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Biochemistry

Affinity of intact *Escherichia coli* for hydrophobic membrane probes is a function of the physiological state of the cells

(probe binding/fluorescent photoreactive membrane probe/colicin Ia/anoxia/substrate depletion)

DAVID NIEVA-GOMEZ* AND ROBERT B. GENNIS*†

Departments of * Chemistry and † Biochemistry, University of Illinois, Urbana, Illinois 61801

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ABSTRACT **The fluorescence parameters of several common membrane probes in the presence of whole *E. coli* have been examined. The probes included electrically neutral lipophilic molecules *N*-phenyl-1-naphthylamine, pyrene, and 1,6-diphenyl-1,3,5-hexatriene as well as the negatively charged molecule 8-anilino-1-naphthalene sulfonate. It is demonstrated in each case that certain fluorescence parameters are a function of the state of energization of the cells.**

All the probes appear to monitor structural changes in the *E. coli* envelope which accompany the energization and de-energization of the cells. The phenomenon is completely reversible as demonstrated by re-energizing anoxic cells by the addition of oxygen, or starved cells by the addition of substrate. All the results are qualitatively consistent with an increased binding of probe by de-energized cells and a subsequent expulsion of probe when the cells are re-energized.

A pyrene substituted with a photosensitive group, 1-azidopyrene, has been synthesized. Photolysis in the presence of a suspension of energized *E. coli* reveals a relatively small amount of probe irreversibly bound to the cells. However, in the presence of cells that have been de-energized the amount of irreversibly bound probe is dramatically increased. This molecule should be useful for localizing the regions of the bacterial envelope that are involved in the structural changes being monitored in these experiments.

The use of fluorescent probes to study membrane structure and processes has become widespread. Electrically charged probes such as 8-anilino-1-naphthalene sulfonate (ANS) and cyanin dyes have been used to monitor membrane potential in liposomes and membrane preparations from mitochondria and bacteria (1–3). Uncharged lipophilic probes such as *N*-phenyl-1-naphthylamine (NPN), pyrene, and 1,6-diphenyl-1,3,5-hexatriene (DPH) have been used primarily to monitor the fluidity or microviscosity of the lipid bilayer (4–6). It has recently been shown that the fluorescence parameters of NPN in the presence of whole *Escherichia coli* cells are a function of the physiological state of the cells (7). Cells that are de-energized bind significantly more NPN than cells that are energized, leading to about a 4-fold increase in the fluorescence intensity when the cells are de-energized by such treatments as substrate or oxygen depletion, addition of electron transport inhibitors, addition of uncouplers, or addition of colicin Ia. The fluorescence changes can be rapidly reversed if the cells are restored to the energized state by addition of oxygen to anoxic cells or the addition of substrate to starved cells. It appears that this lipophilic probe monitors structural changes in the *Escherichia coli* envelope which occur reversibly as the cells are energized and de-energized. These results probably explain at least in part the changes in NPN fluorescence observed with colicin-E1-treated cells (8). The effects of these colicins on the cells resemble in several respects the effects of uncouplers (9, 10).

In an attempt to determine the nature of these structural changes in the cell envelope, the present work reports studies with probes that have fluorescent, structural, and electrical properties different from those of NPN. In addition, a photoreactive probe, 1-azidopyrene, has been used to confirm the conclusion that there is a reversible change in the affinity of the cells for hydrophobic molecules as the cells are energized and de-energized. Upon irradiation, a highly reactive nitrene is generated (11), resulting in covalent, fluorescent adducts presumably with components of the bacterial envelope.

Abbreviations: ANS, 8-anilino-1-naphthalene sulfonate; NPN, *N*-phenyl-1-naphthylamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

MATERIALS AND METHODS

Cell Growth. *E. coli* K-12 strain JK1 was grown at 37° in M9 medium, which consists of glucose at 1.3 g/liter and M9 salts (M9S). M9S is composed of the following (in g/liter): NH_4Cl, 1.0; $MgSO_4$, 0.13; KH_2PO_4, 3.0; Na_2HPO_4, 6.0. Cells were harvested at mid-logarithmic phase (Klett reading of 100 in a Klett colorimeter, 42 filter), washed with M9 salts and resuspended in M9 salts to a density of about 5×10^8 cells per ml (Klett 80, 42 filter) for fluorescence experiments. All measurements were made within 2 hr after harvesting. The cells showed 100% viability after at least 3 hr under these conditions. The presence of the fluorescent dyes or the solvents in which they were added had no effect on either cell growth or viability.

Starvation of Cells. The procedure used for the starvation of cells has been described in detail elsewhere (7) and consisted of a modification of a procedure reported by Berger (12).

Colicin. Purified colicin Ia was prepared and its concentration was determined as previously described (13). For most fluorescence experiments, colicin Ia was added to a final concentration of about 0.7 μg/ml. Survival levels were near 1%.

Dyes and Reagents. DPH, ANS, and NPN were obtained from Eastman, and pyrene was obtained from Aldrich. The purity of the dyes was confirmed by thin-layer chromatography and comparison between observed and reported fluorescence spectra. All other reagents used were of the highest purity commercially available.

Fluorescence Measurements. Fluorescence measurements were made at 37° (unless otherwise specified) with cells suspended to a density of 5×10^8 cells per ml in M9 medium minus glucose (M9S buffer). In most experiments the dyes were added in organic solvents directly to the cell suspension. The NPN, 1-azidopyrene, and ANS were added in ethanol. The DPH was added in a dioxane solution which was prepared fresh before use. The final concentration of organic solvent never exceeded 0.4%. When added directly in this manner to M9S buffer, ANS and NPN form clear, stable solutions. When prepared in this

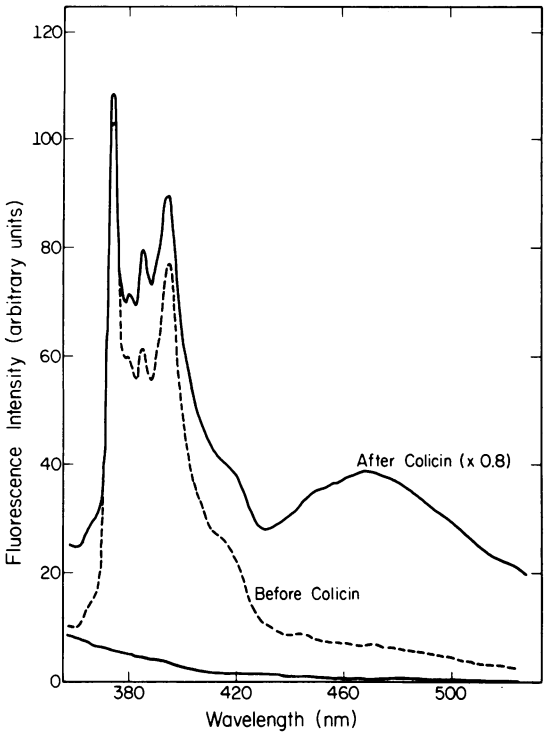

FIG. 1. The effect of colicin Ia on the emission spectrum of pyrene in the presence of *E. coli* cells. Lowest trace corresponds to the cell background. Excitation wavelength was 275 nm. The excitation and emission bandpasses were 10 nm and 2 nm, respectively. A 310 nm cut-off filter was placed in the path of the emission. All spectra are uncorrected. Pyrene concentration was 4 μM. Colicin Ia was added to a final concentration of 0.72 μg/ml.

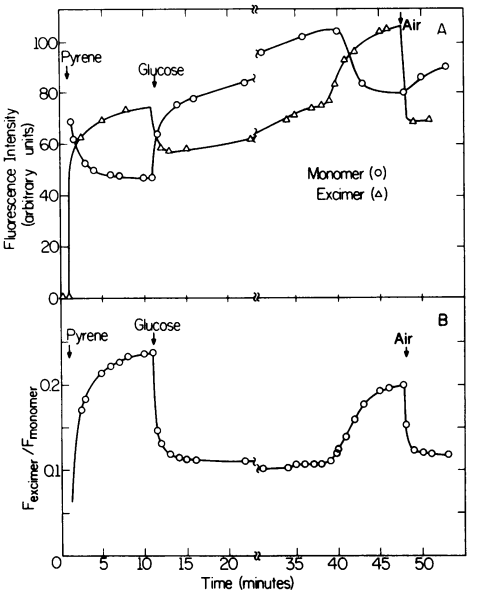

FIG. 2. (*A*) The fluorescence emission intensities of excimer (Δ) and monomer (O) of pyrene in the presence of starved cells as a function of time, through the sequence of substrate addition, anoxia, and oxygenation. The excitation wavelength was 320 nm. The emissions of monomer and excimer were detected at 392 nm and 480 nm, respectively, from the same sample. The excitation and emission bandpasses were 10 nm and 2 nm, respectively. A 350 nm cut-off filter was placed in the path of the emission. Pyrene and glucose were added to final concentrations of 3 μM and 20 mM, respectively. Note that the scales for the monomer and excimer emissions are not the same. (*B*) The ratio of the fluorescence emission intensities of excimer and monomer as a function of time, obtained from the traces in part *A*.

manner, pyrene, which is less soluble in water than NPN, forms a clear, supersaturated solution which is highly fluorescent. After 1 or 2 days at room temperature the pyrene aggregates and precipitates. In a few experiments with starved cells, pyrene or DPH was added using the more common procedure of swirling the cell suspension in a flask in which the probe had been dried. Because of the long times required for equilibration, this procedure was not used routinely. The method of sample preparation did not alter the final results. All measurements of fluorescence intensity, polarization, and lifetime (not reported) were made as previously described (7).

1-Azidopyrene. This probe was synthesized from 1-aminopyrene (Eastman). The details of the synthesis and characterization of this molecule will appear in a subsequent report. The probe was added from a 2 mM stock solution in methanol to a final concentration of 4 μM in a suspension of cells (5×10^8 cells per ml) in M9S buffer. All procedures involving this probe were carried out in the dark up to the actual photolysis. Experiments were performed using 10 ml samples which were kept at 37° in a 250 ml beaker. In all cases, after the addition of the azidopyrene the sample was further incubated for about 4 min with shaking at 37° prior to further treatment (see the caption to Fig. 4). Photolysis was carried out by irradiating the thinly spread suspension for two 20-sec periods using a Sylvania SG-55 lamp type DWY held about 30 cm above the sample. The cells were then washed twice with M9S buffer and resuspended to about 5×10^8 cells per ml in order to record the fluorescence emission from the irreversibly bound probe. The probe is completely removed from the cells by this washing procedure unless the probe is photolyzed in the presence of the cells; photolysis of the probe prior to mixing with the cells results in no irreversibly bound probe. The cells are not perturbed by this entire treatment, remain viable, and fully retain their ability to actively transport proline in the presence of glucose.

RESULTS

Fig. 1 shows the fluorescence emission spectrum of pyrene in the presence of *E. coli* cells before and after treatment with colicin Ia. The highly structured band (360–430 nm) corresponds to the emission of the excited monomer molecule, whereas the broad band (430–560 nm) corresponds to the emission of the excimer. As can be seen, a large increase in the emission intensity of the excimer relative to that of the monomer occurs as a result of treatment with colicin Ia. The phenomenon of excimer formation is well understood (14, 15). The transient excimer species forms upon collision of a molecule in the first singlet excited state and a molecule in the ground state. Under conditions of constant light irradiation, factors that increase the probability of such collisions increase the excimer fluorescence intensity. In the present context, an increase in the excimer:monomer ratio may result from an increase in the local concentration of the bound probe or its translational mobility or both.

Similar qualitative changes in the pyrene emission were observed if the cells were de-energized by starvation or by anoxia. In these cases, however, the physiological changes in the cells could be reversed and monitored by the probe fluorescence. This is illustrated in Fig. 2. In Fig. 2*A* the monomer and excimer intensities are shown, and in Fig. 2*B* the ratio of the two is plotted as a function of time. The cells were starved prior to the addition of pyrene. The excimer contribution is substantial, in contrast to that seen when the cells have not been depleted of substrate (see Fig. 1). After the addition of glucose, however, the excimer emission decreases and that from the

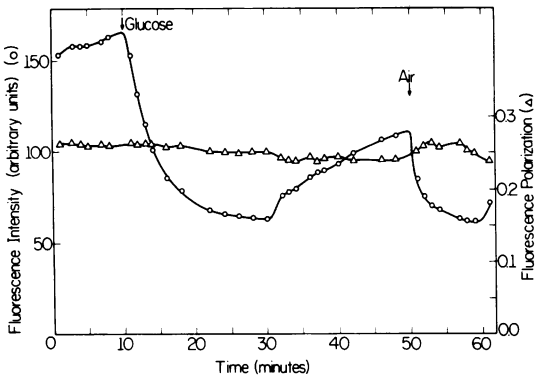

FIG. 3. The fluorescence emission intensity and polarization of DPH in the presence of starved cells as a function of time, through the sequence of substrate addition, anoxia, and oxygenation. The excitation wavelength was 360 nm as defined with a grating monochromator and a Corning 7-60 broad band filter. The emission was defined with a $NaNO_2$ filter (2 M, 2 mm thick) and a Corning 3-73 cut-off filter. DPH concentration was 40 μM. Glucose concentration was 20 mM. Cells were pre-incubated with the dye for 20 min. Polarization values were not corrected for scattering of light.

monomer increases. Addition of substrates such as D-lactate and succinate accomplishes the same result. The excimer:monomer emission intensity ratio drops sharply to a low value and remains nearly constant for about 30 min. In these experiments the addition of glucose resulted in a 10-fold increase in the rate of cell respiration. The gradual depletion of oxygen in the sample is probably responsible for the increase in intensity of the pyrene emission observed following the glucose addition, because oxygen is known to be an effective quencher of pyrene (16). However, it should be noted that the ratio of excimer:monomer emission intensities remains constant until the cells become anoxic, at which point there is a relative increase in the excimer emission as the cells once again become de-energized. This is reversed when air is bubbled into the cuvette.

Other fluorescent probes which were examined were DPH, ANS, and 9-vinylanthracene. Fig. 3 shows the fluorescence intensity and polarization from DPH in the presence of starved *E. coli* as the cells are energized and de-energized as described for pyrene. Upon the addition of glucose there is a substantial decrease in the fluorescence intensity, which remains at a low level until the cells become anoxic and it begins to increase. This increase is reversed when air is bubbled into the cuvette. The fluorescence changes are slower in this case than for the other probes that were examined, which may reflect the slower kinetics of redistribution of the DPH molecules. Clearly, there is very little change in the polarization while the intensity is changing by more than a factor of two. Previous workers have demonstrated that the fluorescence intensity of ANS in the presence of whole *E. coli* changes upon the addition of colicin E1 (17), or upon treatment with cyanide, the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), or upon oxygen depletion (18). ANS fluorescence (not shown) follows the same pattern as demonstrated for DPH and NPN upon the addition of glucose to starved cells and upon anoxia. With 9-vinylanthracene, the polarization value is dramatically increased when the cells are de-energized (not shown). It should be noted that in all cases there was no significant change in the absorption or light scattering by the sample at either the excitation or emission wavelengths.

The strategy of employing a photoreactive probe to determine the locus of the structural changes in the bacterial envelope is illustrated in Fig. 4. 1-Azidopyrene was photolyzed in

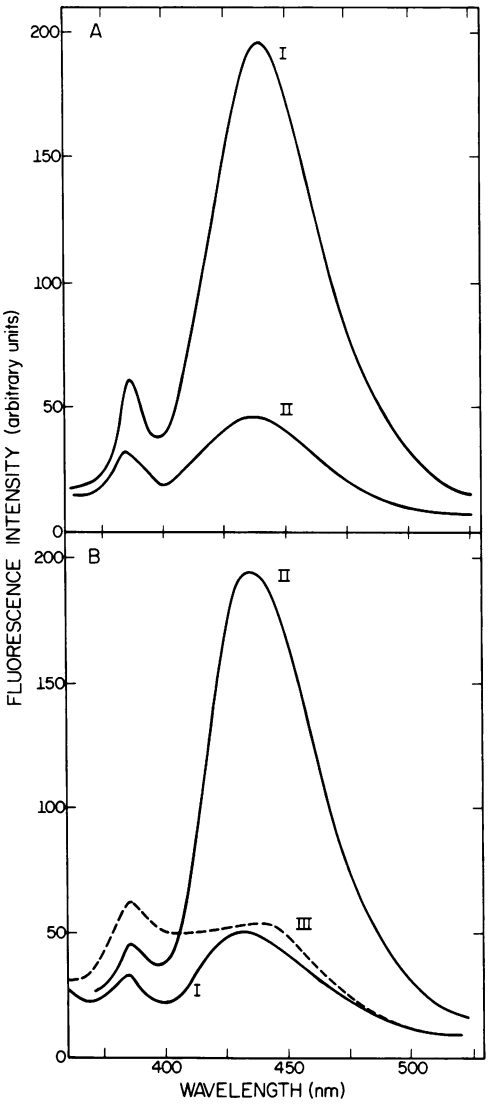

FIG. 4. Fluorescence emission spectra from suspensions of *E. coli* containing the irreversibly bound adduct resulting from the photolysis of 1-azidopyrene. Emission spectra were recorded at room temperature using an excitation wavelength of 340 nm and excitation and emission bandpass widths of 10 nm and 2 nm, respectively. All spectra are uncorrected, and the contribution from the cell background has not been subtracted; the small peak at about 385 nm is largely due to this background and Raman scattering. (*A*) Starved JK1 cells were incubated for 35 min prior to photolysis at 37° under the following conditions: without any further additions (I); after the addition of glucose to a final concentration of 10 mM (II). (*B*) After incubation with 1-azidopyrene for 4 min, unstarved JK1 cells were treated as follows prior to photolysis: I—sample was further incubated for 30 min (37°, with shaking) without any further addition. II—CCCP (2 μM, final concentration) was added and the sample was incubated for 30 min. III—CCCP (2 μM, final concentration) was added and the sample was incubated for 15 min. Glucose (10 mM, final concentration) was then added and the sample was further incubated for 15 min.

the presence of both energized and de-energized *E. coli*, the cells were washed twice with large amounts of M9S and resuspended, and the fluorescence from the irreversibly bound probe was recorded. Starved cells bind a relatively large amount of probe as judged by the intensity of the emission (Fig. 4*A*). However, if glucose is added and the cells are re-energized prior to the photolysis, the amount of bound probe decreases substantially. The addition of glucose subsequent to the photolysis has no immediate effect. The spectrum obtained from the cells

that have been re-energized (Fig. 4A, curve II) is superimposable with the results obtained if the cells have not been starved (see Fig. 4B, curve I). Fig. 4B demonstrates that the results are essentially the same if the cells are de-energized by using an uncoupler, CCCP, rather than by substrate depletion. Photolysis prior to the addition of CCCP results in low probe binding (Fig. 4B, curve I), whereas photolysis subsequent to CCCP addition indicates a dramatic increase in bound probe. Under these conditions CCCP virtually abolishes the ability of the cells to actively transport proline as determined by using a slight modification of the procedure described by Gilchrist and Konisky (9). Using this same criterion, it can be shown that the physiological effects of the uncoupler can be partially reversed by the addition of glucose. The apparent difficulty reported by Cramer *et al.* (19) in substantially reversing the effect of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) under similar conditions was not encountered, perhaps reflecting different assay procedures. Under the conditions described (Fig. 4) the ability to transport proline is restored to about 67% of the value obtained in the presence of glucose alone. This correlates qualitatively with the apparent expulsion of much of the probe: if the photolysis is performed subsequent to the sequential addition of CCCP and glucose, the amount of bound probe is greatly reduced (Fig. 4B, curve III). It should be noted that the changes in the fluorescence parameters of the probes such as NPN in response to these same conditions are all consistent with an alteration in probe binding.

The cells containing the irreversibly bound probe remain viable and can be energized and de-energized as before. However, the fluorescence from the probe, which is now apparently covalently bound to the cells, shows little response to the various treatments.

DISCUSSION

A very conspicuous fact which arises from these studies is the extremely low level of binding to energized *E. coli* cells of hydrophobic molecules such as NPN, pyrene, and DPH, all of which partition very favorably from the aqueous phase into lipid vesicles. It appears that the integrity of the *E. coli* cell envelope plays an essential role in this phenomenon because treatments that disrupt the cell surface, such as extensive washing with a high concentration of Tris buffer in the absence of divalent cations, irreversibly increase the affinity of the cells for NPN (Nieva-Gomez and Gennis, unpublished). Recent studies have led other authors to conclude that pyrene in the presence of whole *E. coli* cells binds almost exclusively to the outer membrane (16) and the ability of hydrophobic molecules to bind to and penetrate the *Salmonella typhimurium* envelope has been shown to correlate with the lipopolysaccharide structure (20) in the outer membrane.

Interactions of bacterial cells with both negatively (17, 18) and positively (21) charged probes have been reported to depend on the energization state of the cell. Those observations are qualitatively similar to those presented for the electrically neutral probes NPN, DPH, pyrene, and 9-vinylanthracene. Evidently, there is a structural change in the bacterial envelope upon de-energization which results in enhanced binding of both charged and neutral lipophilic membrane probes. The results with the photoreactive probe tend to confirm this interpretation. It should be stressed that the increased binding of hydrophobic molecules by the cells upon being de-energized cannot be explained merely as the breakdown of a permeability barrier. The fact that the probes are apparently expelled upon re-energization of the cells cannot be readily explained by such a model.

The locus and nature of the structural changes, and indeed any possible physiological meaning that they may have, have not been determined. Because probes likely to be located in different sites of the cell envelope respond in a similar manner, it appears that the phenomenon is a rather widespread structural alteration, as opposed to a localized change in a particular region of the membrane or involvement of a particular protein species. The increase in the pyrene excimer emission upon de-energization of the cells is indicative of an increase in the local concentration of the probe in some relatively fluid region of the envelope. A fraction of the bound NPN must also be located in lipid regions, because it monitors thermotropic lipid phase transitions in whole bacteria as well as in bacterial membrane preparations (5). On the other hand, the energy transfer from tryptophan to NPN indicates that some of this probe must be near protein (Nieva-Gomez and Gennis, unpublished). The fluorescence polarization studies with DPH indicate that there is probably no large change in the average microviscosity of the environment of the bound probe after addition of glucose to the starved cells, although about half of the bound probe has apparently been expelled.

It is to be hoped that 1-azidopyrene or a similar photoreactive probe will be useful in determining which components of the bacterial envelope are responsible for the observed changes in the affinity for hydrophobic molecules. The initial question is whether the inner membrane, or the outer membrane, or both are involved. It may also be possible to determine the degree to which proteins or lipids are involved in probe binding; however, the resolution offered by this technique will depend upon such factors as the probe mobility within the membrane and the lifetime of the reactive nitrene species. Photoreactive membrane labels have been used previously (22–24), although in the work reported here the fluorescence of the photoproducts is being exploited.

The notion that structural differences exist between an energized and a de-energized membrane is supported by several observations reported in the literature (25–31). Future experimentation may reveal whether the phenomenon reported in this work results from alterations in specific membrane proteins, or alterations in ion distributions such as changes in local pH, or osmotic changes, or perhaps some other effect induced by the manipulation of the bioenergetic state.

Finally, in studying the membranes of intact *E. coli*, it is critical to handle the samples with cognizance of the physiological response of the organism. Clearly, the physiological state of the cells can have a drastic effect on the results of some experiments.

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