press, New York
16 Blok, M.C., Hellingwerf, K.J. and van Dam, K. (1977) FEBS Lett. 76, 45—50
17 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891—2899
18 Kaptein, R., Dijkstra, K., Müller, F., van Schagen, C.G. and Visser, A.J.G.W. (1978) J. Magn. Res., in press
19 De Kruijff, B., Cullis, P.R. and Radda, G.K. (1976) Biochim. Biophys. Acta 436, 729—740
20 Hauser, H., Oldani, D. and Phillips, M.C. (1973) Biochemistry 12, 4507—4517