Use of the pH sensitive fluorescence probe pyranine to monitor internal pH changes in *Escherichia coli* membrane vesicles

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Measurements of the fluorescent properties of 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine) enclosed within the internal space of *Escherichia coli* membrane vesicles enable recordings and quantitative analysis of: (i) changes in intravesicular pH taking place during oxidation of electron donors by the membrane respiratory chain; (ii) transient alkalization of the internal aqueous space resulting from the creation of outwardly directed acetate diffusion gradients across the vesicular membrane. Quantitation of the fluorescence variations recorded during the creation of transmembrane acetate gradients shows a close correspondence between the measured shifts in internal pH value and those expected from the amplitude of the imposed acetate gradients.

Escherichia coli *Membrane vesicle* *Internal pH* *Pyranine* *Fluorescent pH probe*

1. INTRODUCTION

A large body of experimental evidence indicates that, as postulated in [1,2], the electrochemical proton gradient $\Delta\bar{\mu}H^+$ generated during oxidation of electron donors by the respiratory enzymes or ATP hydrolysis by the H^+-ATPase is the immediate driving force for secondary solute transport in many bacterial systems and in membrane vesicles derived from these organisms. Among different strategies developed to define the coupling mechanism between transport activities and $\Delta\bar{\mu}H^+$ or either its electrical ($\Delta\psi$) or chemical (ΔpH) components in membrane vesicles preparations, the use of outwardly directed potassium diffusion gradients in the presence of valinomycin as a generator of transient $\Delta\psi$ (interior negative) or outwardly directed acetate diffusion gradients as a ΔpH generator (interior alkaline) proved to yield valuable information [3–6]. However, the information so far obtained as a result of artificial ΔpH imposition is only qualitative or at best related to theoretical estimates of ΔpH since no adequate technique is available for the determination of the actual magnitude of the ΔpH generated.

Recent studies [7,8] indicate that the pH-dependent fluorescence dye 8-hydroxy 1-3-6 pyrenetrisulfonate (pyranine) is a reliable indicator of the internal pH changes in unilamellar phospholipids vesicles. We here report a detailed characterization of the changes in fluorescence properties of pyranine enclosed in *E. coli* membrane vesicles associated with internal pH changes caused either by oxidation of appropriate electron donors or by creation of outwardly directed acetate diffusion gradients.

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Abbreviations: $\Delta\psi$ and ΔpH, electrical and chemical proton gradients; $\Delta\bar{\mu}$ acetate, transmembrane acetate concentration gradient; TPP^+ Br^-, tetraphenylphosphonium bromide; PMS, phenazine methosulfate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

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2. MATERIALS AND METHODS

2.1. *Growth of cells and preparation of membrane vesicles*

Escherichia coli RA 11 were grown at 37°C in minimal salts medium (medium 63) supplemented with 1% pancreatic digest of casein, 0.5 mg/l thiamine and 10 mM melibiose [9]. Membrane vesicles enclosing pyranine were prepared as in [10]; pyranine (200 μM) was entrapped within the intravesicular space during the osmotic lysis step which converts spheroplasts into vesicles. Subsequent operations (washing and freezing) were performed using solutions containing 200 μM pyranine.

Experiments were performed using concentrated membrane vesicle suspensions containing the dye and previously equilibrated with phosphate-buffered solutions (100 mM K phosphate, 10 mM $MgSO_4$) at different pHs or with media composed of 100 mM K acetate, 10 mM K phosphate and $MgSO_4$ at various pHs.

2.2. *Fluorescence measurements*

The fluorescence experiments were performed by diluting 5-μl aliquots of the concentrated vesicle suspension (~25 mg protein/ml) containing the dye into 2 ml of pyranine-free media of given composition and pH. Mixing was complete in less than 3 s. Fluorescence of pyranine was observed at 90°C from the excitation beam of a Perkin-Elmer MPF 44B spectrofluorimeter using 1 × 1 cm quartz cuvettes thermostated at 21°C. Slit width was adjusted to 5 nm.

The magnitude of $\Delta\psi$ and ΔpH generated during D-lactate oxidation by membrane vesicles with or without pyranine were estimated from [^{14}C]TPP$^+$ (20 μM, 87 mCi/mM) and [^{3}H]benzoic acid (30 μM, 56 mCi/mM) transmembrane distributions using the Flow Dialysis technique [11,12]. [^{14}C]TPP$^+$ and [^{3}H]benzoic acid were from CEA-France, pyranine was purchased from Molecular Probes Inc.

3. RESULTS AND DISCUSSION

Fig.1 shows that the properties and pH dependence of the fluorescence signal recorded from washed *E. coli* membrane vesicles prepared in the presence of pyranine (200 μM) during the osmotic shock procedure are similar to those recorded from the dye in solution [7]. An increase in the pH medium leads to a pronounced red shift in the fluorescence excitation maximum from 405 at pH 5.5 to 460 nm at pH 8 (inset in fig.1) with no concomitant modification of the 508 nm emission maximum (not shown). Furthermore, titration of the amplitude of the 508 nm emission maximum (λ_{ex} at 460 nm) is primarily very similar to that for pyranine in solution except for a slight alkaline shift in pK_a (fig.1). The curve essentially shows an increase in the 508 nm fluorescence intensity (λ_{ex} 460) as the pH is shifted in the alkaline direction.

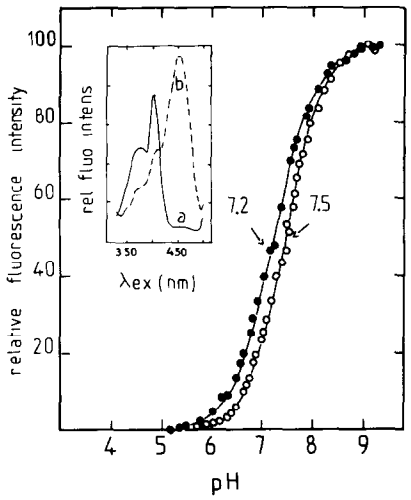

Fig.1. pH-Dependent changes in the fluorescence intensity of pyranine enclosed within *E. coli* membrane vesicles. Inset: excitation spectrum (λ_{em} = 508 nm) of washed membrane vesicles (60 μg protein/ml) enclosing 200 μM pyranine and incubated at pH_{out} 5.5 and 8 (traces a and b, respectively). The vesicles sample contained FCCP (5 μM) to facilitate transmembrane H^+ equilibration. Main curves: variation of the fluorescence intensity (λ_{ex} = 460 nm; λ_{em} = 508 nm) as a function of pH of 1 μM pyranine in solution (●) and washed pyranine-containing membrane vesicles (○) incubated with FCCP. Numbers indicate pK_a values.

3.1. *Effect of D-lactate oxidation on the fluorescence signal*

The fate of this fluorescence signal was first studied during oxidation of appropriate electron donors which leads to alkalization of the intravesicular pH. Importantly, flow dialysis deter-

minations of [^{14}C]TPP$^+$ and [^{3}H]benzoic acid transmembrane distributions indicate that the presence of internal pyranine does not modify the magnitudes of ΔpH and $\Delta\psi$ generated during D-lactate oxidation. Trace a in fig.2A shows that when D-lactate is added to washed membrane vesicles incubated at pH_{out} 6.6 in the presence of valinomycin, i.e., in conditions where only ΔpH is generated [11], a time-dependent increase in fluorescence signal is recorded: the maximal increase (ΔF) is nearly complete in 2 min ($t½$ = 45 s) and corresponds to a 2-fold increase in signal intensity. This trace also shows that subsequent addition of nigericin (0.05 μM) or FCCP (5 μM, not shown) produces a rapid signal return towards the baseline level. A close relationship between the development of ΔF and alkalinization of pH_{in} is further suggested by the following observations. First, trace b in fig.2A shows that addition of succinate, which has been reported to lead to the generation of a smaller pH_{in} change in membrane vesicles [12], elicits a reduced fluorescence increase. Secondly, D-lactate-dependent increases in fluorescence signal are also recorded in vesicles incubated at pH_{out} 5.5 but not 7.5 (not shown): these data fit the pattern of pH_{in} changes in oxidizing vesicles incubated at different pH_{out} [11,12].

Two observations strongly suggest that the D-lactate-induced variations in fluorescence are independent of the presence of external pyranine, either free in the bulk medium or bound onto the external surface of the membrane vesicles. First, the magnitude of ΔF was independent of the concentration of external pyranine (up to 1 μM). Secondly, when vesicles prepared in the absence of pyranine are incubated in solutions containing 200 μM pyranine and then washed, there is a persistence of a fluorescence signal which is not modified upon addition of D-lactate (trace d in fig.2A). This fluorescence signal, which amounts to 0.40 times the signal recorded from washed vesicles enclosing pyranine most likely corresponds to externally bound pyranine.

As detailed in the legend of fig.2, a combination of the fluorescence data shown in fig.2A (trace a) corrected for the contribution of external pyranine and those in the titration curve (fig.1) taken as a calibration curve, enables the determination of the pH_{in} value reached during D-lactate oxidation. In vesicles incubated at pH_{out} 6.6 in the presence of valinomycin (trace a in fig.2A), pH_{in} rose to 7.2; at pH_{out} 5.5, pH_{in} in D-lactate was 6.7. These values are close to those estimated from [^{3}H]benzoic acid distribution (respectively, 7.1 and 6.6). The imprecision of the flow dialysis method at low ΔpH values, however, hindered the study of the detailed correlation between the fluorescence change and weak acid transmembrane distribution following progressive dissipation of the changes with increasing concentration of FCCP. All together, these data indicate that enclosed pyranine conveniently monitors the pH_{in} changes in membrane vesicles associated with D-lactate oxidation.

3.2. *Effects of acetate gradients on enclosed pyranine fluorescence*

Fig.2B illustrates typical transient modifications of fluorescence intensity recorded at pH_{out} 6.6 when outwardly directed acetate diffusion gradients ($\Delta\bar{\mu}$ acetate) of varying magnitude are created across membrane vesicles enclosing pyranine. Thus, upon dilution of membrane vesicles loaded with 100 mM acetate into a medium containing lower concentrations of acetate (0.25 mM in trace a), the fluorescence signal immediately increases, reaches a maximal value after 5 s (ΔF_{max}) and then returns towards a low steady level. Similar transient increases in fluorescence were recorded at pH_{out} 5.5 and 7.5 (not shown). Importantly, no transient changes were recorded:

(i) when no acetate gradient is imposed (trace f);
(ii) if $\Delta\bar{\mu}$ acetate are imposed in the presence of uncouplers (trace similar to trace f);
(iii) in vesicles incubated but not loaded with pyranine (200 μM) (trace g);
(iv) when an outwardly directed potassium diffusion gradient is imposed (generation of $\Delta\psi$) (not shown);
(v) the absolute magnitude of the transient response remains unchanged when the concentration of external pyranine is increased up to 1 μM.

Finally, and as illustrated by traces a–e, the amplitude of the maximal fluorescence change at 5 s (ΔF_{max}) varies in proportion to the magnitude of the $\Delta\mu$ acetate imposed.

Estimation of the shift in intravesicular pH at ΔF_{max} can be calculated from the variation in internal fluorescence signal. This latter was computed,

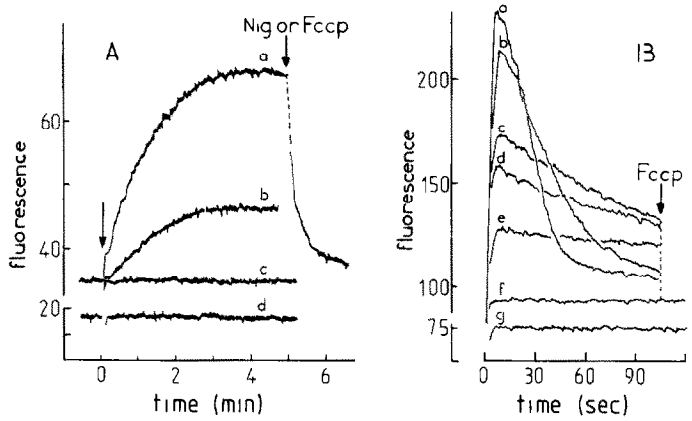

Fig.2. Effects of D-lactate oxidation or outwardly directed acetate diffusion gradients on the fluorescence signal from washed membrane vesicles enclosing pyranine. Panel A, vesicles pretreated with valinomycin (5 μM) were incubated at 60 μg protein/ml in 100 mM phosphate buffer (pH 6.6) in the absence or presence (trace c) of FCCP (5 μM); first arrow: 20 mM D-lactate (traces a and c) or 20 mM succinate (trace b) were added; the second arrow on trace a indicates addition of nigericin (0.05 μM) to vesicle suspension. Trace d: vesicles prepared in the absence of pyranine, then incubated with the dye (200 μM) and washed. At the arrow, D-lactate was added. The D-lactate-induced change in pH_{in} was calculated as follows: correction of the total fluorescence signal recorded before and after D-lactate (trace a) for the contribution of externally located pyranine (trace d) gives an estimate of the intensity of the internal pyranine fluorescence signal at respectively pH_{in} 6.6 (F_{in} (6.6)) and at the pH_{in} reached in D-lactate (F_{in} (D-lactate)). Combining the fractional increase F_{in} (D-lactate)/F_{in} (6.6) and the value of the fluorescence signal at pH (6.6) in the titration curve (fig.1), one obtains a new fluorescence signal which allows the reading from the titration curve of a corresponding pH_{in} value equal to the pH_{in} value reached during D-lactate oxidation. Panel B: 5-μl aliquots of concentrated membrane vesicles (25 mg protein/ml) containing pyranine and 100 mM K acetate (pH 6.6) were diluted into an equimolar medium at the same pH but containing a different ratio of K acetate/K gluconate. Traces a–e correspond to imposed acetate gradients calculated as 58 log $acetate_{in}/acetate_{out}$ of: (a) 150 mV; (b) 90 mV; (c) 60 mV; (d) 50 mV; (e) 30 mV; (f) 0 mV; trace g was recorded from vesicles incubated, but not loaded with pyranine, when a 150 mV acetate gradient was imposed. All experiments were performed in the presence of valinomycin (5 μM).

in all conditions, as the difference between the total fluorescence signal (traces a–f) and the fluorescence contribution of externally located pyranine measured in vesicles equilibrated but not loaded with pyranine (trace g). Then, a combination of the ratio of the internal fluorescence signals measured at ΔF_{max} and in the presence of FCCP and the fluorescence data from the titration curve (fig.1) allows determination of the pH_{in} value reached at ΔF_{max}, for each imposed acetate gradient. The resulting pH_{in} values (pH_{in} (measured)) are plotted in fig.3, as a function of the theoretical changes in pH_{in} (pH_{in} (calculated)) which, in each condition, is calculated as the sum of the pH_{in} value prevailing in FCCP-treated membrane (pH_{in} 6.6) and the magnitude of the imposed acetate gradient (log $acetate_{in}/acetate_{out}$). It can be first observed that the measured and theoretical pH_{in} values are highly correlated over a large range of pH_{in} values. Secondly, the slope of the relationship fits the identity line. Usually, the largest discrepancies between calculated and measured pH_{in} values were observed at high $\Delta\bar{\mu}$ acetate but never exceed 0.2 pH unit. These observations indicate that, in our experimental conditions, the actual change in pH_{in} caused by imposition of an outwardly directed acetate gradient can be directly calculated from the value of the pH of the equilibration solution (or pH_{out}) and the magnitude of the imposed acetate gradient. In summary, the experiments presented above show that pyranine enclosed in *E. coli* membrane vesicles is a reliable indicator of the changes in internal pH caused either during oxidation of electron donors or associated to the generation of artificial ΔpH (interior alkaline) as a consequence of the creation of outwardly directed acetate diffusion gradients. Calibration of the pH_{in} changes elicited by imposed acetate gradients led us to establish quantitative relationships between the activity of the $\Delta\bar{\mu}H^+$-driven Na^+-H^+ antiport

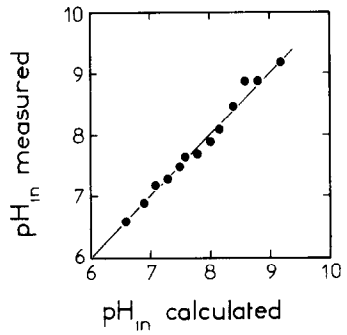

Fig.3. Quantitation of the pH_{in} change produced at ΔF_{max} during imposed acetate gradients in vesicles equilibrated and diluted at pH 6.6. Abscissa: pH_{in} (calculated): theoretical pH_{in} changes calculated as pH_{in} (calc.) = 6.6 + log $acetate_{in}/acetate_{out}$ where 6.6 is the pH_{in} prevailing at the end of the equilibration period. Ordinate: pH_{in} (measured). pH_{in} values estimated at a time the changes in fluorescence signal are maxima (fig.2B). Calculation of the fractional increases in internal pyranine fluorescence and corresponding pH changes were estimated as explained in fig.2.

activity and the chemical proton gradient in *E. coli* membrane vesicles and in particular to show that the internal proton concentration is a major control parameter of the antiport function [13]. Also, kinetics of H^+ movements catalyzed by H^+-symport systems are currently analyzed in *E. coli* membrane vesicles.

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