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Membrane Deenergization by Colicin K Affects Fluorescence of Exogenously Added but Not Biosynthetically Esterified Parinaric Acid Probes in *Escherichia coli*

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Fluorescence of the conjugated polyene fatty acid, parinaric acid (PnA), was studied in membranes of *Escherichia coli* during deenergization by colicin K. The free fatty acid and biosynthetically esterified forms of *cis*-PnA (9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid), both of which are sensitive to *E. coli* lipid-phase transitions, were compared. When free *cis*-PnA was added exogenously to respiring bacteria, dissipation of the energized state of the membrane resulted in a dramatic increase in *cis*-PnA fluorescence; *all-trans*-PnA was much less sensitive. Neither spectral shifts nor a change in *cis*-PnA fluorescence polarization were observed. Analysis of the PnA content of extracellular fractions of deenergized and control cells revealed a difference in probe distribution: the membranes of energy-poisoned *E. coli* bound about 77% of exogenously added *cis*-PnA, whereas membranes of actively respiring controls bound only about 44%. No fluorescence enhancement was observed in cells centrifuged to remove unbound *cis*-PnA before colicin treatment. When *cis*-PnA was biosynthetically esterified to phospholipids of an unsaturated fatty acid auxotroph of *E. coli*, the fluorescence did not change during membrane deenergization. In double-probe experiments, membrane deenergization resulted in fluorescence enhancement of exogenously added *N*-phenyl-1-naphthylamine, without change in esterified PnA fluorescence. We conclude that deenergization of *E. coli* membranes leads to increased binding and fluorescence of exogenously added PnA and cannot be detected from within the inner and outer membranes by PnA esterified in vivo.

Despite the significant contribution of fluorescent probes to the understanding of bacterial membrane energetics, central structural details of deenergization remain unexplained. The transmembrane electrochemical potential (6, 7, 14) of *Escherichia coli* is dissipated by colicins (13, 23, 26), uncouplers of oxidative phosphorylation, electron transport inhibitors, anoxia, or substrate starvation; each causes enhanced binding and fluorescence of many different types of probes (2, 3, 8-10, 15, 16, 25). Unfortunately, little is known about the nature of the underlying structural changes, particularly the identity of the components undergoing a change. It would be useful to examine this problem systematically, studying each class of membrane component individually. However, two serious problems are encountered with exogenously added probes: partitioning among aqueous, membrane, and intracellular environments, and uncertain location and orientation once associated with the membrane.

In recent years the naturally occurring conjugated polyenes (18-20) have gained attention as sensitive probes of lipid phase transitions and lateral phase separation in model membranes (18, 20, 21), bacterial membranes (22), and animal cell membranes (17). In a previous study (22) we found conditions for the biosynthetic esterification of small amounts of the parinaric acid (PnA) probes, *cis*-PnA (9,11,13,15-*cis-trans-trans-cis*-octadecatetraenoic acid) and *trans*-PnA (9,11,13,15-*all-trans*-octadecatetraenoic acid) (18-20) to membrane phospholipids of an unsaturated fatty acid auxotroph of *E. coli*. Similar lipid phase transitions were detected by the free fatty acid and esterified forms of PnA in cells supplemented with oleic or elaidic acid. Here we discuss the utility of free and esterified PnA as probes of bacterial membrane energetics. We have used free *cis*-PnA to demonstrate the sensitivity of the exogenously added probe to *E. coli* membrane deenergization. Upon biosynthetic esterification, the partitioning problems cited above are eliminated, and probe fluorescence reveals the properties of an intrinsic membrane phospholipid during the deenergization event.

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MATERIALS AND METHODS

Growth of bacteria. Growth was carried out in the E medium (24) supplemented with 1% Casamino Acids (Difco) and 5 μg of thiamine per ml. Strain 30Eβox⁻ (12), derived from MO(F⁻*rpsL*) (5), is an unsaturated fatty acid auxotroph (*fabB*) of *E. coli* K-12 deficient in β-oxidation of fatty acids and was provided by C. Linden and C. F. Fox. Growth medium for the auxotroph was supplemented with 35 μg of oleic acid per ml, 0.5% Triton X-100, and either 70 μg of *cis*-PnA (added as a 10-mg/ml solution of ethanol) per ml or the equivalent amount of ethanol. Biosynthetic incorporation of PnA was determined from the absorbance spectrum of extracted (1) phospholipids as described (22). After four generations of logarithmic growth at 37°C, cells were harvested at approximately 150 Klett units (Klett-Summerson colorimeter, no. 54 filter), washed three times with medium E plus 0.5% Triton X-100 to remove free fatty acids, and washed twice with medium E to remove detergent. Samples were resuspended with medium E to 100 Klett units (5×10^8 cells per ml) for fluorescence measurements. Wild-type K-12 strain 1100 (obtained from R. D. Simoni) was treated in the same manner, except that growth was carried out in rich medium without further supplement and washing of harvested cells was omitted.

Samples of purified colicin K and the colicinogenic strain K235 of *E. coli* were kindly supplied by S. E. Luria; strain K235 was grown as described (11) for the preparation of colicin K.

Energy poisoning. To maintain active respiration during fluorescence measurements, 2.5 mM glucose was added and samples were aerated continuously through a syringe needle at 32 ± 2°C. Samples were then treated with 4 to 20 μM carbonylcyanide *m*-chlorophenylhydrazone (Sigma), 6 mM KCN or 3 μg of a crude colicin K preparation (11) per ml. Cell survival was determined from plating efficiency on rich agar medium containing 1.5% agar (strain 1100) or rich agar medium spread with 2 mg of oleic acid per 20-ml plate (strain 30Eβox⁻).

Fluorescence measurements. *cis*-PnA and *trans*-PnA were obtained from L. A. Sklar or prepared from the seeds of *Parinari glabberimum* as described previously (20). Free *cis*-PnA was added to samples at 1.1 to 1.5 μM; *N*-phenyl-1-naphthylamine (NPN) (Eastman) was added at 2 μM. Fluorescence intensity was recorded as a function of time with a Perkin-Elmer MPF44 fluorescence spectrophotometer (set in the energy mode) equipped with an Omnigraph 2000 X-Y recorder. In double-probe (esterified *cis*-PnA and NPN or esterified *cis*-PnA and free *cis*-PnA) experiments, difference spectra were recorded with a Perkin-Elmer model 512 fluorimeter (set in the subtract mode) by using a sample containing only one probe (esterified *cis*-PnA) as a reference. In preliminary fluorescence polarization measurements, the ratio $I_{\parallel}/I_{\perp}$ was measured with an Hitachi-Perkin-Elmer model MPF2A fluorimeter equipped with Glan-Thompson UV transmitting polarizers. $I_{\parallel}$ (polarizing filters oriented parallel) and $I_{\perp}$ (second filter rotated 90°) were recorded as a function of time by rotating filters every 20 to 30 s during colicin killing of cells containing free *cis*-PnA.

To determine the extent of free PnA partitioning, 50-ml samples (approximately 2.5×10^{10} cells) of colicin-treated cells and untreated controls were collected by centrifugation at 32 ± 2°C. The amount of PnA remaining in the supernatant solutions was determined as follows: 400 μg of dimyristoylphosphatidylcholine (DMPC) (prepared by sonication at 20 mg per ml in 50 mM potassium phosphate buffer, pH 7.0) was added per ml of supernatant of energized or deenergized cells. An excitation spectrum of PnA fluorescence was recorded at 10°C at an emission wavelength of 410 nm. At 10°C, DMPC is solid, and the resulting high quantum yield of PnA (20) permits quantitation of small amounts of probe. The magnitude of peak PnA fluorescence in each sample was compared with a standard curve prepared by plotting PnA fluorescence intensity as a function of concentration under identical conditions in 400 μg of DMPC per ml of medium E.

Heating or cooling of samples utilized a circulating water bath connected to a cuvette holder adapted for temperature control.

RESULTS

Fluorescence of free *cis*-PnA as a function of energy state in *E. coli* cells. In experiments using exogenously added *cis*-PnA, the results obtained with *E. coli* strains 1100 and 30Eβox⁻ were essentially equivalent. Cells exhibited very little background fluorescence at wavelengths of PnA fluorescence. Upon addition of 1.1 μM *cis*-PnA, distinctive PnA fluorescence peaks were observed in the excitation spectrum (Fig. 1B, lower and middle curves). With constant aeration and mixing during measurements and a small band pass for excitation (1 to 3 nm), the fluorescence of *cis*-PnA was stable for at least 40 min. Figure 1A illustrates the fluorescence of *cis*-PnA as a function of time during colicin K treatment at 32 ± 2°C. The time course of the fluorescence increase is in keeping with that of colicin K deenergization (13, 23, 26). Plating efficiency of colicin-treated cells was less than 1% of that of untreated controls. Final values of PnA fluorescence ranged from 1.5 to 3 times the value observed before the addition of colicin K. (The small decrease in fluorescence seen in Fig. 1A after colicin addition was due to absorbance properties of the colicin solution and is not a relevant feature of the experiment.) An excitation spectrum measured after colicin treatment (Fig. 1B, upper curve) demonstrates that the fluorescence increase was due specifically to an increase in *cis*-PnA fluorescence. We also compared the time course of *cis*-PnA fluorescence during treatment of cells with two other classes of membrane energy poisons, CCCP (carbonyl cyanide-*m*-chlorophenyl hydrazone; an uncoupler of oxidative phosphorylation) and KCN (an inhibitor of electron transport). Each poison promoted an increase in the fluorescence

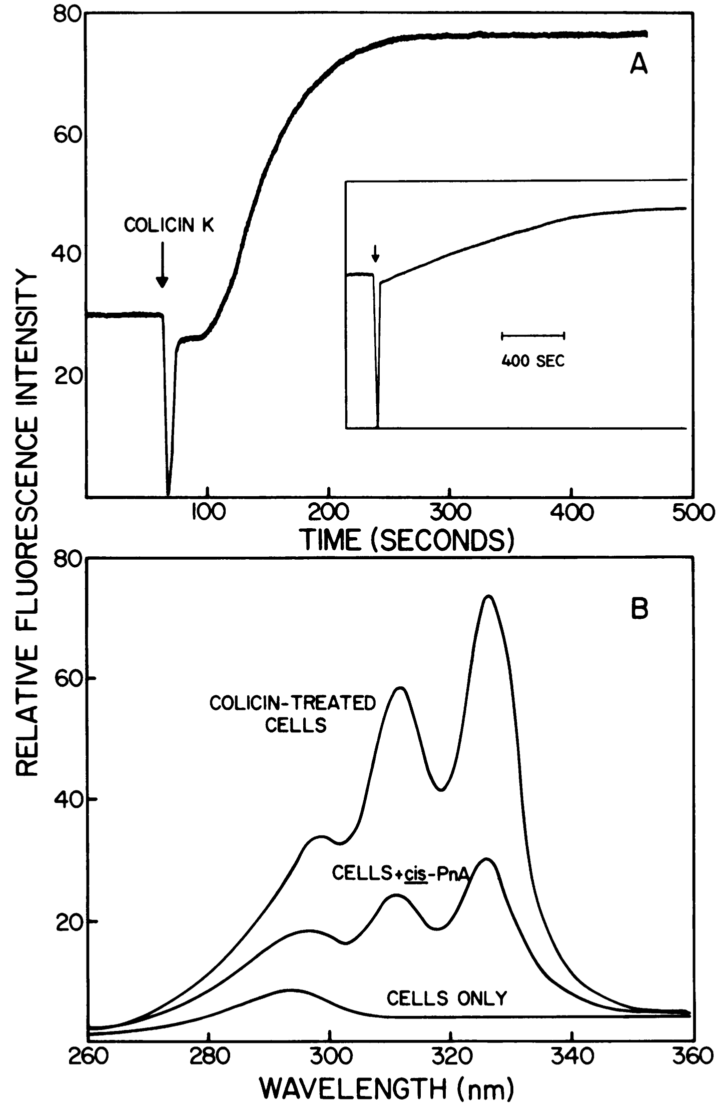

FIG. 1. *Effect of colicin K upon cis-PnA fluorescence. A sample containing 5×10^8 cells of strain 1100 per ml was provided with air, 2.5 mM glucose, and 1.1 μM cis-PnA. (A) Time course of fluorescence upon addition (arrow) of 3 μg of colicin K per ml. Inset: Time course of fluorescence of 1.1 μM trans-PnA under the same conditions. PnA emission was measured at 410 nm (slit setting, 20 nm) during excitation of the sample at 324 nm for cis-PnA and 318 nm for trans-PnA (slit setting, 1 nm). (B) Uncorrected excitation spectra before (lower curve) and after (center curve) the addition of cis-PnA and 15 min after colicin treatment (upper curve).*

of exogenously added *cis*-PnA, similar to that shown in Fig. 1A.

Early in this study the isomers *cis*-PnA and *trans*-PnA were compared as probes in this system, and substantial differences were observed. Colicin treatment of cells containing free *trans*-PnA caused a much slower rise in fluorescence than that observed with *cis*-PnA (Fig. 1A); after 10 to 15 min, the fluorescence leveled off at only 1.2 to 1.4 times the initial values (inset to Fig. 1A). Thus, *cis*-PnA appeared to be far more sensitive to the effects of colicin K. Because the *cis* isomer is also more readily incorporated into phospholipids of strain 30Eβox$^-$ than the *trans* isomer (22), comparisons of the free and esterified forms of PnA as energy probes of *E. coli* were made with *cis*-PnA.

As seen from the excitation spectra (Fig. 1B), energized and deenergized cells have the same fluorescence maxima despite the large fluorescence increase due to colicin treatment. In a preliminary investigation of PnA polarization, the polarization ratio was measured at 32 ± 2°C during colicin treatment of cells containing free *cis*-PnA. $I_{\parallel}/I_{\perp}$ remained constant, at the value typical of fluid *E. coli* cell membranes (E. Tecoma, unpublished data). The phase transitions characteristic of 30Eβox⁻ cells supplemented with oleic acid (data not shown; see reference 22) were reproducible after colicin treatment with proportionately higher fluorescence intensity of *cis*-PnA at all temperatures. These observations suggest that the increase in fluorescence intensity is due to a phenomenon other than a gross change in the physical state of lipid molecules which constitute the probe environment.

Analysis of bound/unbound PnA. Because the fluorescence of PnA in an aqueous solution is negligible compared with that in a hydrophobic membrane environment (20), the partitioning of PnA between cells and aqueous media during the course of membrane deenergization is of primary importance to the analysis of fluorescence enhancement. We compared the amount of unbound *cis*-PnA in the supernatant of control and colicin-treated cells of strain 1100 containing 1.5 μM *cis*-PnA (Fig. 2). The supernatants of untreated and colicin-treated cells in this experiment contained 225 and 95 ng of *cis*-PnA per ml, respectively. The increase in *cis*-PnA binding, from 44 to 77%, is sufficient to account for a large increase in fluorescence. A range of values observed in different experiments (35 to 50% of available *cis*-PnA bound to untreated cells and 70 to 85% bound to colicin-treated cells) was reflected in the range of values obtained for the fluorescence ratio of colicin-treated to colicin-untreated cells (1.5 to 3 in different experiments). The absolute increases in fluorescence and binding usually were not identical (see Discussion).

To separate the fluorescence contributions of *cis*-PnA originally bound to untreated cells and *cis*-PnA newly bound upon deenergization, we treated cells in the absence of free aqueous *cis*-PnA. Bacterial samples equilibrated with 1.5 μM *cis*-PnA were collected by centrifugation (32 ± 2°C) and resuspended in buffer without PnA. Cells were then treated immediately with colicin K. The fluorescence of *cis*-PnA already associated with the cells remained constant (Fig. 3, curves B and B′). However, when 0.75 μM PnA was provided in the resuspension buffer, fluorescence enhancement (Fig. 3, curves C and C′) was observed upon colicin treatment. These data support the hypothesis that newly bound probe is responsible for fluorescence enhancement upon deenergization.

Fluorescence of biosynthetically esterified *cis*-PnA as a function of membrane energy state. Under the growth conditions described in Materials and Methods, *cis*-PnA was biosynthetically esterified (22) to approximately 1 to 3% of membrane phospholipids of strain 30Eβox⁻. The washing procedure effectively removed all free *cis*-PnA present in the growth medium (22). The excitation spectrum of cells containing esterified PnA is shown in Fig. 4A; curve I in Fig. 4C shows PnA fluorescence as a function of time during colicin treatment. There was no increase in esterified PnA fluorescence due to colicin addition; an excitation spectrum recorded 30 min after colicin treatment was identical to that shown as curve I in Fig. 4A.

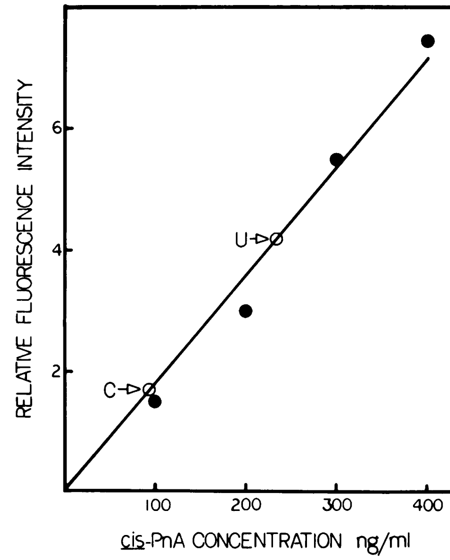

FIG. 2. *Fluorescence of cis-PnA in DMPC (dimyristoylphosphatidylcholine) vesicles. Colicin-treated (C) and untreated control (U) cells in medium E containing 1.5 μM cis-PnA were centrifuged at 32 ± 2°C to compare the amount of unbound probe.* (○) *Fluorescence of cis-PnA in the supernatant solutions upon addition of 400 μg of DMPC vesicles per ml.* (●) *Fluorescence of aliquots of cis-PnA added to 400 μg of DMPC vesicles per ml of medium E. Fluorescence was measured at 10°C. Other settings were the same as those in the legend to Fig. 1.*

Although cell survival, determined by plating efficiency, was found to be less than 1% after colicin treatment, we wised to demonstrate that the time course of colicin action was not altered in 30Eβox⁻ cells containing esterified *cis*-PnA. Therefore, a second fluorescent probe was added before treatment with colicin K. NPN was chosen because it has been shown to be sensitive to membrane deenergization by colicin K (8–10, 15, 16–25), and the fluorescence spectra of NPN and PnA are reasonably distinct. The excitation spectrum of cells containing both esterified PnA

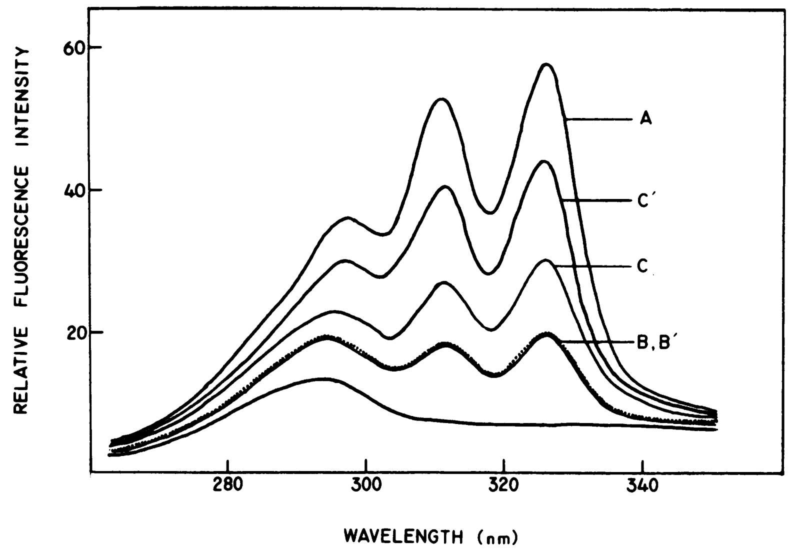

FIG. 3. *Effect of colicin K on fluorescence of resuspended cells. All samples except those represented by the lower curve contained 1.5 μM cis-PnA at the outset and were aerated continuously at 32 ± 2°C while one sample was treated with colicin K. After 10 min, all samples were centrifuged (32 ± 2°C) and resuspended without cis-PnA for spectra and further treatment. (A) Cells treated with colicin before centrifugation; (B) untreated cells; (B′) sample B, 5 min after colicin treatment; (C) same as B, after addition of 0.75 μM cis-PnA; (C′) sample C, 5 min after colicin treatment; lower curve: control cells without cis-PnA. Other conditions are given in the legend to Fig. 1.*

and 2 μM NPN is shown in Fig. 4A. In Fig. 4C, curve II shows the fluorescence of cells containing both probes during colicin treatment. The large fluorescence enhancement upon addition of colicin K is similar in magnitude as well as time course to that reported for NPN by other investigators (8–10, 15, 16, 25) and to that of exogenously added *cis*-PnA in the present study. Fig. 4B contains a difference spectrum of cells containing both esterified *cis*-PnA and NPN before and after colicin treatment. Comparison of the difference spectrum and the excitation spectrum of NPN alone (curves I and II in Fig. 4B) demonstrates that fluorescence enhancement observed in the double-probe experiments is due specifically to an increase in NPN fluorescence. (The small shoulders in the region of PnA fluorescence are attributable to minor differences in cell density—hence endogenous *cis*-PnA—of the sample pair used to record the illustrated difference spectrum.)

In analogous double-probe experiments, 1.5 μM *cis*-PnA was added to cells containing esterified *cis*-PnA. Approximately half the total PnA fluorescence was derived from the free fatty acid. Whereas, cells with only esterified PnA showed no fluorescence change upon colicin treatment, cells with both free and esterified PnA showed an increase in PnA fluorescence, similar in magnitude and time course to the curve shown in Fig. 1A.

DISCUSSION

It is well documented that the affinity of *E. coli* membranes for many hydrophobic probes increases upon deenergization. Examples of probes which show fluorescence enhancement during *E. coli* membrane deenergization include 8-anilino-1-naphthalene-sulfonate (ANS) (8, 9, 15), NPN (8–10, 15, 16, 25), 1,6-diphenyl-1,3,5-hexatriene (15), 3,3′-dihexyloxacarbocyanine (3), chlorotetracycline (2), and pyrene (15).

cis-PnA, a fatty acid which serves as both fluorescent probe and substrate for phospholipid biosynthesis, readily detects membrane deenergization by colicin (Fig. 1). The basis for fluorescence enhancement was found to be increased association of unbound *cis*-PnA with deenergized cells (Fig. 2). The increase in fluorescence intensity and the increase in apparent binding were not identical, the former being somewhat higher. For example, in the experiment of Fig. 2, binding of free *cis*-PnA to cells increased by a factor of 1.7, whereas fluorescence intensity (similar to that of Fig. 1A) increased by a factor of 2.3. The reason for this discrepancy is not obvious, but the values are consistent with a sigmoidal curve of PnA fluorescence as a function of PnA bound (not added) to whole cells. It is plausible that probe access to two or more classes of binding sites is a function of the energy state of cell. Fluorescence lifetime and quantum

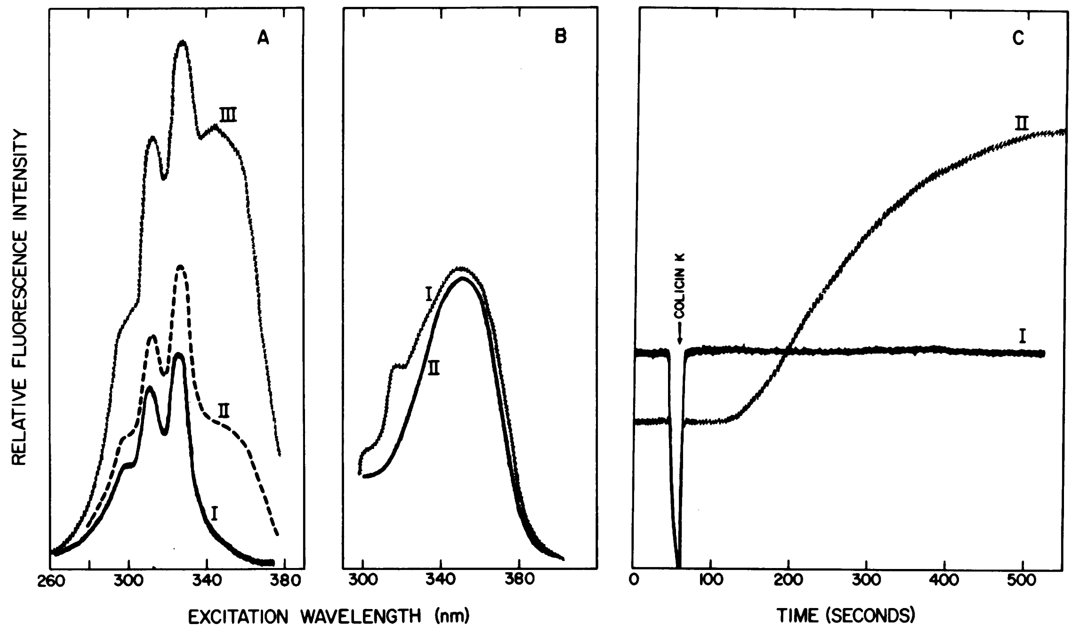

FIG. 4. *Fluorescence of biosynthetically esterified cis-PnA and exogenously added NPN (N-phenyl-1-naphthyl amine). Samples of strain 30Eβox⁻ containing esterified cis-PnA (in 3% of membrane phospholipids) were prepared as described in the legend to Fig. 1. (A) Uncorrected excitation spectra before (curve I) and after (curve II) addition of 2 μM NPN, and after subsequent treatment with colicin K (curve III). (B) Difference spectrum of samples II and III in panel A (curve I). Excitation spectrum of NPN in lipid vesicles (curve II). (C) Fluorescence response to colicin K of cells containing esterified cis-PnA (curve I) or both esterified cis-PnA and 2 μM NPN (curve II). Excitation and emission slit settings were 3 nm and 20 nm, respectively. Wavelengths of excitation were 324 nm (cis-PnA) and 345 nm (NPN); fluorescence emission of either probe was measured at 420 nm.*

yield may be higher, on the average, for probe molecules bound to sites newly available after colicin treatment than for molecules originally bound to the energized cell. This explanation invokes a heterogeneity in the physical properties of the composite of membrane microenvironments which serve as *cis*-PnA binding sites, but does not invoke a change in the properties of any given binding site as a result of colicin treatment. Because the data given in Fig. 2 do not eliminate the possibility of a change in the properties of initially existing binding sites, another type of analysis was used to emphasize the requirement for newly bound probe (Fig. 3). There was no fluorescence enhancement in cells containing *cis*-PnA when unbound *cis*-PnA was removed from the buffer by centrifugation and resuspension of cells before colicin treatment. Although in preliminary experiments we did not detect any change in the polarization ratio measured isothermally during colicin treatment, further analysis, including measurements of the fluorescence lifetime components before and after treatment, may help to resolve the properties of old and new *cis*-PnA binding sites.

Sklar et al. (21) recently reported the partitioning of *cis*-PnA and *trans*-PnA among solid lipid, fluid lipid, and aqueous phases. The mole fraction of bound/free *cis*-PnA in 1.33×10^{-4} M lipid dispersions was found to be 56/44 for a solid lipid and 69/31 for a (different) fluid lipid. Expressed in an analogous fashion from the data of Fig. 2, the mole fraction of *cis*-PnA bound to 5×10^{8} cells per ml was about 44/56 for energized and 77/25 for deenergized cells. Our values are, therefore, in good agreement with their observation that a significant amount of *cis*-PnA remains in the aqueous phase of a lipid (cell) dispersion (suspension). The values observed (21) for binding of *trans*-PnA to phospholipid vesicles were higher than the values observed for *cis*-PnA. Bound/free *trans*-PnA was 87/13 for solid and 80/20 for fluid lipid. Although we did not examine the binding of *trans*-PnA to *E. coli*, with this information we suspect that the low sensitivity of *trans*-PnA to deenergization was due to the relatively small number of probe molecules present in the buffer before treatment. It is also worth noting that *trans*-PnA, by virtue of its partitioning from fluid to solid lipid, is sensitive to the formation of a few percent solid lipid (21). Thus, if lateral phase separation or formation of local clusters of solid lipid were a feature of bacterial deenergization, *trans*-PnA would detect the phenomenon with greater efficiency than *cis*-PnA, the opposite of our observations.

It remains difficult to demonstrate convincingly with any exogenously added probe that a change in the physical properties of membrane lipids accompanies deenergization, or that such a change can be ruled out completely. Studies with exogenously added probes necessarily measure the specific properties of probe binding sites. The extent to which the measurements are physiologically meaningful is dependent upon the extent to which the binding sites are known and can be shown to be representative of the average membrane domain. Because the location of most probes is uncertain and heterogeneous, attempts to isolate changes in, e.g., microviscosity, from qualitative changes superimposed upon quantitative changes in probe binding have led to conflicting reports. For example, Helgerson and Cramer and their colleagues have examined numerous fluorescence parameters of exogenously added probes (8–10, 25) in an effort to distinguish changes in probe binding from dynamic structural changes, particularly changes in microviscosity of membrane components. Their conclusions from a study of the rotational relaxation time of NPN and ANS (8) emphasize that deenergization promotes both changes in probe binding and (unspecified) structural changes in the environment of probe sampling the outer membrane. Nieva-Gomez and Gennis (15) have also used the ANS and NPN probes, in addition to 1,6-diphenyl-1,3,5-hexatriene, pyrene, and its photoactivable derivative 1-azido-pyrene, to arrive at a different conclusion. They reported that quantitative changes in dye binding alone were responsible for fluorescence changes upon deenergization and that membrane microviscosity was not affected. It is of interest to note that 1-azido-pyrene, photolyzed in situ in untreated cells, was relatively insensitive to subsequent deenergization (15). However, the problems attendant in the use of azido compounds severely restrict the interpretation of this type of experiment. Upon photoactivation, the nitrene radical forms covalent adducts nonspecifically with nearest-neighbor molecules in the binding site, which may be protein, lipoprotein, or lipopolysaccharide, as well as phospholipid components of the cell envelope.

In contrast, the present study provides specific information about the physical properties of the average microenvironment of an intrinsic membrane phospholipid during deenergization. In *E. coli*, exogenously supplied fatty acids are esterified to phosphatidic acid and distributed to inner and outer membranes during phospholipid biosynthesis (4). The esterified *cis*-PnA probe itself is an inert membrane component. It does not partition between cells and buffer, and it is not likely to sample any specialized microenvironment within either the outer or inner membrane. The unaltered fluorescence of esterified *cis*-PnA (Fig. 4C) clearly indicates that membrane phospholipids exhibit steady-state fluidity during energy poisoning. Although additional fluorescence parameters of esterified *cis*-PnA await further examination, it is unlikely that a change in either fluorescence lifetime or polarization would escape detection as a change in fluorescence intensity. The fluorescence increased of a second probe (NPN in Fig. 4) added exogenously to these cells illustrates the important point that enhanced binding and fluorescence of exogenous probe persist as a result of deenergization, whereas the same event cannot be detected from within the membrane by an endogenous probe.

A complex combination of quantitative and qualitative changes in binding of exogenously added probes accompanies deenergization of *E. coli*. The rationale for the present study was to clarify the basis for the observed fluorescence enhancement. Our comparison of free and esterified *cis*-PnA reveals that lipid structural changes known to affect esterified *cis*-PnA fluorescence (20, 22) are not a major feature of deenergization. We feel that further elucidation of the details of structural changes during membrane deenergization lies in the continued development of techniques which distinguish between apparently related phenomena. The loss of sensitivity to colicin treatment upon biosynthetic esterification of *cis*-PnA provides a general method to distinguish the deenergized state from conditions which decrease the fluidity of bulk *E. coli* membrane phospholipids. It will be useful to focus now upon other membrane components, for example, proteins of the outer membrane, by using methods which provide specific information about their structure, function, organization, or display under different physiological conditions.

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