The Protonmotive Force in *Pseudomonas aeruginosa* and its Relationship to Exoprotease Production

By MICHAEL A. WHOOLEY AND AIDEN J. McLOUGHLIN*

Department of Industrial Microbiology, University College, Dublin 4, Ireland

(Received 7 May 1982; revised 13 July 1982)

In *Pseudomonas aeruginosa* ATCC 10145 a negative correlation was observed between the protonmotive force (ΔP) and the amount of exoprotease produced, with a decrease in ΔP resulting in an increase in exoprotease. The two components of ΔP , the transmembrane pH gradient (ΔpH) and the membrane potential ($\Delta\psi$) were examined independently and it was observed that $\Delta\psi$ varied very little under the conditions which influenced the activities of exoprotease. However, a positive correlation existed between pH and exoprotease production although the intracellular pH varied very little with either changes in growth rate or changes in extracellular pH. It was observed that with a decrease in growth rate, ΔpH became more alkaline and increased exoprotease activities were recorded. Furthermore, an increase in extracellular pH to give an artificial alteration in ΔpH , and, consequently, a decrease in ΔP , increased exoprotease production, thus confirming the importance of ΔpH in exoprotease production.

INTRODUCTION

Previous investigations in this laboratory into the regulation of exoprotease production by *Pseudomonas aeruginosa* indicated the importance of the energy status of the cells. Exoprotease production was found to be repressed in energy-sufficient cells and derepressed in energy-deficient cells (Whooley *et al.*, 1983); regulation of exoprotease production by another Gram-negative organism, *Vibrio* sp., appeared to be under a similar control (Wiersma, 1978). In order to determine the exact nature of this energy-linked regulation of exoprotease production, the energy generating systems of *P. aeruginosa* were examined.

Pseudomonas aeruginosa is a strict aerobe (Stanier *et al.*, 1966) and is therefore mainly dependent on respiratory electron transport for energy generation. This energy is in the form of an electrochemical potential for protons across the membrane generated by proton-translocating pumps. The energy in this electrochemical potential is utilized as a driving force for several membrane-related processes such as oxidative phosphorylation (Wilson *et al.*, 1976; Maloney, 1977; Tsuchiya, 1977), nutrient transport (for reviews see Harold, 1974; Dills *et al.*, 1980) and motility (Goulbourne & Greenberg, 1980; Shioi *et al.*, 1980).

In this paper extracellular pH (i.e. the pH of the culture) and growth rate were examined for their effects on the protonmotive force (and its two components) and exoprotease production.

METHODS

Organism and growth. *Pseudomonas aeruginosa* ATCC 10145 was maintained and grown as described previously (Whooley *et al.*, 1982).

Enzyme assay. Protease activity was assayed as described in the previous paper (Whooley *et al.*, 1983). Units of activity (U) were calculated from a standard curve and are expressed as μg tyrosine released from casein per ml of cell-free supernatant per hour at 30 °C.

Abbreviations: pH_{ex} , extracellular pH (i.e. pH of medium or culture); pH_{in} , intracellular pH; ΔpH , transmembrane pH gradient ($\Delta pH = pH_{\text{in}} - pH_{\text{ex}}$); $\Delta\psi$, membrane potential; ΔP , protonmotive force ($\Delta P = \Delta\psi - 59\Delta pH$).

Measurement of intracellular volume. The intracellular volume was determined by a modification of the method of Collins & Hamilton (1976). To triplicate 3 ml samples of culture 0.1 ml (hydroxy[^{14}C]methyl)inulin ($11.3 \text{ mCi mmol}^{-1}$, $418 \text{ MBq mmol}^{-1}$), dissolved in distilled water, was added to give a final concentration of $73.75 \text{ }\mu\text{M}$ and 0.1 ml $^3\text{H}_2\text{O}$, to give a final concentration of $15 \text{ }\mu\text{Ci ml}^{-1}$ (555 KBq ml^{-1}). Samples were incubated on a roller drum at 27°C for 20 min and then centrifuged at 13000 g for 15 min and the supernatants poured off. The insides of the tubes containing the pellets were swabbed dry with cotton buds to remove any adhering droplets of the cell-free supernatant (Kashket & Wong, 1969) and the pellets were resuspended in 2 ml water. The resuspended pellet (0.5 ml) was added to 5 ml of a scintillation fluid containing: PPO, 4 g; POPOP, 100 mg; Triton X-100, 333 ml; toluene, 667 ml. Cell-free supernatant (0.1 ml) along with 0.4 ml water was added to another vial containing 5 ml of the toluene/Triton scintillation fluid. The vials were stored at ambient temperature for 12 h, which allowed the establishment of a stable gel with the radioactivity evenly distributed, and were then counted on a Packard model 2002 Tri-Carb liquid scintillation spectrometer.

Since hydroxymethyl inulin does not enter the cells, the ^{14}C activity represents the extracellular water trapped in the pellet, and the ^3H activity represents the intracellular plus the extracellular water. The intracellular volume (V_i) was calculated from the equation

$$V_i = (^3\text{H in pellet}/^3\text{H in supernatant}) - (^{14}\text{C in pellet}/^{14}\text{C in supernatant})$$

Measurement of transmembrane pH gradient (ΔpH) and intracellular pH (pH_i). pH was determined from the distribution of methylamine hydrochloride (Rottenberg *et al.*, 1972). A sample was taken from the culture vessel and to triplicate 3 ml samples, 0.1 ml [^{14}C]methylamine hydrochloride, ($40.4 \text{ mCi mmol}^{-1}$, $1.49 \text{ GBq mmol}^{-1}$) dissolved in distilled water was added to give a final concentration of $20.625 \text{ }\mu\text{M}$. The tubes were placed on a roller drum at 27°C for 20 min (to allow equilibration of methylamine across the membrane) and were then centrifuged at 13000 g for 15 min and the supernatants poured off. Samples of the resuspended pellet and the supernatant were assayed for radioactivity as described for measurement of intracellular volume.

The value of ΔpH (inside acidic) was calculated from $\log(\text{methylamine}_i/\text{methylamine}_{\text{out}})$. The values for (methylamine_i) were corrected using the calculated value for intracellular volume in order to exclude the counts due to methylamine in the extracellular pellet water.

Intracellular pH was taken as extracellular pH minus ΔpH , where extracellular pH was the pH of the culture.

Measurement of membrane potential ($\Delta\psi$). The membrane potential of *P. aeruginosa* cells was determined using $14.125 \text{ }\mu\text{M}$ -potassium[^{14}C]thiocyanate (59 mCi mmol^{-1} , $2.18 \text{ GBq mmol}^{-1}$). The procedure followed was the same as that used in intracellular pH determinations. The protonmotive force was calculated from the equation

$$\Delta\text{P} = \Delta\psi - 59 \Delta\text{pH}$$

Chemicals. Antifoam A, PPO and POPOP were obtained from Sigma. [^{14}C]Methylamine hydrochloride, (hydroxy[^{14}C]methyl)inulin, potassium[^{14}C]thiocyanate and $^3\text{H}_2\text{O}$ were obtained from Amersham. All other chemicals used were BDH AnalaR grade.

RESULTS

Extracellular pH and exoprotease production

Previous investigations of exoprotease production in a medium containing 1% (w/v) Casamino acids as the sole source of carbon and energy (Whooley *et al.*, 1983) revealed that maximum production occurred in the decelerating phase of growth and continuous culture studies revealed that as growth rate decreased, exoprotease production increased linearly. However, it was also noted that the pH of the medium, the extracellular pH (pH_{ex}), increased with decreasing growth rate, paralleling the effect of growth rate on exoprotease production (Fig. 1).

Intracellular pH, ΔpH and $\Delta\psi$

The influence this variation in extracellular pH had on the internal environment of the cell was examined. Before measuring the intracellular pH, the postulate that the cell membrane was much more permeable to the undissociated form of the base had to be tested. This was accomplished using the method of Harold *et al.* (1970). Osmotically sensitive cells of the organism were prepared by the Spizizen (1957) method and were incubated in 0.5 M-sucrose solutions at different pH values. Some of these cells were also added to 0.5 M unlabelled methylamine hydrochloride at pH values of 11.0 and 8.0 (i.e. above and below its pK value of 10.6).

Fig. 1. The influence of growth rate on extracellular pH and exoprotease production by *P. aeruginosa* in continuous culture at 27 °C under conditions of carbon-limitation (Casamino acids). Exoprotease activity (○), extracellular pH (●).

Fig. 2. The effect of extracellular pH on intracellular pH (○) and membrane potential (●) of *P. aeruginosa* in continuous culture at 27 °C under conditions of carbon limitation (Casamino acids). Dilution rate was 0.1 h^{-1} . Values of pH were controlled by the automatic addition of NaOH or HCl.

After 10 min the absorbance at 650 nm was measured and a decrease was taken to be indicative of lysis. Methylamine hydrochloride was found to stabilize these cells at pH 8.0. Above its pK value (i.e. at pH 11.0) methylamine hydrochloride caused lysis.

When intracellular pH values of cells growing at different controlled extracellular pH values were determined, intracellular pH was found to be more acidic at all the values studied (Fig. 2). The intracellular pH increased with increasing extracellular pH up to an intracellular pH value of approximately 7.0 (which corresponded to an extracellular value of approximately 7.5), and thereafter any further increase in extracellular pH resulted in very little change in intracellular pH, suggesting a buffering effect of the cytoplasm.

The membrane potential was found to be negative inside. No relationship was apparent between membrane potential and extracellular pH and, indeed, over the range of pH values studied, the $\Delta\psi$ was found to remain within -165 to -190 mV (Fig. 2).

Fig. 3. The influence of growth rate on extracellular pH ($\circ$), intracellular pH ($\bullet$) and membrane potential ($\square$) of *P. aeruginosa* in continuous culture at 27 °C under conditions of carbon limitation.

Fig. 4. Correlation between the transmembrane pH gradient (ΔpH) and the production of exoprotease by *P. aeruginosa* under controlled pH conditions.

Intracellular pH was found to be unaffected by growth rate, remaining relatively constant at approximately pH 7.0 (Fig. 3) confirming the buffering effect of the cytoplasm suggested in Fig. 2. However, the magnitude of the transmembrane pH gradient was found to increase (becoming more acidic internally relative to external pH), with decreasing growth rate. The intracellular pH was only determined at those growth rates where exoprotease was produced i.e. less than 0.15 h^{-1} . At all these growth rates $\text{pH}_{\text{in}} < \text{pH}_{\text{ex}}$, therefore, the ΔpH had a negative value.

The value of the membrane potential also appeared to be relatively unaffected by growth rate over the range examined, with only a difference of approximately 10 mV between the values at growth rates of 0.02 h^{-1} and 0.15 h^{-1} (Fig. 3).

Since it was shown in Fig. 3 that ΔpH decreased (i.e., became less acidic) with increasing

Fig. 5. The effect of the magnitude of the transmembrane pH gradient on exoprotease production by *P. aeruginosa* at different controlled growth rates in continuous culture at 27 °C under conditions of carbon limitation. D (h^{-1}) = 0.033 (○), 0.1 (●), 0.133 (□).

Fig. 6. The effect of extracellular pH on protonmotive force (○) and the relationship between transmembrane pH gradient (●) and protonmotive force of *P. aeruginosa* cells in continuous culture at 27 °C under conditions of carbon limitation ($D = 0.1 \text{ h}^{-1}$).

growth rates (in a similar fashion to extracellular pH) and that exoprotease production increased with increasing growth rate (Fig. 1), the relationship between ΔpH and exoprotease production was investigated. Figure 4 demonstrates that exoprotease production increased with increasing (negative value) ΔpH . Therefore, it is evident that there is a relationship between exoprotease production and ΔpH .

To determine if the ΔpH effect on exoprotease production is independent of growth rate, the latter was kept constant at three different values (0.033, 0.1 and 0.133 h^{-1}) and a ΔpH was imposed by controlling the extracellular pH using a 0.5 M-NaOH or HCl. The pH range examined was between 7.25 and 8.0 since it was over this range that maximum exoprotease production occurred (Fig. 1). Exoprotease production increased with increasing ΔpH at all the growth rates (Fig. 5). The effect of ΔpH on exoprotease production was more pronounced as the growth rate decreased. Therefore, it appears that an increase in ΔpH (negative value) enables

Fig. 7. The influence of the magnitude of the protonmotive force on the production of exoprotease by *P. aeruginosa*.

increased exoprotease production. Also, since the rate of exoprotease production per change in ΔpH increased with increasing growth rate, it is evident that both ΔpH and growth rate exert independent effects on exoprotease production.

The protonmotive force

The protonmotive force was calculated from the measured values of $\Delta\psi$ and ΔpH . Because the membrane potential remained constant within limits (-165 to -190 mV) irrespective of extracellular pH or growth rate, the protonmotive force decreased with increasing extracellular pH (Fig. 6). Indeed, because of the constancy of the membrane potential under the conditions studied, the protonmotive force was directly dependent on the ΔpH . It was found that as the ΔpH increased (i.e. became more acidic), the protonmotive force decreased.

The values of the protonmotive force for the ΔpH values of Fig. 5 were calculated and plotted against the corresponding exoprotease levels and it was found that as the protonmotive force decreased, exoprotease production increased in a linear fashion (Fig. 7).

DISCUSSION

Most of the determinations in bacteria of the protonmotive force and its two components, ΔpH and $\Delta\psi$, have been carried out on 'resting' cell suspensions (usually exponential phase cells resuspended in buffer) and on spheroplasts and vesicles (for reviews see Rottenberg, 1975; Maloney *et al.*, 1975). Recently, however, some investigations of ΔP in growing cells have been reported (Kashket, 1981*a, b*). In our investigation, growing cells were also used and, indeed, using continuous culture the ΔP and its components at different growth rates were examined as we supported the view held by Kashket (1981*a*) that 'it was important to examine the status of energy metabolism in the physiological condition of growth'.

According to the chemiosmotic theory the intracellular pH should be more alkaline than the extracellular pH resulting in a positive ΔpH (i.e. inside alkaline). However, the cells used here were at low growth rates, and, consequently, high extracellular pH values. In order for these cells to maintain a positive ΔpH , the intracellular pH would have to be in the region of 8.0 to 9.0. At these postulated intracellular pH values, the functioning of intracellular enzymes would probably be severely retarded. Therefore, it appears that in *P. aeruginosa* the cytoplasm has the ability to buffer the intracellular pH around neutrality (Fig. 3) to optimize the function of these enzymes. This close regulation of the intracellular pH has been reported in various bacteria (Kashket, 1981*a, b*; Kihara & Macnab, 1981; Felle *et al.*, 1980; Khan & Macnab, 1980; Padan *et al.*, 1976; Hsung & Haug, 1975). Most of the previous research, where the ΔpH is found to be

positive, has dealt with cells removed from cultures at high growth rates (i.e. exponential phase cells) and it is probable that, if one extrapolated back the lines in Fig. 3, one would also find that in this organism a positive ΔpH exists at higher growth rates.

The membrane potential ($\Delta\psi$) was found to be unaffected by extracellular pH over the range examined (Fig. 2). Similar results were found for *Escherichia coli* (Padan *et al.*, 1976; Kashket, 1981*b*) and *Staphylococcus aureus* (Kashket, 1981*a*). It was also found to be unaffected by growth rate over the range examined (Fig. 3) a result also supported by similar findings for *S. aureus* (Kashket, 1981*a*) and *E. coli* (Murastugu *et al.*, 1979). Thus as the $\Delta\psi$ remained constant, the ΔP was dependent on the ΔpH .

It was evident that as the ΔP decreased, exoprotease production increased (Fig. 7). It must be emphasized, however, that the presence of a negative ΔpH (i.e. inside acidic) in this study was only at low growth rates. The ΔpH was not determined at higher growth rates because no exoprotease was produced at these growth rates. It is postulated that the situation that prevails in cells of low growth rates (where exoprotease is produced) is that as the extracellular pH increases (above 7.0) the ΔpH becomes negative due to the fact that the intracellular pH remains constant at approximately 7.0 at these extracellular pH values. However, the $\Delta\psi$ remains constant and cannot compensate further for the decrease in the ΔpH . Therefore, the ΔP decreased. These results are supported by recent findings by other investigators using *S. aureus* (Kashket, 1981*a*), *E. coli* (Padan *et al.*, 1976; Kashket, 1981*b*), *Mycoplasma gallisepticum* (Rottem *et al.*, 1981), *Bacillus subtilis* (Shioi *et al.*, 1980) when grown at high extracellular pH values. On the other hand, we postulate that, in *P. aeruginosa* cells at high growth rates, the ΔP remains relatively constant but the relative contributions of the $\Delta\psi$ and the ΔpH vary with varying extracellular pH, and the findings of Shioi *et al.* (1980) for *B. subtilis* support this.

According to the chemiosmotic theory, the formation of a ΔP is a result of the expulsion of protons from the respiratory chain and this ΔP promotes the influx of protons back into the cell through the membrane-bound ATPase (Harold, 1972). However, the equilibrium of the ATPase is very delicately poised and the presence of a ΔP shifts the equilibrium towards ATP formation. When the ΔP decreased the equilibrium shifts and the ATPase breaks down ATP due to the net flowing out of protons through the ATPase. Therefore, as the ΔP decreases so too does the ATP concentration of the cell which suggests a possible role for ATP in the regulation of exoprotease production.

Results presented in this paper also indicated a possible physiological explanation behind the fact that micro-organisms have pH optima for product formation that do not always correspond to their pH optimum for growth, and, emphasizes the importance of pH control in product formation.

REFERENCES

- COLLINS, S. H. & HAMILTON, W. A. (1976). Magnitude of the protonmotive force in respiring *Staphylococcus aureus* and *Escherichia coli*. *Journal of Bacteriology* **126**, 1224–1231.
- DILLS, S. S., APPERSON, A., SCHMIDT, M. R. & SAIER, M. H. (1980). Carbohydrate transport in bacteria. *Microbiological Reviews* **44**, 385–418.
- FELLE, H., PORTER, J. S., SLAYMAN, C. L. & KABACK, H. R. (1980). Quantitative measurements of membrane potential in *Escherichia coli*. *Biochemistry* **19**, 3585–3590.
- GOULBOURNE, E. A. & GREENBERG, E. P. (1980). Relationship between protonmotive force and motility in *Spirochaeta aurantia*. *Journal of Bacteriology* **143**, 1450–1457.
- HAROLD, F. M. (1972). Conservation and translocation of energy by bacterial membranes. *Bacteriological Reviews* **36**, 172–230.
- HAROLD, F. M. (1974). Chemiosmotic interpretation of active transport in bacteria. *Annals of the New York Academy of Sciences* **227**, 297–311.
- HAROLD, F. M., PAVLASOVA, E. & BAARDA, J. R. (1970). A transmembrane pH gradient in *Streptococcus faecalis*: origin and dissipation by proton conductors and *N,N'*-dicyclohexylcarbodiimide. *Biochimica et biophysica acta* **196**, 235–244.
- HSUNG, J. C. & HAUG, A. (1975). Intracellular pH of *Thermoplasma acidophila*. *Biochimica et biophysica acta* **389**, 477–482.
- KASHKET, E. R. (1981*a*). Protonmotive force in growing *Streptococcus lactis* and *Staphylococcus aureus* cells under aerobic and anaerobic conditions. *Journal of Bacteriology* **146**, 369–376.
- KASHKET, E. R. (1981*b*). Effects of aerobiosis and nitrogen source on the protonmotive force in growing *Escherichia coli* and *Klebsiella pneumoniae* cells. *Journal of Bacteriology* **146**, 377–384.
- KASHKET, E. R. & WONG, P. T. S. (1969). The

- intracellular pH of *Escherichia coli*. *Biochimica et biophysica acta* **193**, 212–214.
- KHAN, S. & MACNAB, R. M. (1980). Proton chemical potential, proton electrical potential and bacterial motility. *Journal of Molecular Biology* **138**, 599–614.
- KIHARA, M. & MACNAB, R. M. (1981). Cytoplasmic pH mediates pH taxis and weak-acid repellent taxis of bacteria. *Journal of Bacteriology* **145**, 1209–1221.
- MALONEY, P. C. (1977). Obligatory coupling between proton entry and the synthesis of adenosine 5'-triphosphate in *Streptococcus lactis*. *Journal of Bacteriology* **132**, 564–575.
- MALONEY, P. C., KASHKET, E. R. & WILSON, T. H. (1975). Methods for studying transport in bacteria. In *Methods in Membrane Biology*, vol. 5, pp. 1–59. Edited by E. D. Korn. New York & London: Plenum Press.
- MURASTUGU, M., KAMO, N., KOBATAKE, Y. & KIMURA, K. (1979). Determination of membrane potential of *Escherichia coli* with use of an electrode sensitive to tetraphenyl phosphonium. *Journal of Electroanalytical Chemistry (Bioelectrochemistry and Bioenergetics)* **104**, 477–491.
- PADAN, E., ZILBERSTEIN, D. & ROTTENBERG, H. (1976). The proton electrochemical gradient in *Escherichia coli* cells. *European Journal of Biochemistry* **63**, 533–541.
- ROTTEM, S., LINKER, C. & WILSON, T. H. (1981). Protonmotive force across the membrane of *Mycoplasma gallisepticum* and its possible role in cell volume regulation. *Journal of Bacteriology* **145**, 1299–1309.
- ROTTENBERG, H. (1975). The measurement of transmembrane electrochemical proton gradients. *Bioenergetics* **7**, 61–74.
- ROTTENBERG, H., GRUNWALD, T. & AVRON, M. (1972). Determination of ΔpH in chloroplasts. I. Distribution of [^{14}C]methylamine. *European Journal of Biochemistry* **25**, 54–63.
- SHIOI, J., MASUURA, S. & IMAR, Y. (1980). Quantitative measurements of protonmotive force and motility in *Bacillus subtilis*. *Journal of Bacteriology* **144**, 891–897.
- SPIZIZEN, J. (1957). Infection of protoplasts by disrupted T2 virus. *Proceedings of the National Academy of Sciences of the United States of America* **43**, 694–701.
- STANIER, R. Y., PALLERONI, N. J. & DOUDOROFF, M. (1966). The aerobic pseudomonads: a taxonomic study. *Journal of General Microbiology* **43**, 159–271.
- TSUCHIYA, T. (1977). Adenosine 5'-triphosphate synthesis driven by a protonmotive force in membrane vesicles of *Escherichia coli*. *Journal of Bacteriology* **129**, 763–769.
- WHOOLEY, M. A., O'CALLAGHAN, J. A. & McLOUGHLIN, A. J. (1983). The effect of substrate on the regulation of exoprotease production by *Pseudomonas aeruginosa* ATCC 10145. *Journal of General Microbiology* **129**, 981–988.
- WIERSMA, M. (1978). *Regulation of extracellular proteolytic enzyme synthesis in Vibrio SA1*. Ph.D. thesis, University of Groningen, Holland.
- WILSON, D. M., ALDERETE, J. F., MALONEY, P. C. & WILSON, T. H. (1976). Protonmotive force as the source of energy for adenosine 5'-triphosphate synthesis in *Escherichia coli*. *Journal of Bacteriology* **126**, 327–337.