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INTRACELLULAR pH OF HALOBACTERIA CAN BE DETERMINED BY THE FLUORESCENT DYE 2', 7'-BIS(CARBOXYETHYL)-5(6)-CARBOXYFLUORESCEIN

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Determination of the internal pH of halobacterial cells grown in 4M salt solution has proven to be a difficult problem. We now report the steady state cytosolic pH of Halobacterium halobium S-9 to be 7.2. Intracellular pH was determined after the cells were loaded with the membrane permeable precursor of the pH sensitive dye 2', 7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein-acetyxymethyl ester - (BCECF/AM). In order to minimize light-scattering in the measurement of the fluorescence, a thin cuvette was newly devised. This method should be suitable for studies of the cytosolic pH in other bacteria.

The measuring of cytosolic pH is important in Halobacteria in order to understand the various ion transport and bioenergetic systems (bacteriorhodopsin, electron transport, (Na^+/H^+ exchange) which affect the proton gradients and electrochemical potentials. Halobacteria have an obligatory requirement for high salt media. So far most information considering bioenergetics of halobacteria comes from studies on vesicles derived from bacterial cytoplasmic membrane [1,2], in which pH gradients were also studied [3]. In order to carry out such experiments in intact cells, it was important to develop a method which would allow for following the changes of cytoplasmic pH under conditions of high salinity. We

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have utilized the pH sensitive dye BCECF which has been used for monitoring of cytoplasmic pH in various cells, including cyanobacteria [4,5].

The dye contains two carboxyl groups which are capped by acetoxymethyl groups to yield an uncharged, membrane-permeable molecule, which apparently can enter intact cells of H. halobium S-9 in media containing 4 M NaCl. The permeable precursor is then cleaved by cell esterases and in effect the charged, pH-sensitive dye remains trapped inside [6].

In this investigation, we demonstrate that this method is a reliable indicator of internal pH in intact cells of H. halobium. We expect that this method should also allow for studying of the dynamic changes of cytosolic pH in response to changes in metabolism and environmental conditions.

MATERIALS AND METHODS

The cells of H. halobium were grown as described in [7]. pH_o of the solutions was determined with a Corning 130 pH meter using the Orion combination electrode 9115. Centrifugation was performed either on a Beckman or Sorval preparative centrifuge. Fluorescence Spectra were recorded on a Perkin-Elmer Spectrometer (MPF-44A). Thin cuvettes for the fluorescence measurements were fabricated as follows: A microscope cover glass was cut to a dimension of 11x22 mm. Then two glasses were located at each end of the cover slips between parafilm spacers of 0.6 mm thickness so as to form a sample compartment. The rims were sealed with a piece of parafilm that was extended-out. The volume of the cuvette thus formed accomodated about 100 ul. For detecting fluorescence spectra, the thin cuvette was placed so as to equally divide at an angle (45^{o}) the direction of the exciting light and the direction of the detector.

BCECF/AM was purchased from Molecular Probes Inc. (Or., USA) The 10mM stock solution of the dye in DMSO was kept at -20°C. Porcine liver esterase, monensin, nigericin, and HEPES were obtained from the Sigma Chemical Company.

Dye-Loading and pH Titration

A 0.6 ml of the suspension of H. halobium S-9 in 4M NaCl (pellet/suspension=2/3 v/v) was resuspended 10 ml of 4M NaCl containing 25 mM HEPES at the pH indicated. To this cell suspension 10 ul of a 10mM BCECF/AM in DMSO was added. After incubating the cells with the dye for four days in the dark at room temperature, the suspension was centrifuged at 5,000 rpm for 5 min at 10°C, then the pellet was resuspended and washed twice with 2 ml of 4M NaCl/25 mM HEPES solution at pH 7.70. After washing, the pellet was resuspended in 1 ml of 4M NaCl/25 mM HEPES solution at pH 7.70.

For measurements of the intracellular fluorescence intensity 100 ul of the the cell suspension was injected into the thin cuvette with a microsyringe. Spectra were recorded at two excitation wavelengths, 490 and 450 nm by scanning fluorescence from 510 to 580 nm and the ratio of the fluorescence intensities at 530 nm (490nm/450nm) was calculated. After the scanning, the suspension was withdrawn from the cuvette with a syringe and then merged with the stock suspension to conserve the sample.

Measurement of pH_i using monensin and NaCl

The stock suspension was mixed with 2.5 ul of 10 mM monensin in DMSO to give 25 uM final concentration of monensin. After incubating the suspension for 10 min, the pH of the suspension and the ratio of fluorescence intensity were measured as described above. Then the suspension was titrated by adding small aliquots of 1M HCl in 4M NaCl/25 mM HEPES solution to give

three or four points at different pH_o for which the fluorescence intensity ratios were measured in order to obtain points for a calibration curve.

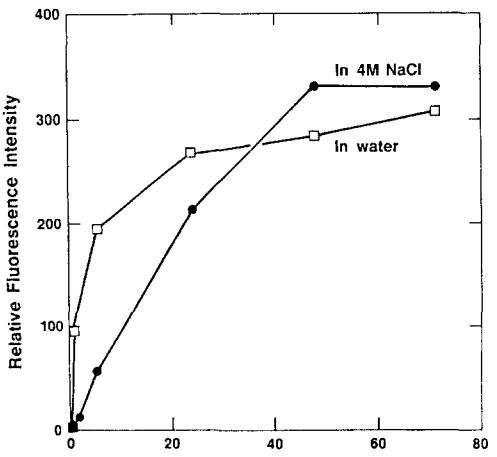
Measurement of pH_i using nigericin and KCl

In this case 2ul of a 10mM nigericin solution instead of monensin was added into the loaded cells to give a final concentration of nigericin = 20 uM. The cells were previously washed with 4M KCl/25 mM HEPES at pH 7.70, resuspended in 1 ml KCl/HEPES, and then the suspension was titrated with aliquots of 1M HCl in 3M KCl/25 mM HEPES to obtain the points for the pH calibration.

RESULTS

In initial experiments we tested the effect of 4M NaCl on the BCECF/AM fluorescence intensity changes in the presence of commercially available esterase. As shown in figure 1, the fluorescence intensity increases with time in the presence of esterase. Dye cleavage is more rapid in ddH_2O than in 4M NaCl. In the presence of 4M NaCl, the condition needed for studies with Halobacteria, it takes almost 50 hours for complete cleavage of the dye to occur: the reaction is clearly slower in the presence of the high concentration of NaCl but still proceeds.

Intact cells of H. halobium were loaded with the dye in a time period up to 150 hours. Fluorescence intensity during this time period progressively increases. When the supernatant from the intact cells suspensions was separated from the cells by centrifugation, it was seen that the supernatent contained fluorescent dye. This could be due to the presence of the extracellular esterases or because of the active release of the cleaved dye. However, when the cells are free of extracellular dye after washing and then broken by osmotic lysis or by sonication (both methods give the same results), relatively high



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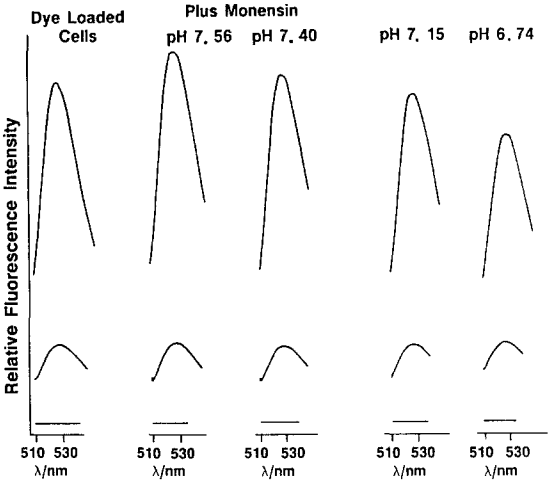

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Figure 1: Time course of cleavage of BCECF/AM to fluorescent BCECF by porcin liver esterase in ddH_2O and in 4M NaCl.

Figure 2: Calibration of intracellular pH of H. halobium S-9 cells with monensin - NaCl.

fluorescence is seen in supernatant, indicating that the dye is intracellularly trapped. After completion of the loading, which is confirmed by the stabilization of the intracellular fluorescence signal, pH_i is shown to be constantly maintained for periods of time up to one week.

Typical results of pH_i calibration with monensin - NaCl are shown in figure 2 where a ratio of fluorescence intensities for two exitation wavelengths 490nm/450nm are compared. Figure 3 shows a linear pH_o - dependent intracellular dye florescence over a wide range of external pH when the loaded cells, washed and resuspended in 4M KCl/25mM HEPES, are titrated with 1M HCl in 3M KCl/25mM HEPES in the presence of 20 uM nigericin.

When the initial external pH of the cell suspension was 6.3, after one week the intercellular pH was found to be about 7.0. In another series of experiments when the external pH was set at 7.70, the intracellular pH was found to be 7.15 +/- 0.05 after nine days.

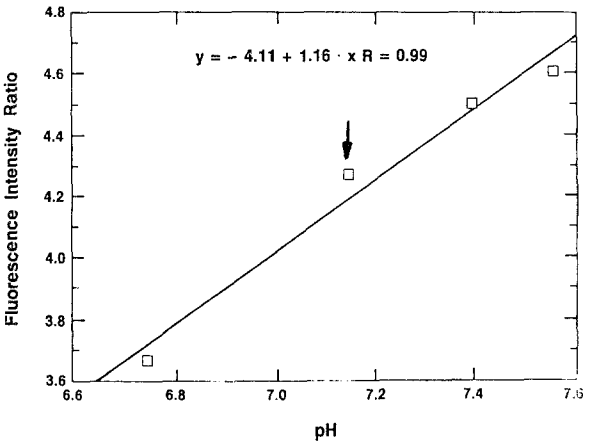

Figure 3: Ratio of fluorescence intensities in intact cells suspensions of H. Halobium S-9, in the prescence of nigericin - 4M KCl, replotted from the trace obtained as in Fig. 2. Florescence intensity ration of the steady state cell sample (without nigericin) is indicated by the arrow.

DISCUSSION

Despite the long loading time, after BCECF/AM is taken up and processed by H. halobium, it becomes a useful intracellular pH indicator for at least a week after the loading is completed. Apparently, H. halobium S-9 contains esterases that are able to cleave the BCECF precursor in the conditions of high intracellular KCl and NaCl concentration into its membrame impermeable form, and this esterase activity can be located both intra- and/or extra-cellularly. BCECF was found to be a reliable pH indicator in either 4M NaCl or 4M KCl. The intracellular pH of H. halobium loaded with the dye is stable and is maintained at about a pH of 7.20 when cells are kept in the dark at room temperature for one week or longer.

However, in other experiments our attempts to attach H. halobium cells to a poly-L-lysine coated glass surface have failed. Thus, the method is still unsuitable for examining individual cell responses to changes in pH_o or other factors in conditions of a perfusion chamber. However, additional methods are being explored, such as the use of different glass surfaces

to find conditions that will allow for attaching Halobacteria to glass surfaces. This will enable us to observe in a fluorescence microscope the effects of exchanging solutions that would be used to alter ion transport, metabolic and environmental conditions and to explore the individual cellular responses to pH change. Such an approach has been found to be successful for the fresh water Cyanobacterium Synechococcus 6311 which was tested under low salt conditions (Huflejt et al.).

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