Biochimica et Biophysica Acta, 389 (1975) 477–482

BBA 76945

INTRACELLULAR pH OF *THERMOPLASMA ACIDOPHILA*

J. C. HSUNG and A. HAUG

MSU/AEC Plant Research Laboratory, Michigan State University, East Lansing, Mich. 48824 (U.S.A.)

(Received October 31st, 1974)

SUMMARY

Thermoplasma acidophila, a mycoplasma-like organism, was grown at 56 °C and pH 2. The intracellular pH of this organism is close to neutral as measured by the distribution of a radioactive weak organic acid, 5,5-dimethyl-2,4-oxazolidinedione, across the plasma membrane. The cell can maintain the pH gradient when subjected to heat or to metabolic inhibitors. Our experiments seem to indicate that a major portion of the pH gradient is not maintained by active processes, but rather by a Donnan potential across the cell membrane.

INTRODUCTION

Thermoplasma acidophila is a mycoplasma-like organism which grows optimally at 59 °C and pH 2 [1]. We are interested in the problem of how these cells can live in such a hostile environment without the protection of the cell wall. Since the organism grows under acidic conditions, the question of the intracellular pH becomes significant. If the intracellular pH value should lie in the acidic range, how does the metabolic machinery function? On the other hand, if the intracellular pH lies in the neutral region, how can the cell maintain such a huge gradient across the membrane?

The intracellular pH was measured by the distribution of a radioactive weak organic acid ^{14}C-labeled 5,5-dimethyl-2,4-oxazolidinedione (DMO). This method was first developed for determining the intracellular pH of muscle cells [2], and was also applied to the study of pH changes in mitochondria [3] and bacteria [4].

MATERIAL AND METHODS

Thermoplasma acidophila was grown in a medium [1] containing 0.02 % $(NH_4)_2SO_4$, 0.05 % $MgSO_4$, 0.025 % $CaCl_2 \cdot 2H_2O$, 0.3 % KH_2PO_4, 1 % glucose, and 0.1 % yeast extract (Difco). The pH was adjusted to 2 with concentrated H_2SO_4 and the medium then autoclaved [5]. Cells were harvested at late log phase, after 22 h of growth, by centrifugation at room temperature, at $9000 \times g$ for 5 min [5]. Then the cells were washed once and resuspended in 0.02 M KCl, 0.05 M sucrose, and 0.01 M glycine buffer at pH 2. This buffer had the same osmolarity and ionic strength as the

Abbreviation: DMO, 5,5-dimethyl-2,4-oxazolidinedione.

growth medium. An 18-l culture usually yielded 35–40 ml of a cellular suspension containing 20–30 mg/ml protein. In aliquots of 1 ml, that cellular suspension was distributed into a set of centrifugation tubes (size: 3.5 ml), followed by addition of 1 ml of buffer.

In a typical run 10 tubes were used to determine gravimetrically the total pellet water, V_t. Another 12 tubes were incubated with [^{14}C]dextran (molecular weight 16 000) to measure extracellular pellet water, V_e. An additional 12 tubes were incubated with 0.1 μCi of [^{14}C]DMO to monitor the intracellular pH. Within experimental accuracy the intracellular pH did not depend upon the length of incubation which was varied from 2 min to 2 h. After incubation for 1 h at 56 °C or at room temperature, all 34 tubes were chilled to 4 °C, then centrifuged simultaneously at $9000 \times g$ for 10 min. Then the supernatant was transferred to test tubes, each pellet was resuspended in 2 ml of buffer in a separate test tube. Then 1 ml from each test tube was dried separately in a scintillation counting vial. After drying, 0.075 ml water and 0.5 ml NCS tissue solubilizer (Amersham/Searle Corp.) was added, followed by addition of 10 ml of PPO (3 g/l) dimethyl-POPOP (0.1 g/l) toluene solution, and counted in a Packard Tricarb scintillation instrument.

The principle to employ the distribution of a weak organic acid as a monitor for intracellular pH is based on the assumption that the non-ionized form of the acid permeates passively across the cell membrane and attains equilibrium during incubation, while the ionized form of the weak acid remains practically impermeant to the cellular membrane [2–4].

Because DMO has a pK value of 6.1 at 56 °C [3], almost all DMO molecules are in the non-ionized form at pH 2 or 4. If the molecule permeates to the interior of the cell, and if the intracellular pH is higher than the pK value, some of the molecules will dissociate from the non-ionized form into the ionized form. The ratio of the two forms at equilibrium can be described by the Henderson-Hasselbach equation [2, 3]. Moreover, if DMO passively enters the cell, the intracellular concentration of the non-ionized form will be the same as that of the extracellular molecular form. The net result will be an accumulation of DMO in the cell, provided the intracellular pH is higher than the pK value.

The following relationships can be used to calculate the intracellular pH value, if the extracellular pH $\ll$ pK:

Intracellular water volume $V_i = V_t - V_e$, where $V_e = C_p(\text{dextran})/C_s(\text{dextran})$ (1)

Here, C_p are the counts of [^{14}C]dextran per pellet, and C_s are the counts of [^{14}C]-dextran per unit volume of supernatant.

Intracellular concentration of ionized form:

$$C_i = [C_p(\text{DMO}) - C_s(\text{DMO}) \times V_t]/V_i \quad (2)$$

Intracellular concentration of non-ionized form $C_n = C_s(\text{DMO})$ (3)

Intracellular pH = pK + log (C_i/C_n) (4)

1 h sonication of cells was carried out with a Raytheon Sonic Oscillator, model DF-101, 0.92 A. Direct pH measurements of the sonicated material was performed with a Beckman micro blood assembly, No. 46 850, attached to an Orion digital pH meter, model 801.

TABLE I

DETERMINATION OF THE INTRACELLULAR pH IN *THERMOPLASMA ACIDOPHILA* CELLS BY MEASURING THE DISTRIBUTION OF RADIOACTIVE DMO ACROSS THE PLASMA MEMBRANE

C_s are the counts of 1 ml supernatant; C_p are the counts of 1 ml of resuspended pellet material. V_t is the total pellet water measured gravimetrically; V_e is the extracellular pellet water; $V_i = V_t - V_e$ is the dextran-impermeable space. The intracellular pH was calculated from the ratio of the concentration of the intracellular ionized form to that of the non-ionized form of DMO (Eqns 2 and 3).

	Expt 1*	Expt 2*	Control*	Cells boiled for 5 h**	Control*	Cells with 0.1 mM 2,4-dinitrophenol**
C_s ([^{14}C]dextran) (cpm $\times 10^4$)	5.77±0.12	12.46±0.37	9.07±0.61	8.26±0.16	5.81±0.06	5.73±0.08
C_p ([^{14}C]dextran) (cpm $\times 10^3$)	2.70±0.06	7.74±0.40	4.67±0.19	3.79±0.19	2.66±0.08	2.65±0.05
V_t (ml)	0.132±0.003	0.133±0.004	0.122±0.001	0.1030±0.0005	0.118±0.001	0.118±0.001
V_e (ml)	0.103±0.004	0.117±0.005	0.103±0.004	0.0906±0.002	0.0915±0.003	0.0924±0.002
V_i (ml)	0.028±0.007	0.015±0.009	0.0186±0.005	0.0123±0.0025	0.026±0.004	0.025±0.003
C_s ([^{14}C]DMO) (cpm $\times 10^4$)	3.33±0.07	20.96±0.53	5.08±0.13	4.59±0.76	13.90±0.81	14.78±0.40
C_p ([^{14}C]DMO) (cpm $\times 10^3$)	4.48±0.01	18.60±0.41	36.6±0.75	27.41±0.67	13.31±0.13	13.00±0.09
Ionized form (cpm $\times 10^3$)	4.47±0.01	9.27±0.23	19.9±1.8	12.44±0.57	12.93±0.87	11.07±0.57
Nonionized form (cpm $\times 10^3$)	0.94±0.05	3.21±0.11	9.42±0.24	5.67±0.09	3.58±0.21	3.73±0.10
Intracellular pH	6.84±0.04	6.56±0.02	6.41±0.06	6.46±0.05	6.66±0.06	6.57±0.04

	Control*	Cells with 10 mM iodoacetate**	Control*	Cells with 10 mM NaN_3**
C_s ([^{14}C]dextran) (cpm $\times 10^4$)	6.61±0.16	6.64±0.16	5.01±0.09	4.84±0.18
C_p ([^{14}C]dextran) (cpm $\times 10^3$)	4.06±0.19	4.08±0.20	3.51±0.16	3.46±0.17
V_t (ml)	0.1373±0.0016	0.137±0.003	0.156±0.004	0.156±0.004
V_e (ml)	0.124±0.056	0.123±0.006	0.140±0.007	0.142±0.006
V_i (ml)	0.0136±0.057	0.0145±0.009	0.0161±0.011	0.015±0.01
C_s ([^{14}C]DMO) (cpm $\times 10^4$)	8.24±0.35	8.24±0.33	9.71±0.35	8.75±0.55
C_p ([^{14}C]DMO) (cpm $\times 10^3$)	6.57±0.06	6.40±0.18	9.24±0.15	8.69±0.29
Ionized form (cpm $\times 10^3$)	3.18±0.13	2.77±0.14	4.68±0.14	5.47±0.30
Nonionized form (cpm $\times 10^3$)	1.12±0.05	1.19±0.05	1.41±0.09	1.31±0.07
Intracellular pH	6.56±0.06	6.47±0.09	6.62±0.07	6.72±0.06

* Typical experiments and controls showing the range and variability of the intracellular pH from different batches of cells.

** Influence of heat and inhibitor treatments upon intracellular pH, measured parallel to a control from the same cell batch.

RESULTS AND DISCUSSION

The intracellular pH lies in the range from 6.4 to 6.9 (Table I). The average variation within the same set of experiments is about 0.05 pH unit. However, the variation between different sets of experiments amounts to 0.5 pH unit maximally. This may result from biological variation or from errors in the estimation of the intracellular water, V_i. A 100 % error of V_i would change the pH value by 0.3 unit. Therefore, we conclude that the intracellular pH is close to neutral.

To test whether DMO molecules are passively transported across the cell membrane, dilution experiments were undertaken. If the cells were initially loaded with radioactive DMO (60 000 cpm in the supernatant, and 24 000 cpm in the pellet of 20 mg protein, after centrifugation), the counts in the supernatant C_s (DMO) and in the pellet C_p(DMO) decrease linearly and parallel to each other, when plotted on semi-logarithmic paper, and within three washes by three orders of magnitude. Additional experiments were carried out to determine the intracellular DMO to extracellular DMO ratio. Using six different DMO concentrations ($1.7 \cdot 10^4$–$3.1 \cdot 10^5$ cpm/ml), the ratio remained at a constant value of about 2.5. These results support the assumption that DMO is passively transported into the cell.

There exists no direct way to verify whether the ionized form is actually impermeant to the cell membrane. Comparing the rate of lyzing *Streptococcus faecalis* protoplasts at pH 5.3 and 7.5, it was found that the ionized form of DMO penetrates at least 200 times more slowly than the non-ionized form [4]. However, the difference of the concentration of the non-ionized DMO form at pH 5.3 and 7.5 amounts to $10^{2.2} = 158$-fold. The rate of passive penetration of non-ionized DMO is proportional to the concentration. Thus, the ratio of the permeability coefficients of the non-ionized form to that of the ionized form of DMO should be significantly slower than 200-fold. This argument is consistent with our experimental finding that the ratio C_i(DMO)/C_s(DMO), and consequently the intracellular pH value, do not depend upon the length of incubation (2 min–2 h).

There are three independent lines of evidence that the intracellular pH lies in the neutral region. Firstly, after harvest *Thermoplasma acidophila* cells were washed ten times with distilled water, then the pellet was sonicated for 1 h. After sonication pH values varying from 6.3 to 6.8 were recorded. Transmission electron micrographs showed broken cells [5]. Secondly, a curve of cells (10 mg/ml protein) titrated with 0.04 M NaOH has an inflection point around pH 6.5 to 6.9. At pH 9.3 the cells lyze, the cytoplasm can be collected, and it starts to precipitate when the pH becomes close to neutral [5]. Thirdly, we also demonstrated that *Thermoplasma acidophila* has malate dehydrogenase activity associated with the cytoplasma fraction. The enzymatic activity was assayed in four different buffers ranging from pH 3 to 12 [6]. A pH profile of that enzyme has a broad optimum from pH 8.5 to 10 (Fig. 1), and the activity is less than 25 % for pH values lower than 6.5. Such a profile is typical for that enzyme in higher organisms [6–8]. These facts also indicate that the intracellular pH is near neutral.

Within our experimental accuracy, the intracellular pH is not affected by temperature changes (56 °C, 24 °C), or by alteration of the extracellular pH (2, 4 and 6). At pH 4 and 6, the cells were suspended in a buffer, where glycine was replaced by citrate. Moreover, to calculate the intracellular pH of cells in citrate buffer

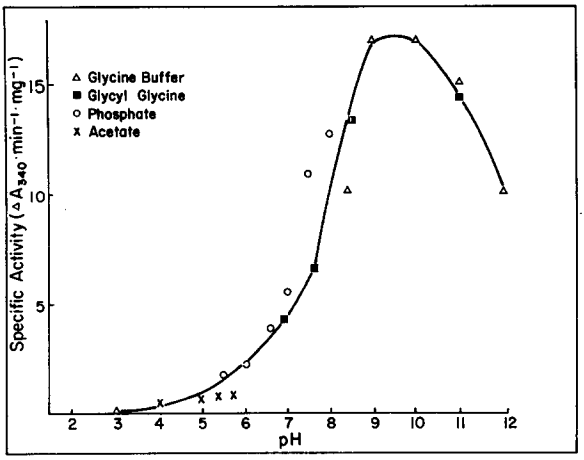

Fig. 1. pH profile of cytoplasmic malate dehydrogenase from *Thermoplasma acidophila*, assayed in four different buffers at 56 °C. The enzymatic activity was measured by reduction of oxaloacetate and monitoring the absorbance decrease of NADH at 340 nm.

at pH 6, we assumed that in the extracellular medium half of the number of DMO molecules are ionized, and the other half are in the non-ionized form. Thus, the cells are capable to maintain the intracellular pH when subjected to those environmental alterations.

Next we would like to explore whether *Thermoplasma acidophila* cells require active metabolic processes to maintain the pH gradient of 4.5 units, or whether passive mechanisms play an important role. When *Thermoplasma* cells were boiled for 5 h at 100 °C, the intracellular pH measured at the end of the harsh treatment was still neutral. These boiled cells were no longer viable as demonstrated by inoculating them into a culture medium. By exposing viable cells to general metabolic inhibitors, as listed in Table I, growth of cultured cells was prevented. None of these inhibitors altered the intracellular pH within experimental accuracy (Table I). On the basis of these heat and inhibitor treatments, we therefore conclude that the major portion of the pH gradient is not maintained by active processes, but rather by passive properties of the cell. A Donnan potential across the membrane generated by an internal charged macromolecule impermeant to the cell membrane, can account for maintenance of the huge pH gradient without participation of active mechanisms. For rat-liver mitochondria, ions are also distributed between the mitochondrial interior and the surrounding medium by way of a Donnan potential [9].

ACKNOWLEDGEMENT

This work was supported by United States Atomic Energy Commission Contract No. AT-(11-1)-1338.

REFERENCES

1 Darland, G., Brock, T. D., Samsonoff, W. and Conti, S. F. (1970) Science 170, 1416–1418
2 Waddell, W. J. and Butler, T. C. (1959) J. Clin. Invest. 38, 720–729

3 Addanki, S., Cahill, F. D. and Sotos, J. F. (1968) J. Biol. Chem. 243, 2337–2348
4 Harold, F. M., Pavlosova, E. and Baarda, J. R. (1970) Biochim. Biophys. Acta 196, 235–244
5 Ruwart, M. J. and Haug, A. (1975) Biochemistry, in the press
6 Yang, N. S. and Scandalios, J. G. (1974) Arch. Biochem. Biophys. 161, 335–353
7 McReynolds, M. S. and Kitto, G. B. (1970) Biochim. Biophys. Acta 198, 165–175
8 Kitto, G. B. and Kaplan, N. O. (1966) Biochemistry 5, 3966–3980
9 Harris, E. J. (1974) in Membrane Structure and Mechanisms of Biological Energy Transduction (Avery, J., ed.), pp. 381–387, Plenum Press, London