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The role of efflux systems and the cell envelope in fluorescence changes of the lipophilic cation 2-(4-dimethylaminostyryl)-1-ethylpyridinium in *Escherichia coli*

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Abstract

The interaction of the fluorescent dye 2-(4-dimethylaminostyryl)-1-ethylpyridinium cation (DMP^+) with cells of *Escherichia coli* AN120 (*uncA*) and AS-1 (*acrA*) was studied to elucidate the role of the envelope and of efflux systems in the uptake of lipophilic cations. DMP^+ bound to the two strains in a different manner. With AS-1 the bound dye was displaced only to a small extent by addition of Mg^{2+} or other divalent cations. By contrast, 50% of the DMP^+ was displaced by micromolar concentrations of Mg^{2+} from resting cells of AN120. Energization of the cells by substrate oxidation resulted in the loss in AN120 of 50% of the bound dye and a decrease of the fluorescence in the cell suspension. With AS-1, energization caused more DMP^+ to be taken up from the medium. This was associated with an increase in fluorescence in the cell suspension. The extent of the quenching by addition of Mg^{2+} was not increased. Right-side out vesicles from AN120, like those of AS-1, showed DMP^+ fluorescence behaviour which resembled that of intact cells of AS-1. Transformation of AS-1 with plasmids encoding the *E. coli* Mvr and EmrAB efflux systems resulted in the DMP^+ fluorescence response of this strain becoming like that of AN120. It is suggested that with strain AN120 the changes in binding of DMP^+ and fluorescence intensity were associated with activation of efflux systems on cell energization. With AS-1, it is suggested that the observed fluorescence and binding changes are due to inactivation of the AcrAB efflux system by the *acrA* mutation. Thus, the net entry of lipophilic cations is facilitated. Energization of dye uptake and release is driven by an electrochemical gradient of protons. ATP is not directly involved in energizing the movement of the dye.

Keywords: 2-(4-Dimethylaminostyryl)-1-ethylpyridinium; DMP^+; Lipophilic cation; Efflux system; Energization; Envelope; (*E. coli*)

1. Introduction

The permeation of lipophilic substances across the envelope of Gram-negative organisms such as *Escherichia coli* is not well understood but clearly has great potential interest for the development of chemotherapeutic agents [1]. The outer membrane is considered to constitute a partial barrier to permeation due to the presence of lipopolysaccharide in its outer monolayer [1]. It has also become apparent recently that several types of efflux systems having a broad specificity for lipophilic substances may have a role in determining the extent of net permeation of substances [1,2] and indeed may have a primary role in resistance to toxic compounds [3,4].

The fluorescent dye 2-(4-dimethylaminostyryl)-1-ethylpyridinium cation (DMP^+) was introduced by Bereiter-Hahn [5] to measure the metabolic state of mitochondria in situ. Rafael and coworkers [6,7] concluded that this dye responded to the transmembrane potential of mitochondria. Midgley and coworkers [8–10] have examined the response of DMP^+ with bacterial cells. They observed that the dye was apparently taken up by the cells but was extruded on addition of glucose. They proposed that extrusion was driven by a lipophilic cation efflux system, possibly energized by cation/proton antiport [8–12].

We have shown that DMP^+ responds to the imposition of a transmembrane potential in phospholipid vesicles by moving from the headgroup region of the bilayer into the region of the fatty acyl chains [13]. We have also studied the uptake of DMP^+ by *E. coli acrA* strain AS-1 [14]. We

Abbreviations: DMP^+, 2-(4-dimethylaminostyryl)-1-ethylpyridinium; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; PCP, pentachlorophenol; SDS, sodium dodecyl sulfate; TPP^+, tetraphenylphosphonium.

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found that uptake of DMP^+ in AS-1 was driven by the transmembrane potential. This strain is highly sensitive to methylene blue and detergents which inhibit growth. Until recently, it was believed that *acrA* mutations led to increased rates of permeation of nonpolar substances through the outer membrane [15,16]. However, it now seems more likely the increased susceptibility of *acrA* mutants to nonpolar substances is due to inactivation of a component of an efflux system for nonpolar substances [1,2,17].

In the present paper we have examined the role of the envelope and of efflux systems in the DMP^+ response. The fluorescence behaviour of DMP^+ in wild-type strains revealed a distinct difference when compared to AS-1. Energization resulted in an increase in fluorescence intensity with AS-1 but a decrease was observed with wild-type strains. The fluorescence changes occurring on energization have been rationalized in terms of alterations in the environment of the probe and changes in the amount bound to the cells. ATP has been shown to have no direct role in the changes in DMP^+ fluorescence.

2. Materials and methods

2.1. Chemicals

DMP^+, 2-(4-dimethylaminostyryl)-1-ethylpyridinium, and CCCP, carbonylcyanide *m*-chlorophenylhydrazone were obtained from Sigma. PCP, pentachlorophenol, was obtained from Calbiochem. TCS, 3,3′,4′,5-tetrachlorosalicylanilide, was obtained from Eastman. TPP^+, tetraphenylphosphonium, was obtained from ICN. Other chemicals were of reagent grade obtained from commercial sources.

2.2. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. Bacterial cultures were grown to stationary phase (to diminish the contribution of endogenous substrates as energy sources) at 37°C with aeration from a 1% inoculum on Penassay Broth (Difco) medium. The cells were harvested by centrifugation and washed once by centrifugation from 50 mM Hepes buffer, pH 7.5.

2.3. Transformation of strains

Competent cells were prepared from AS-1 by the calcium chloride method described by Sambrook et al. [18] or by electroporation. Minipreparations of plasmid DNA were obtained by the alkaline lysis method described by Sambrook et al. [18]. Transformants with pASP505 were selected on the basis of their resistance to ethidium bromide (80 μg/ml) and ampicillin (50 μg/ml) on Penassay Broth (Difco) agar plates. Transformants with other plasmids (pUC151A and pEMR2.1) were selected initially for ampicillin resistance and examined subsequently for resistance to other appropriate agents (see Table 4).

2.4. DMP^+ fluorescence with intact cell preparations

The fluorescence of DMP^+ was measured at 22°C with an SLM-Aminco SPF-500C spectrofluorometer interfaced to a Hewlett Packard Model 7470A plotter, using an excitation wavelength of 467 nm and an emission wavelength of 557 nm. Washed cell pellets obtained by harvesting 40 ml of cultures were resuspended in 3 ml of 50 mM Hepes buffer, pH 7.5. 50 μl of these cell suspensions were added to 2 ml of 50 mM Hepes buffer (pH 7.5) in a cuvette of 1 cm path length. The resulting suspensions contained 1 mg of cell protein. Assays were started by the addition of DMP^+ at the concentration indicated in the legends to the figures.

The wavelength of maximum fluorescence emission of DMP^+ was determined by scanning emission from 500 nm to 700 nm with the excitation wavelength set at 467 nm.

2.5. Binding assay for DMP^+

DMP^+ fluorescence assays were terminated at the desired time points by transferring 1.5 ml of the contents of the cuvettes to microfuge tubes and centrifuging at 13 000 $\times g$ for 3 min. The supernatants were pooled and their absorbance scanned from 350 nm to 550 nm using a

Table 1
E. coli strains used in this study

Strain or plasmid designation	Genotype	Source
Strains		
W3110	K-12	Dr. Yasuo Imae, Ngoya University, Japan
AS-1	isogenic with W3110, acrA	Dr. Yauo Imae, Ngoya University, Japan
AN180	argE3 thi mtl xyl rpsL704	Dr. F. Gibson, Canberra, Australia
AN120	isogenic with AN180, uncA401	
Plasmids		
pUC151A	contains *acrAE* genes	Dr. Z. Ma, University of California, Berkeley
pEMR2.1	contains *emrAB* genes	Dr. O. Lomovskaya, Massachusetts Institute of Technology
pASP505	contains *mvr* gene conferring ethidium resistance	Dr. M. Midgley, University of Hull, UK

Perkin-Elmer Lambda 3A spectrophotometer. DMP^+ (free) content was calibrated by comparison with absorbance from known concentrations of DMP^+.

2.6. Preparation of membrane vesicles

Right-side out membrane vesicles were prepared by osmotic lysis of spheroplasts. Spheroplasts were prepared by the method of Witholt et al. [19] and were converted to right-side out vesicles by osmotic lysis in distilled water. The vesicles were collected by centrifugation at 144 000 × *g* for 2 h, washed, and resuspended in 50 mM Hepes, pH 7.5. Aliquots of right-side out membrane vesicle suspensions were added to DMP^+ fluorescence assay systems to give a final protein concentration of 0.5–1.0 mg/ml. DMP^+ fluorescence was measured as described for intact cell experiments.

2.7. Determination of ATP content by the luminometric method

Duplicate fluorescence assay systems were established to allow sampling for ATP content during the course of the experiment. At the desired time points, 0.1 ml of the assay mixture was removed and added to 0.4 ml of distilled water at 100°C. After 3 min, the samples were cooled on ice and then centrifuged briefly to remove insoluble material. Samples (0.1 ml) of the quenched supernatants were added directly to luminometer cuvettes. The assay was initiated by adding 0.1 ml of buffered firefly extract (Sigma), mixing briefly, and placing the cuvette in the chamber of an LKB 1250 Luminometer linked to a LKB 1250 display and recorder unit. Light intensity was calibrated by comparison with light emission from known concentrations of ATP.

2.8. Determination of protein

Protein was measured by the method of Lowry et al. [20] using bovine serum albumin as a standard.

2.9. Determination of oxygen depletion

Oxygen depletion coincident with fluorescence changes was confirmed by cytochrome reduction as previously described [21].

3. Results

3.1. Energization of fluorescence intensity changes in E. coli AN120 and AS-1

We have shown previously [14] that fluorescence intensity of the dye DMP^+ increases on energization of intact cells of *E. coli* strain AS-1. We attributed the fluorescence

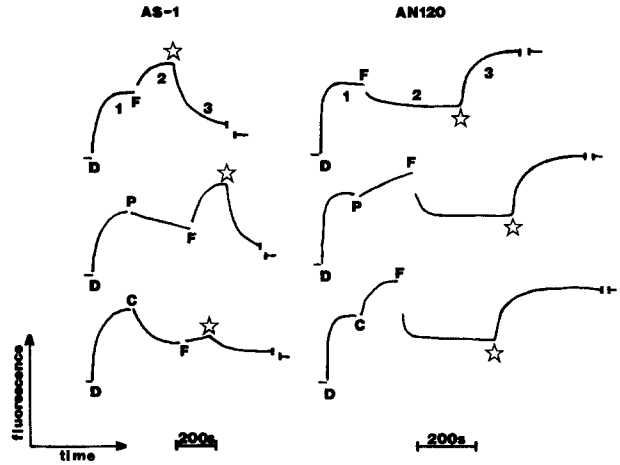

Fig. 1. Effect of substrates and lipophilic compounds on the fluorescence of DMP^+ in cell suspensions of AS-1 and AN120. Additions to the cell suspension were as follows: D, DMP^+ (AN120, 6.25 μM; AS-1, 2.5 μM); F, formate (10 mM); P, pentachlorophenol (12.5 μM); C, CCCP (12.5 μM). The star indicates the point at which dissolved oxygen in the system is exhausted. 1, 2 and 3 indicate phases 1, 2 and 3. Changes in fluorescence are shown on the vertical axis. The final equilibrium fluorescence intensity is shown at the end of each trace.

increase to increased uptake of the dye from the medium although the extent of binding was not measured. It was suggested that uptake was driven by the establishment of a membrane potential across the cytoplasmic (inner) membrane. The possible role of ATP in energization was not examined.

E. coli AN120 is an *uncA* strain. It has a defect in the α subunit of the F_1F_0 ATPase and thus cannot use an electrochemical gradient of protons to energize the formation of ATP [22]. The fluorescence intensity of DMP^+ was followed after addition to a cell suspension of strain AN120 (Fig. 1). A stable level of fluorescence was quickly established after addition of the dye (phase 1). Fluorescence was partially quenched when formate was added (phase 2). Succinate, DL-lactate, glucose and glycerol were effective also (results not shown). Following depletion of dissolved oxygen in the system there was an abrupt increase in fluorescence intensity to phase 1 levels (phase 3). By contrast, addition of formate (or glucose) to a cell suspension of strain AS-1 gave an increase in fluorescence intensity (Fig. 1). This increase declined to phase 1-like levels on anaerobiosis (phase 3). Removal of dissolved oxygen prevented substrate-induced changes in fluorescence intensity. Similar results to those with AN120 were obtained with AN180 (*unc*$^+$ parent of AN120) and with W3110 (parent of AS-1).

The results show that DMP^+ responds differently with strains AN120 and AS-1. However, with both strains the changes in fluorescence intensity respond to the availability of substrate and oxygen suggesting that they are mediated through the establishment of an electrochemical gradient of protons by the respiratory chain.

The role of the proton electrochemical gradient in ener-

Table 2
ATP levels during substrate utilization

Strain	Substrate	ATP (nmol/mg protein)		
		phase 1	phase 2	phase 3
AS-1	glucose	2.4±0.4	4.5±0.1	7.5±0.5
	formate	30.3±0.15	4.9±0.1	3.8±0.6
AN120	glucose	1.7±0.6	6.3±0.35	5.8±0.8
	formate	1.6±0.4	3.5±0.36	1.4±0.6
	glucose(+arsenate)	0.91	0.84	0.33

Samples of the assay systems were removed before the addition of substrate (phase 1), during the aerobic phase following addition of substrate (phase 2), or after the system had become anaerobic (phase 3). ATP was determined as described in Section 2. When arsenate was present, the cells were preincubated with 0.5 mM sodium arsenate for 3 min before addition of DMP^+.

gizing changes in fluorescence intensity of DMP^+ was further investigated using uncoupling agents. These agents are known to dissipate the proton gradient. As shown in Fig. 1 with AN120, PCP, and CCCP caused an increase in fluorescence intensity which could be quenched by subsequent addition of formate. These results are consistent with the uncouplers acting to dissipate the proton electrochemical gradient established by oxidation of endogenous substrate and which had led to partial quenching of dye fluorescence. In contrast, addition of PCP and CCCP to AS-1 caused a decrease in fluorescence intensity which could be reversed on energization by formate. This is consistent with the proton electrochemical gradient driving uptake of DMP^+. It was also apparent that some DMP^+ had been taken up in response to the electrochemical gradient created by metabolism of endogenous substrates. This was released by the addition of uncouplers.

The lack of direct involvement of ATP in the fluorescence changes was examined by measurement of the ATP concentrations during the sequence of fluorescence changes (Table 2). Changes in fluorescence intensity and ATP concentration did not run in parallel. The lack of a role for ATP in the fluorescence changes was also supported by the effect of arsenate on ATP levels and fluorescence intensity in strain AN120 (Table 2). Glucose induced normal fluorescence quenching in the treated cells in spite of the drastic reduction in ATP levels in the presence of arsenate. These results do not entirely exclude a role for ATP in DMP^+ movement but the dye would have to respond to ATP in the 50 μM range.

3.2. Effect of Mg^{2+} on DMP^+ fluorescence

Addition of low concentrations of $MgCl_2$ or $MgSO_4$ to a suspension of cells of strain AN120 in the presence of DMP^+ resulted in a 50% decrease in fluorescence intensity (Fig. 2a). By contrast, $MgCl_2$ and $MgSO_4$ had a much smaller effect on the fluorescence of DMP^+ with strain AS-1 (Fig. 2c). Other divalent cations (Mn^{2+}, Ca^{2+}) at a concentration of 62.5 μM had similar effects to Mg^{2+}. Na^+ and K^+ were effective only at much higher concentrations (100 mM range).

The effect of Mg^{2+} ions under different physiological states of the cells was also investigated (Fig. 2). Under all conditions, Mg^{2+} had a greater effect on the fluorescence of DMP^+ in AN120 than in AS-1. The effect of Mg^{2+} on AN120 appeared to be similar before (phase 1) or after (phase 3) addition of formate (Fig. 2a,b). Addition of Mg^{2+} during the aerobic phase 2 (results not shown) caused only a small further decrease in fluorescence. In the case of AS-1, the small effect of Mg^{2+} (Fig. 2c) decreased still further on energization by formate oxidation (Fig. 2d).

A possible explanation of the effect of Mg^{2+} is that DMP^+ is being displaced from cation-binding sites on the surface of the cells and that more of the dye is at the surface, and therefore accessible to Mg^{2+}, in AN120 compared with AS-1. We have confirmed experimentally that Mg^{2+} causes release of bound DMP^+ from AN120 (see later).

3.3. Binding of DMP^+ during membrane energization / deenergization

Binding of DMP^+ to cells in suspension was assessed by removing the cells by sedimentation in a microcentrifuge and measuring by spectrophotometry the amount of the dye remaining in the supernatant. As shown in Table 3, approx. 20% of the DMP^+ added to the medium was bound by cells of both AN120 and AS-1 under the conditions of this experiment (phase 1). Energization by formate oxidation (phase 2) increased the amount of DMP^+ bound by AS-1 but decreased that bound by cells of strain AN120. Following the deenergization occurring on anaerobiosis (phase 3), bound dye was lost with strain AS-1 and regained with strain AN120. Thus, the changes in the binding of DMP^+ parallel the changes in fluorescence

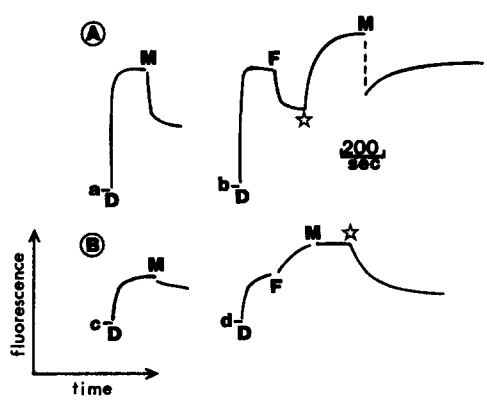

Fig. 2. Decrease in the fluorescence of DMP^+ in cell suspensions of AN120 (A) and AS-1 (B) produced by exogenous Mg^{2+}. Additions to the cell suspensions were as follows: D, DMP^+ (AN120, 12.5 μM; AS-1, 3.75 μM); F, formate (10 mM); M, $MgSO_4$ (62.5 μM). The star indicates the point at which dissolved oxygen in the system is exhausted. Changes in fluorescence are shown on the vertical axis. The bar indicates 200 s on the time scale.

Table 3
Extent of binding of DMP^+ to cells following the addition of substrate

Strain	Addition	% added DMP^+ bound		
		phase 1	phase 2	phase 3
AN120	formate	20	13	26
AS-1	formate	21	54	28

Cells were sedimented from suspensions containing DMP^+ before the addition of formate (phase 1), during the aerobic phase after the addition of formate (phase 2), and after the system had become anaerobic (phase 3). The amount of DMP^+ in the supernatants was determined by absorption spectroscopy as described in Section 2. The extent of variation in the measurements was 2–3%.

intensity observed during the different phases. It should be noted that the amount of dye bound by AN120 in phase 1 represents binding during the state of partial energization generated by oxidation of endogenous substrates.

3.4. Effect of Mg^{2+} and membrane deenergization on the binding of DMP^+

The extent of binding of DMP^+ was measured as described in the previous section. Addition of Mg^{2+} during phase 1 resulted in loss of bound DMP^+ to a greater extent in AN120 (60%) than in AS-1 (26%) indicating that a greater proportion of the DMP^+associated with cells of AS-1 was less accessible to Mg^{2+} than in AN120 (Fig. 3).

Addition of 25 μM PCP, an uncoupler which causes membrane deenergization, to AN120 cells resulted in increased binding of DMP^+ but there was no increase in the amount of DMP^+ which could be displaced by Mg^{2+}. A similar result was obtained with AS-1, although the extent of the change was less (Fig. 3).

3.5. The role of efflux systems in the DMP^+ response

To investigate the involvement of an efflux system in the DMP^+ response, strain AS-1 was transformed by plasmids encoding the products of the *acrAE* genes (pUC151A), *mvrC* gene (pASP505) or *emrAB* genes (pEMR2.1). The *emr* genes, which map at 57.5 min, encode a system which confers resistance to various nonpolar compounds including CCCP and nalidixic acid but not to lipophilic cations such as ethidium or triphenylphosphonium [23]. The *mvrC* gene (at 12.3 min on the *E. coli* genome) causes the efflux of ethidium and methyl viologen [24–26]. The plasmid encoding the *acrAE* genes conferred resistance to sodium dodecyl sulfate and methylene blue, but not to CCCP, on AS-1. However, the *mvrC* plasmid did not provide resistance to these agents (Table 4). Plasmid pEMR2.1 conferred resistance to CCCP and SDS and methylene blue (Table 4).

The effect of these plasmids on the DMP^+ response was examined. As shown in Fig. 4, the presence of pASP505, pUC151A or pEMR2.1 did not alter the DMP^+

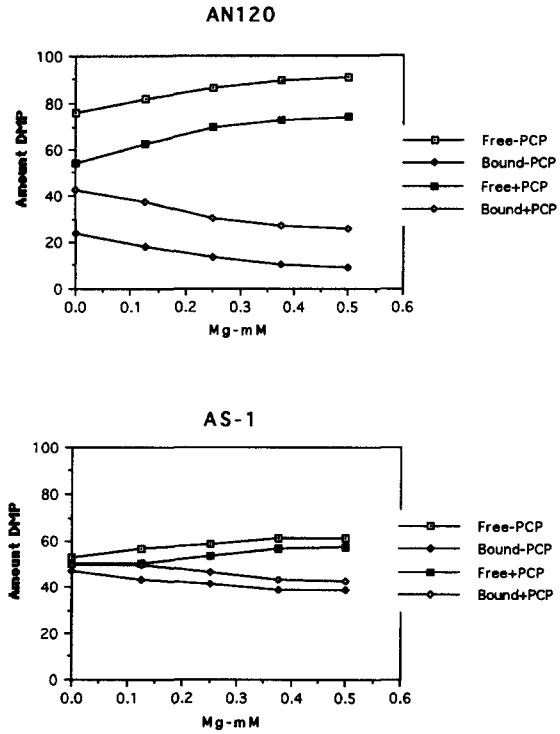

Fig. 3. Effect of Mg^{2+} on the binding of DMP^+ to intact cells of strains AN120 and AS-1 in the presence or absence of PCP. Cells incubated with DMP^+ in the presence and absence of PCP (25 μM) were treated with the indicated concentration of Mg^{2+}, sedimented by centrifugation, and the amount of DMP^+ in the supernatant estimated by absorption spectroscopy. The amount of bound or free DMP is expressed as a percentage of the total DMP^+ added.

response of strain W3110 (curves 1–5), parent of AS-1, and was typical of that shown by AN120 and other wild-type strains. However, all three plasmids converted the response of AS-1 to that typical of AN120 and W3110 (curves 7–9).

These results indicate that the presence and activity of efflux systems for nonpolar compounds profoundly affects the DMP^+ response. Experiments were now directed towards determining the environment of the probe in the envelope during membrane energization and deenergization.

Table 4
Growth sensitivity of transformed strains to various compounds

Strain	Sensitivity (S) or resistance (R) to			
	SDS	Methylene blue	CCCP	Ampicillin
W3110	R	R	R	S
AS-1	S	S	S	S
AS-1 pASP505	S	S	S	R
AS-1 pEMR2.1	R	R	R	R
AS-1 pUC151A	R	R	S	R

Sensitivity or resistance to the agents indicated was determined by growth on Penassay Broth agar plates containing 50 μg/ml methylene blue, 80 μM CCCP, 50 μg/ml ampicillin or with 2% SDS on a filter paper disc.

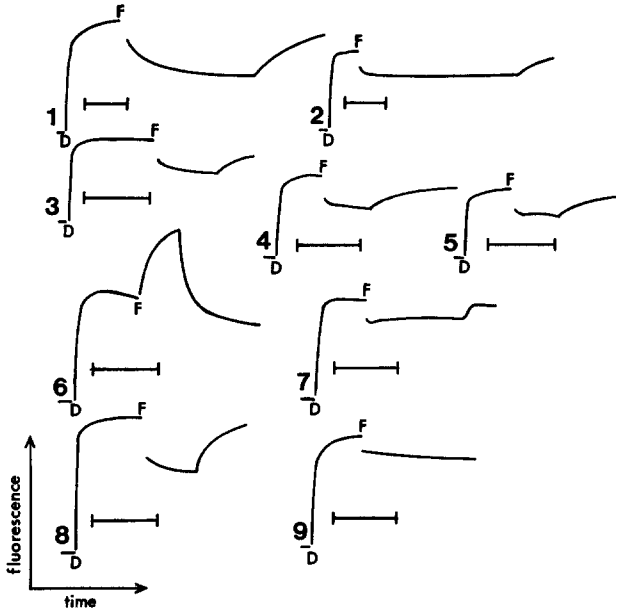

Fig. 4. Effect of substrate on the fluorescence of DMP^+ in cell suspensions of W3110 (curves 1–5), and AS-1 (curves 6–9) transformed with pASP505 (curves 2, 7), pEMR2.1 (curves 4, 8) or pUC151A (curves 5, 9). Curves 1, 3 and 6 were obtained with nontransformed cells. Curve 1 is the control for curve 2. Curve 3 is the control for curves 4 and 5. Curve 6 is the control for curves 7–9. Additions to the cell suspension were as follows: D, DMP^+ (2.5 μM); F, formate (10 mM). Changes in fluorescence are shown on the vertical axis. The bars indicate 200 s on the time scale.

3.6. Changes in the environment of DMP^+ during membrane energization / deenergization

The wavelength of maximum fluorescence emission of DMP^+ is sensitive to the polarity of the medium in which the probe is located. The following emission maxima were measured in a series of solvents of decreasing polarity: water, 598 nm; methanol, 598 nm; ethanol, 593 nm; chloroform, 572 nm. A wavelength of 584 nm was observed in non-energized liposomes of egg phosphatidylcholine. In a solution (5 mg/ml) of lipopolysaccharide, the emission maximum was at 574 nm.

The wavelength of the fluorescence emission maximum of DMP^+ in the presence of a cell suspension under different conditions is shown in Table 5. DMP^+ in the presence of a suspension of cells of strain AS-1 gave an emission maximum at 577–582 nm, suggesting that the dye was located in a medium of polarity intermediate between that of egg phosphatidylcholine and chloroform. Energization by formate oxidation (phase 2) resulted in an increase in the fluorescence emission maximum to 585–588 nm, indicative of a more polar location of the dye. In phase 3, on exhaustion of dissolved oxygen, and a decrease in the fluorescence intensity, the wavelength of maximum fluorescence emission returned to the initial phase 1 range. By contrast, DMP^+ with AN120 showed

Table 5
Changes in the wavelength of maximum fluorescence emission by DMP^+ following addition of substrate

Strain	Addition	Emission maximum (nm)		
		phase 1	phase 2	phase 3
AN120	formate	576–579	571–573	581–583
AS-1	formate	577–582	585–588	579–582

The fluorescence emission spectrum of the system containing DMP^+ and cells in buffer was measured prior to the additions indicated (phase 1), during the aerobic phase after the addition of formate (phase 2), and after the system had become anaerobic (phase 3).

fluorescence emission maxima suggesting that the dye was located in a less polar environment on energization by formate oxidation. It is likely that the values for the emission maxima in phase 1 for AN120 are somewhat lower than they would be in non-energized cells due to partial energization by oxidation of endogenous substrates.

3.7. DMP^+ fluorescence in inner membrane vesicles

The behaviour of the dye was examined using right-side out vesicles of wild-type (W3110) and AS-1 strains. The wild-type vesicles gave a similar response to AS-1. Substrate induced an increase in fluorescence in both instances (Fig. 5). Fluorescence declined on anaerobiosis. The presence of TPP^+, a putative inhibitor of the *mvrC* efflux system [8,12], had little effect on fluorescence behaviour in both strains.

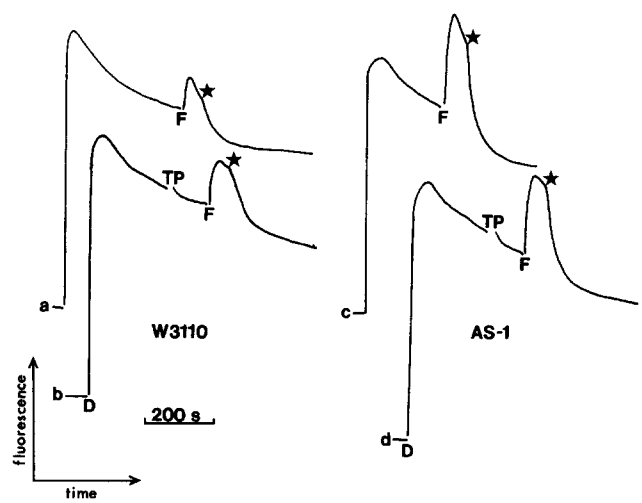

Fig. 5. Effect of substrate oxidation on the fluorescence of DMP^+ in suspensions of right-side out vesicles of W3110 and AS-1. Additions to the vesicle suspensions were as follows: D, DMP^+ (12.5 μm); F, formate (10 mM); TP, TPP^+ (12.5 μM). The star indicates the point at which dissolved oxygen in the system is exhausted. Changes in fluorescence are shown on the vertical axis. The bar indicates 200 s on the time scale.

4. Discussion

The fluorescence intensity of DMP^+ in cell suspensions of AN120, an *uncA* strain, and AS-1, an *acrA* strain, responds to cell energization. Addition of DMP^+, which has only a weak fluorescence in aqueous solutions, to a cell suspension resulted in binding of dye and an enhancement of fluorescence. The establishment of the proton electrochemical gradient in AS-1 increased the uptake of dye from the medium. This was associated with an increase in fluorescence intensity and with apparent movement of the dye into a more polar environment, as shown by the change in the wavelength of maximum fluorescence emission. Deenergization of AS-1 on anaerobiosis led to a decrease in fluorescence intensity, loss of dye from the cells, and the indication that the dye was now in a less polar environment. By contrast, AN120 (AN180 and W3110) showed the inverse response. On energization, DMP^+ was lost from the cells and the probe was in a less polar environment. Deenergization on anaerobiosis produced a reversal of these changes. Thus, the direction of the changes in fluorescence intensity was paralleled by the changes in the amount of bound dye. Energization of the fluorescence changes could not be related to the utilization of ATP.

In a previous study [14] we had suggested that the difference in the responses of AS-1 and AN120 were due largely to the different permeability properties of the outer membranes of these strains. It was suggested that AS-1, an *acrA* mutant, had a defect in the outer membrane which permitted more ready access of nonpolar substances to the inner membrane [16] and that efflux systems for such compounds could not cope with the amount of probe available. Thus, net uptake occurred in response to the membrane potential. The outer membrane was presumed to be a more effective barrier in the parent W3110 and other wild-type strains. The recognition that the *acrA* mutation affects a component of an efflux system, and indeed that efflux systems may be responsible for the apparent barrier effect of the outer membrane in *Pseudomonas* [3,4], indicates the need to reexamine our previous explanation.

Transformation of AS-1 with pUC151A which carries the *acrAE* genes converts the DMP^+ response of AS-1 to that typical of wild-type strains such as W3110 (or AN120). Whereas this response might be due to repair of the effects of the defect caused by the *acrA* mutation, a similar result was obtained when AS-1 was transformed with pASP505 carrying the *mvrC* gene or with pEMR2.1, which carries the *emrAB* genes. Both of these plasmids result in an increase in the activity of efflux systems for nonpolar components. The proton electrochemical gradient appears to energize both uptake and efflux of DMP^+ since both processes were inhibited by uncouplers. During uptake the dye equilibrates across the membrane in response to the transmembrane potential (interior negative). Efflux is likely driven by a H^+/DMP^+ antiport mechanism [8,9,14,27]. The net direction of movement of the dye probably depends on the activity of the efflux systems. Thus, the diminished activity of an efflux system for nonpolar compounds as in strain AS-1, or if the efflux system is damaged [1,2] as in right-side out vesicles of AN120, results in net uptake of dye. By contrast, the levels of efflux activity of AN120 (and W3110) are sufficient to remove DMP^+ entering in response to the membrane potential such that energization results in net efflux of the dye.

The effect of Mg^{2+} on bound DMP^+ was used to investigate the location of the probe within the envelope. Only a fraction of the bound DMP^+ was released on addition of Mg^{2+} (Fig. 3). As can most clearly be seen with AN120, the amount released was independent of the state of energization of the cells. Thus, although more than twice as much DMP^+ was bound by AN120 in the presence of the uncoupler PCP than in its absence, the amount of probe released by Mg^{2+} was about the same. An essentially similar result was obtained with AS-1 (Fig. 3). These results suggest that there exists a pool, of DMP^+, affected by addition of Mg^{2+}, which is a significant proportion of the total probe bound in energized AN120 but which is a smaller fraction in deenergized AN120 or in AS-1. The location of the Mg^{2+}-displaceable pool of DMP^+ within the cell envelope is not known. Although the outer membrane is the most likely site, it is possible that it might be associated with the outer leaflet of the inner membrane. However, under these circumstances added Mg^{2+} would have to readily penetrate the outer membrane to enter the periplasm.

We propose that DMP^+ added to AS-1 equilibrates from the external medium into the Mg^{2+}-displaceable pool. On energization there is a re-equilibration of the dye under the influence of the transmembrane potential. DMP^+ is taken up from the medium and more dye binds to the inner membrane. This results in an increase in fluorescence intensity. Since the quantum yield is increased in a nonpolar environment [7], it is difficult to reconcile the observed increase in fluorescence intensity with the observed red-shift in the emission maximum which suggests that the dye has moved into a more polar environment. A possible explanation is that the red-shift is due to aggregation of the accumulated dye. In the presence of active efflux (e.g., in AN120), the accumulated DMP^+ is discharged into the medium. This results in a decrease in fluorescence intensity. The observed blue-shift in the emission maximum could be due to the reversal of the cause of the red-shift observed above and would be consistent with the remaining bound DMP^+ being located in a nonpolar environment.

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