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Respiration-Linked Proton Transport, Changes in External pH, and Membrane Energization in Cells of *Escherichia coli*

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The kinetics of respiration-dependent proton efflux and membrane energization have been studied in intact cells of logarithmic-phase *Escherichia coli*. Parallel measurements of the rate and extent of proton efflux into the external medium (half-time, about 10 s; ratio of H^+ to O, about 0.5) and the oxidation of *E. coli* cytochrome *b* (half-time, ≤1 s; about 6% oxidized) after a pulse of 5.5 ng-atoms of O indicate that the rate of proton efflux is at least 10 times slower than expected from the time required for the cells to reduce the oxygen added in the pulse. The kinetics of formation and dissipation of the transmembrane electric potential ($\Delta\psi$) after an O_2 pulse were estimated from changes in the fluorescence properties of the cell envelope-bound probe *N*-phenyl-1-naphthylamine. Under anaerobic conditions, a small pulse of oxygen induced a rapid (half-time, ≤1 s) partial decrease in the fluorescence intensity of the probe, followed by a slower relaxation of the fluorescence change to the original intensity. The extent of the initial rapid decrease was linearly dependent upon the amount of oxygen added in the pulse (0 to 11 ng-atoms of O per pulse), whereas the rate of the subsequent relaxation was accelerated by the uncoupler *p*-trifluoromethoxycarbonylcyanidephenylhydrazone and the K^+ ionophore colicin E1. This suggests that the initial fluorescence decrease after an O_2 pulse reflects the energization of the membrane, whereas the relaxation of the fluorescence decrease reflects the subsequent deenergization of the membrane arising from counterion redistributions. The fact that the efflux of H^+ into the external medium after an O_2 pulse was inefficient and much slower (half-time, about 10 s) than the reduction of the added O_2 (half-time, ≤1 s) and the energization of the membrane (half-time ≤1 s) suggests that some of the protons translocated across the cytoplasmic membrane during a brief pulse of respiratory activity are accumulated in a region of the cell which is not in rapid equilibrium with the external medium.

The notion that transmembrane proton fluxes play an important and obligatory role in the mechanism of energy transduction in bacterial membranes is supported by a large body of experimental data (for recent reviews, see references 12, 13, and 30). According to the chemiosmotic hypothesis proposed by Mitchell (19, 20), the electrically uncompensated transfer of a proton from the cytoplasm of the cell into the external medium, catalyzed by either the respiratory chain or by an H^+-pumping adenosine triphosphatase, results in the formation of a transmembrane pH gradient (ΔpH) and an electrical potential ($\Delta\psi$), which serve as the energy sources driving endergonic reactions such as ATP synthesis and active transport. These endergonic reactions are often coupled to the inward movement of protons (20, 27, 35-38), so that an effective H^+ current is established across the bacterial membrane. In *Escherichia coli* the primary form of membrane energy appears to be the electrical potential, and steady-state values for $\Delta\psi$ of 100 to 140 mV have been measured (11, 16, 24, 26).

Williams has proposed that the actual charge separation and proton movement occur within the membrane and that the functional current of protons does not necessarily include the bulk aqueous phases (39). Recent studies on energy transduction in chloroplast membranes have lent support to this model (23).

In a previous paper (9) we studied the properties of the respiration-dependent proton efflux observed when a small oxygen pulse was rapidly injected into an anaerobic suspension of intact *E. coli* cells. We found that, under normal conditions, where counterion fluxes are minimal, the appearance of protons in the medium (half-time, about 10 s) was 5 to 10 times slower than

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expected, based upon the calculated reduction time for the oxygen added in the pulse. Furthermore, the number of protons extruded for each atom of oxygen added (H^+/O ratio) was rather small (typically about 0.5 for logarithmic-phase cells grown on succinate). Increasing the membrane permeability to charge compensating counterions resulted in much larger H^+/O ratios (>2.0) and much faster H^+ efflux kinetics (half-time, ≤1 s). However, these effects could not be easily explained by a respiratory control phenomenon (28) since (i) respiratory control by conditions which enhance counterion permeability could not be demonstrated under steady-state conditions of oxygen consumption in either the presence or absence of substrate, (ii) the rate of oxygen consumption needed to explain the slow H^+ efflux under normal conditions would be at least 1/5 to 1/10 the steady-state rate observed in the absence of substrate, and (iii) the observed H^+/O ratio was independent of both the cell concentration and the amount of oxygen present in the pulse over a 600-fold variation and down to levels of O_2 per cell sufficient to generate a maximum $\Delta\psi$ of only 16 mV. These results led us to the tentative conclusion that the transmembrane H^+ current associated with membrane energy transduction in *E. coli* need not include the bulk phase of the external medium.

In this paper I have extended our previous investigation by using the energy-dependent changes in the fluorescence properties of the lipophilic fluorescence probe *N*-phenyl-1-naphthylamine (NPN) in an *E. coli* suspension. Cramer et al. (3, 4, 25) were the first to show that the deenergization of the *E. coli* inner membrane by colicin E1 or colicin K led to a structural change in the cell envelope resulting in a two- to three-fold increase in NPN fluorescence intensity. This fluorescence increase was found to arise from both an increase in the number of probe molecules bound to the cells (14, 21, 22) and a change in the microviscosity of the probe binding site(s) (14, 15). Furthermore, it is not specific for membrane deenergization induced by colicins, since very similar fluorescence changes were found to be associated with membrane deenergizations resulting from the addition of uncouplers such as *p*-trifluoromethoxycarbonylcyanidephenylhydrazone (FCCP) or from the inhibition of respiration by KCN or anaerobiosis (21, 22). Nieva-Gomez et al. (21, 22) also showed that the fluorescence increase induced by anaerobiosis could be completely reversed by oxygen.

I report here a further characterization of NPN as an indirect indicator of the energy state of the *E. coli* cell membrane. Under certain limiting conditions, the fluorescence intensity of NPN was found to be linearly proportional to the energy level of the cytoplasmic membrane. When the membrane was completely deenergized by an anaerobic incubation, the oxygen pulse-induced, partial, transient reenergization of the membrane, estimated from the NPN fluorescence response, was very much faster than the efflux of protons into the external medium. Measurements of the kinetics of the partial oxidation of *E. coli* cytochrome *b* after an oxygen pulse indicate that the reduction of the oxygen in the pulse is well correlated with the NPN fluorescence decrease and is very much faster than the observed H^+ efflux.

(A preliminary account of a portion of this work has been presented [7]).

MATERIALS AND METHODS

Bacteria. Cells of *E. coli* strain B/1, 5 were grown on a minimal salts medium (pH 7.0) as described earlier (9), with the addition of trace metals according to Anraku (1). Sterile 250-ml Erlenmeyer flasks containing 50 to 60 ml of minimal medium were inoculated from overnight cultures and incubated at 37°C with vigorous shaking for 4.5 to 5.5 h (midlogarithmic phase of growth). Cells were harvested by centrifugation at 4°C, washed twice in a solution containing 150 mM KCl and 0.5 mM 3-(*N*-morpholino)propane sulfonic acid-KOH (pH 7.0), and resuspended in this medium to a final concentration of about 3×10^9 cells per ml. For experiments involving colicin E1 treatment of cells, the cells were grown as described above, except that 1% (wt/vol) glycerol replaced succinate as the carbon source. This was necessary since colicin E1 does not affect succinate-grown cells under anaerobic conditions, presumably because of an energy requirement for colicin action. After the addition of colicin E1 to the cell suspension, the cells were incubated for 5 to 10 min at 37°C before the addition of oxygen pulses. Cell survival after colicin E1 treatment was less than 0.1%.

Fluorescence measurements. Changes in the fluorescence intensity of the probe NPN were monitored with a Perkin-Elmer Hitachi MPF-4 spectrofluorimeter essentially as described by Helgerson and Cramer (14). Sample temperature was maintained at 33°C by a constant-temperature circulating water bath. The instrument amplifier time constant was set for either a 0.7- or 0.1-s half-response time.

Measurements of fluorescence lifetime, polarization, and, occasionally, changes in fluorescence intensity were performed with an SLM subnanosecond cross-correlation-phase spectrofluorimeter interfaced with a Nova model 1220 minicomputer. The sample compartment was modified to permit continuous stirring with a magnetic stirrer and to allow for thermostatting at 33°C by a constant-temperature circulating water bath. Fluorescence lifetimes (τ) were measured by the phase-delay technique of Spencer and Weber (31), with the exciting light modulated at 10 MHz. The rotational relaxation time (ρ) was calculated on line from the measured lifetime (τ) and polarization (p)

according to the Perrin equation: $(1/p - 1/3) = (1/p_0 - 1/3)(1 + 3\tau/\rho)$, where p_0 is the limiting polarization value (0.39 for NPN).

To achieve anaerobiosis, samples were sealed in 3-ml fluorescence cuvettes with several layers of Parafilm. A small Teflon tube passing through the Parafilm allowed a continuous stream of water-saturated N_2 or Ar gas to be passed over the surface of the sample. A second small hole allowed the escape of the gas and admitted the needle of a microliter syringe, which was used for making additions to the sample. Oxygen pulses generally consisted of 10 μl of air-saturated distilled water containing 5.5 ng-atoms of O (assuming a dissolved oxygen concentration in distilled water of 275 μM at 23°C). The time required to inject a 10-μl pulse was much less than 1 s. The mixing kinetics in the cuvette, measured as the fluorescence increase after a rapid injection of NPN into buffer, showed a half-time of 0.5 to 1 s.

pH measurements. Changes in the pH of the medium after an oxygen pulse were recorded as described earlier (9).

Cytochrome measurements. Changes in the level of reduced cytochrome *b* were monitored with an Aminco-Chance dual-wavelength spectrophotometer. Measurements were routinely performed in the Soret band by using an isobestic point of 450 nm. The percentage of total cytochrome undergoing the spectral change was estimated from a reduced minus oxidized (anaerobic minus aerobic) difference spectrum recorded under similar conditions on a Shimadzu scanning spectrophotometer. Reduction of the cytochrome by dithionite (minus aerobic cells) gave a spectrum similar to that obtained by anaerobic reduction. The optical light path for all spectrophotometric measurements was 10 mm.

Miscellaneous. Colicin E1, with an activity of about 1:50 on a protein basis, was prepared from the colicinogenic *E. coli* strain JC411 (Col E1) by the method of Schwartz and Helinski (29). FCCP, kindly supplied by P. G. Heytler, was dissolved in ethanol. NPN (Eastman Organic Chemicals), recrystallized once, was dissolved in methanol. The *E. coli* B/1,5 cell strain was obtained from S. Silver.

RESULTS

When the lipophilic fluorescent probe NPN was added to an aerobic suspension of intact, logarithmic-phase *E. coli* cells, there were large increases in the fluorescence intensity, polarization, and lifetime of the probe compared with the values observed in buffer alone (Table 1). The increase in fluorescence intensity was accompanied by a pronounced blue shift in the emission spectrum (Fig. 1a and b); also, about 90% of the total fluorescence intensity at 405 nm arose from probe molecules associated with the cells (Fig. 1b and c).

If the cell suspension was incubated under an oxygen-free atmosphere, there was, after an initial lag period, a further (approximately threefold) increase in the fluorescence intensity (Fig. 2), which was associated with increases in the fluorescence lifetime and polarization and an approximately twofold increase in the apparent rotational relaxation time (ρ) of the probe mol-

TABLE 1. *Dependence of fluorescence parameters of NPN on aerobic-anaerobic transitions*[a]

Conditions	I (relative)	τ (ns)	p	ρ (ns)
NPN in buffer[b]	0.14	2.2	0.01	0.2
NPN + cells (aerobic)[c]	1.0	4.4	0.12	5.1
NPN + cells (anaerobic)	2.8	7.0	0.15	11.4
NPN + cells (aerobic)[d]	1.1	5.1	0.12	5.9

[a] I, Intensity; τ, lifetime; p, polarization; and ρ, rotational relaxation time.

[b] The buffer contained 50 mM KCl and 0.5 mM 3-(*N*-morpholino)propane sulfonic acid-KOH (pH 7.0); the concentration of NPN was 4 μM.

[c] 3.2×10^9 cells per ml.

[d] After being anaerobic for about 20 min.

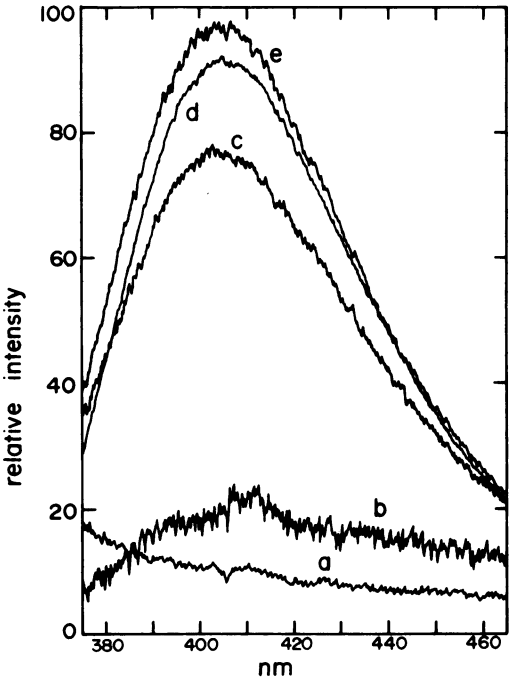

FIG. 1. *Fluorescence emission spectra of NPN under various conditions. Cells of E. coli strain B/1,5 were grown to midlogarithmic phase and resuspended to a final concentration of* 4.3×10^9 *cells per ml. The fluorescence cuvette contained a final volume of 2.5 ml. Spectrum a, Cells in buffer (no NPN) (×3.3); spectrum b, 4 μM NPN in buffer (no cells; ×10); spectrum c, 4 μM NPN plus cells under aerobic conditions (×3.3); spectrum d, conditions as in spectrum c, except under anaerobic conditions (×1); spectrum e, as in spectrum d, except the cell suspension was returned to aerobic conditions before the spectrum was recorded (×3.3).*

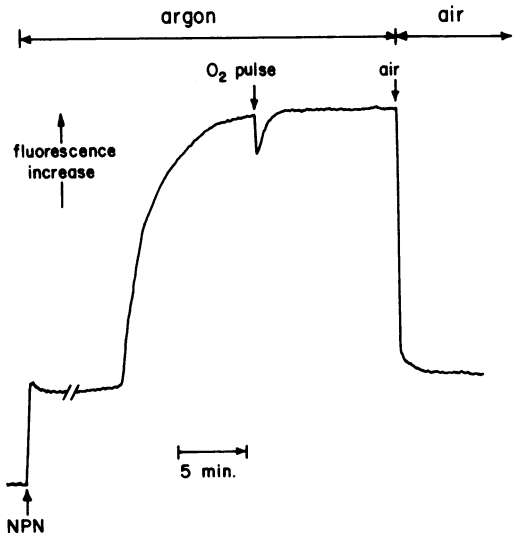

FIG. 2. *Changes in fluorescence intensity of NPN associated with changes in oxygen tension. Reaction conditions were generally as described in the footnotes to Table 1. The cell concentration in the cuvette was 3 × 10^9 cells per ml. The atmosphere in the cuvette was changed from water-saturated air to water-saturated argon as indicated. The time between the hash marks represents about 12 min in this experiment. The oxygen pulse given after the fluorescence increase contained 5.5 ng-atoms of O.*

ecules (Table 1). The length of the lag period preceding these changes in fluorescence seemed dependent upon the endogenous substrate levels in the cells and ranged from about 30 min for freshly harvested cells to about 3 min for cells mildly starved by an aerobic incubation for 1 h at 33°C in the absence of a carbon source. Nivea-Gomez et al. (21, 22) found similar increases in NPN fluorescence and polarization associated with anaerobiosis. These authors also found a 40-nm irreversible red shift in the NPN emission spectrum for anaerobic cell suspensions. However, under the conditions used in this study, there was no significant change in the shape or position of the emission spectrum under anaerobic conditions (Fig. 1). If an anaerobic cell suspension was made aerobic by replacing the N_2 or Ar atmosphere in the cuvette with air, the fluorescence intensity returned to a value close to the original aerobic level, as did the fluorescence lifetime, polarization, and rotational relaxation time (Fig. 1 and 2 and Table 1).

Once the fluorescence increase associated with anaerobiosis reached a maximum, it was possible to induce a transient, partial reversal of the fluorescence by injecting a small oxygen pulse into the cell suspension (Fig. 2), which was followed by a slower relaxation of the fluorescence to the original high level. The maximum extent of the fluorescence decrease was linearly proportional to the amount of oxygen added in the pulse for pulses containing 11 ng-atoms of O or less (Fig. 3). The rate constant for the relaxation of the rapid fluorescence decrease induced by an oxygen pulse was independent of the amount of oxygen present in the pulse (Fig. 3).

As reported earlier (9), the half-time for the efflux of H^+ ions into the medium after a pulse of 5.5 ng-atom of O is about 10 s, or at least 5 to 10 times longer than the half-time expected from the calculated reduction time of the oxygen pulse, assuming the steady-state respiration rate (9). Figure 4a shows the cytochrome *b* oxidation kinetics for an anaerobic suspension of *E. coli* cells pulsed with 5.5 ng-atoms of O. The oxidative absorbance change was complete within the mixing time (1 s or less) and relaxed to the original, anaerobic level with a half-time of about 7 s in this experiment. The half-time for the reductive relaxation was somewhat variable from experiment to experiment, ranging up to about 1 min. The oxidative change after an oxygen pulse was always complete in less than 1 s, however. The spectrum for the oxygen pulse-induced absorbance change confirms that these changes were due predominantly to cytochrome *b* (Fig. 4b) (2, 33).

The total amount of cytochrome *b* participating in the oxygen pulse-induced absorbance changes at 427 minus 450 nm was determined by comparing the extent of the absorbance change with the total absorbance change determined from anaerobic (reduced) minus aerobic (oxidized) difference spectra (Fig. 4c). Only a small portion of the total cytochrome *b* was oxidized by the small oxygen pulses, and the amount of cytochrome oxidized by a pulse was linearly dependent upon the amount of oxygen present in the pulse over the range of 0 to 11 ng-atoms of O per pulse.

The energy-dependent decrease in NPN fluorescence, like the oxidation of cytochrome *b*, exhibited a very short half-time, which was probably limited by the mixing time in the cuvette (about 1 s) (Fig. 5). This was in marked contrast to the relatively slow acidification of the external medium (half-time, about 10 s) measured under identical conditions (Fig. 6a).

Increasing the permeability of the cytoplasmic membrane to H^+ ions by the addition of the uncoupler FCCP largely eliminated the net efflux of protons into the medium after an oxygen pulse (Fig. 6b). Interestingly, the maximum extent of the rapid decrease in NPN fluorescence was only inhibited by about 50%. However, the rate of the subsequent relaxation of the fluores-

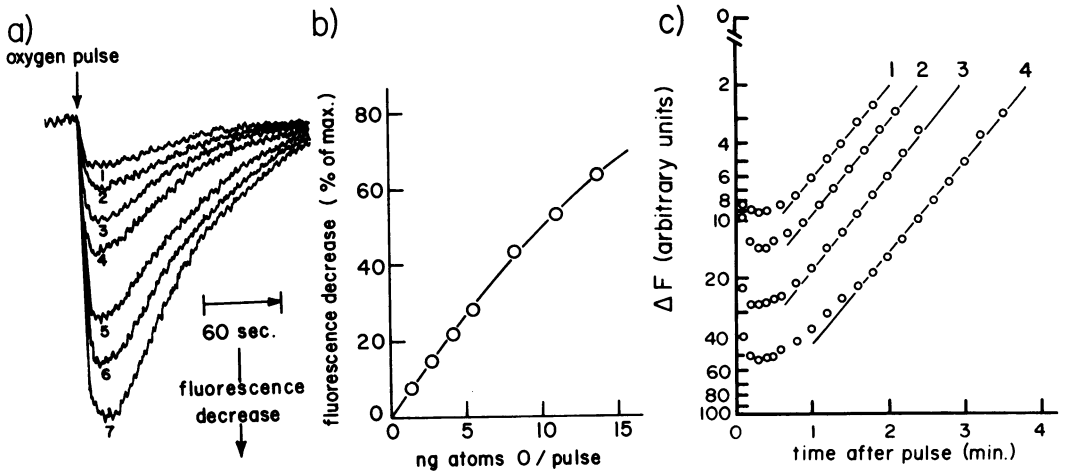

FIG. 3. *Dependence of the oxygen pulse-induced decrease in NPN fluorescence on the amount of oxygen present in the pulse. The final E. coli cell concentration in the cuvette was 4.2 × 10^9 cells per ml. The concentration of NPN was 4 μM. The fluorimeter amplifier half-response time was 0.7 s in this experiment. (a) Superimposed traces for the fluorescence decreases and subsequent increases induced by oxygen pulses containing 1.38 (line 1), 2.75 (line 2), 4.13 (line 3), 5.5 (line 4), 8.3 (line 5), 11 (line 6), and 13.75 (line 7) ng-atoms of O. (b) Maximum extent of the fluorescence decrease as a function of the amount of oxygen added in the pulse. (c) Semilogarithmic plot of the decay of the rapid fluorescence decrease after an oxygen pulse, replotted from (a). The oxygen pulses contained 1.38 (line 1), 2.75 (line 2), 5.5 (line 3), and 11 (line 4) ng-atoms of O.*

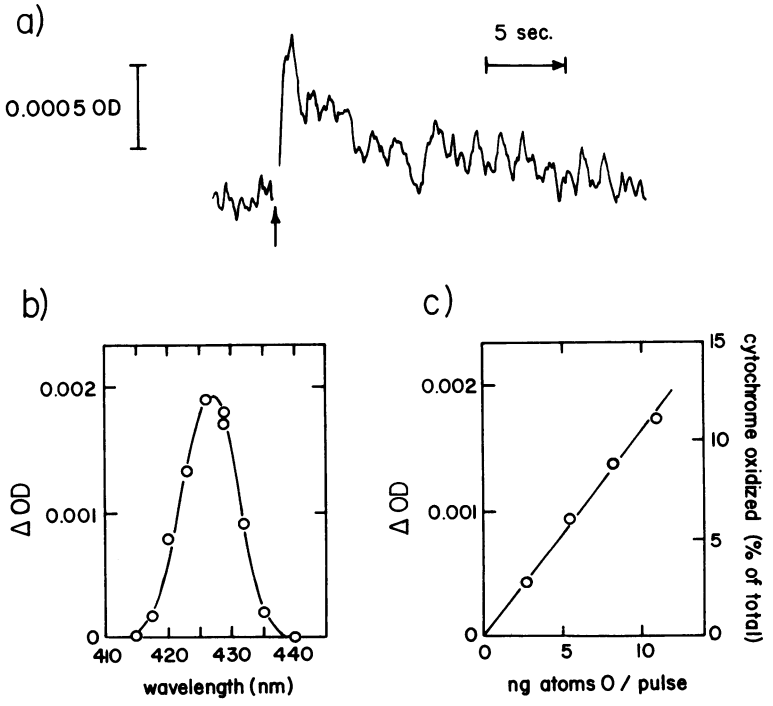

FIG. 4. *Oxygen pulse-induced oxidation of E. coli cytochrome b. The amplifier time constant for the dual-wavelength spectrophotometer was 100 ms. An upward deflection in the trace represents an absorbance decrease. (a) Oxygen pulse-induced (5.5 ng-atoms of O; arrow) decrease in the absorbance (429 minus 450 nm) of E. coli cytochrome b (oxidation). OD, Optical density units. (b) Spectrum for the oxygen pulse-induced changes in the Soret band absorbance of an anaerobic E. coli suspension. The oxygen pulses contained 5.5 ng-atoms of O. The reference wavelength was 450 nm. (c) Dependence of the extent of the oxygen pulse-induced absorbance decrease (427 minus 450 nm) on the amount of oxygen present in the pulse.*

cence decrease was greatly increased. When the permeability of the cell to K^+, but not H^+, was increased by treating the cells with colicin E1 (8, 10) or valinomycin (29), both the rate and the extent of the proton efflux were enhanced (Fig. 6c). However, the oxygen pulse-induced changes in NPN fluorescence were very similar to those observed in cells treated with FCCP (Fig. 6c and d).

The concentration of FCCP used in the experiment shown in Fig. 6b (4.15 μM) was sufficient to cause complete inhibition of net H^+ efflux (8) and active transport (14). Similarly, the concentration of colicin E1 used in the experiment shown in Fig. 6c was sufficient to completely inhibit active transport and to allow survival of less than 0.1% of the cells. Even in the presence of these high levels of energy poisons, however, a significant portion of the fluorescence decrease observed after an oxygen pulse persisted. The relaxation of the fluorescence decrease was now very much more rapid than in untreated cells, and changing the atmosphere in the cuvette from N_2 or Ar to air caused only a very small (~10%) reversal of the fluorescence increase (data not shown). This indicates that the steady-state energy level of the membrane was low in the poisoned cells, even in the presence of excess oxygen.

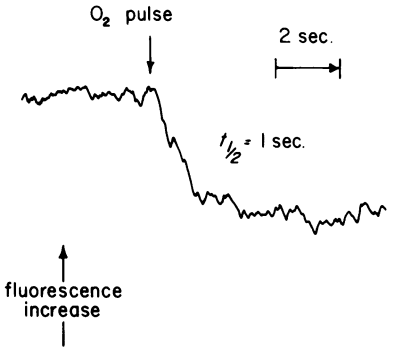

FIG. 5. *Kinetics of the decrease in NPN fluorescence intensity after the addition of an oxygen pulse to an anaerobic E. coli suspension (3 × 10^9 cells per ml). The fluorimeter amplifier half-response time was 0.1 s in this experiment. The concentration of NPN was 4 μM. At the downward arrow an oxygen pulse containing 5.5 ng-atoms of O was rapidly injected from a microliter syringe.*

DISCUSSION

These data, taken in conjunction with those presented elsewhere by others (14, 21, 22), support the notion that the structural change in the *E. coli* envelope which is reflected by changes in NPN fluorescence is modulated by the energy level of the cytoplasmic membrane. This structural change involves alterations in an outer membrane permeability barrier to large hydrophobic molecules, leading to changes in the access of lipophilic probe molecules to hydrophobic binding sites within the cell envelope.

Energy-dependent changes in NPN fluores-

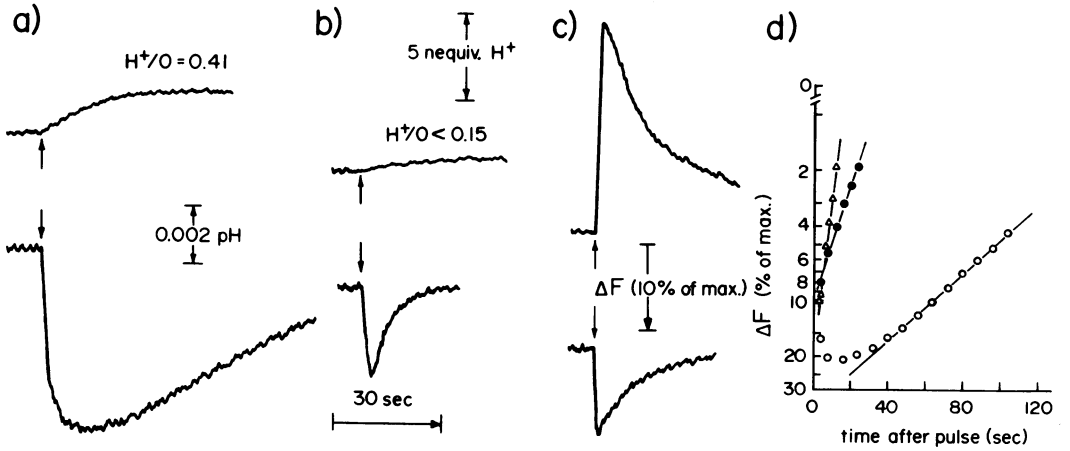

FIG. 6. *Effects of colicin E1 and FCCP on the kinetics of the oxygen pulse-induced changes in the medium pH (proton efflux) and in NPN fluorescence intensity in an anaerobic E. coli suspension. The reaction conditions were essentially as described in the legend to Fig. 3 and in reference 21. The cell concentration was 3 × 10^9 cells per ml. The upper traces in (a), (b), and (c) show the changes in medium pH, whereas the lower traces show the changes in NPN fluorescence intensity (ΔF). The oxygen pulses (arrows) contained 5.5 ng-atoms of O. (a) Untreated cells. (b) Cells treated with 4.2 μM FCCP. (c) Cells treated with 1 μg of colicin E1 per ml. (d) Semilogarithmic plot of the relaxation kinetics of the rapid fluorescence decrease induced by the oxygen pulses given in (a) through (c). Symbols: ○, untreated cells; ●, cells treated with 1 μg of colicin E1 per ml; △, cells treated with 4.2 μM FCCP.*

cence are clearly not linearly related to the actual energy level of the membrane over the entire range of the fluorescence change, because Cramer et al. (5) have shown that the addition of glucose to cells deenergized with colicin E1 caused a transient decrease of approximately 80% in the fluorescence intensity, which was not accompanied by any increased capacity of the cells to catalyze proline transport. However, there is a range of oxygen concentrations where the extent of the transient reversal of the fluorescence increase is linearly proportional to the amount of oxygen added and, hence, to the respiratory energy generated (Fig. 3). At higher levels of O_2 (>11 ng-atoms of O per pulse), the relationship becomes nonlinear, with larger and larger amounts of oxygen required to cause a given incremental change in the fluorescence intensity (data not shown). Apparently NPN fluorescence can act as a convenient amplifier of small changes in the energy level of a mostly deenergized membrane.

It should be noted that the changes in NPN fluorescence studied here do not result directly from the effects of an electric field upon the dye molecule, as has been shown for some cyanine fluorescence probes in other systems (34). Previous studies have indicated that in intact *E. coli* the changes in NPN fluorescence intensity associated with the energization and deenergization of the cell membrane can be attributed mostly to changes in the amount of dye bound to the surface of the cell (14, 21, 22) and to a change in the rotational freedom of probe molecules at their binding sites (14, 15). In intact *E. coli* cells the binding of merocyanine and oxycarbocyanine probes is also limited by the outer membrane permeability barrier (S. M. Hammond, unpublished data).

One indication of the sensitivity of the NPN fluorescence changes observed in these experiments to small levels of membrane energization is that in the presence of high levels of FCCP or colicin E1 a transient energization of the membrane can still be observed (Fig. 6b and c). The maximum extent of the fluorescence decrease is somewhat lowered by these agents (Fig. 6), but their major effect seems to be on the relaxation of the fluorescence decrease (i.e., on the loss of membrane energy). This result is not unexpected since both colicin E1 and FCCP do not directly affect energizing reactions per se, but rather act by accelerating deenergizing reactions. It is interesting to note that the effects on the NPN fluorescence response are the same for both FCCP and colicin E1, indicating that the relaxation of the transient NPN fluorescence decrease is reflecting the rate of deenergization and not the mechanism.

Since the changes in NPN fluorescence are only responding indirectly to the energization of the cytoplasmic membrane, the actual rate of energization must be at least as fast as the observed changes in fluorescence. The similarity in the half-time for the fluorescence decrease after an oxygen pulse (about 1 s; Fig. 5) and the mixing time in the cuvette (about 0.9 s when measured by the rapid injection of NPN into buffer) indicates that the fluorescence changes are probably occurring at a rate faster than that shown here.

The fact that the fluorescence decrease induced by an oxygen pulse is very much faster than proton efflux again points to the conclusion that the energizing event associated with the cytoplasmic membrane preceeds the appearance of translocated protons in the external aqueous phase. The half-time measured for the fluorescence change is similar to the half-time calculated for the reduction of the oxygen added in the pulse (10) and the measured half-time for the oxidation of cytochrome *b*.

The findings are also consistent with our earlier conclusion that the effects of permeant counterions on the rate and extent of proton efflux were not the result of respiratory control, since the changes in NPN fluorescence indicate that the degree of energization induced by the small oxygen pulses used in these studies is very much less than the steady-state energy level of the membrane under aerobic conditions.

All of the data presented here and elsewhere (9) are consistent with a model for bacterial membrane energization in which the primary energizing event is the transfer of a proton from the cytoplasm to some location closely associated with the cell envelope and not accessible to the external pH electrode. The slow equilibration of protons which remain associated with the envelope with the aqueous medium would account for the observed small H^+ efflux. The low H^+/O ratio observed in log-phase cells (9) implies that the cell envelope does in fact have the capacity to retain nearly all of the protons translocated after a small O_2 pulse, so that they may be utilized in endergonic energy transduction reactions (e.g., active transport) or leak back into the cell without ever entering the external osmotic phase. It is only when the H^+ translocation-generated $\Delta\psi$ is rapidly collapsed by the movement of permeant counterions that apparently all of the protons actually transported appear in the external medium (Fig. 6c) (8–10).

At the moment one can only speculate on where the protons apparently translocated but not detected by the pH electrode are actually going. One possibility is the periplasmic space between the inner and outer membranes. Stock

et al. (32) have found that the periplasm in *E. coli* can account for up to 40% of the total cell volume and that it contains a large number of nondiffusable negative charges. Furthermore, a Donnan potential (outside positive) probably exists across the periplasmic membrane (32), which would tend to resist the movement of protons from the periplasm to the medium. Acidification of the periplasm could actually serve to increase the transmembrane pH gradient across the energy-transducing cytoplasmic membrane, leading to an increase in the total protonmotive force available to drive endergonic membrane reactions, such as active transport and ATP synthesis.

Besides the periplasm, other reservoirs for the missing protons are also possible. Williams (39) has suggested that the protons remain within the lipid phase of the energy-transducing membrane and do not enter the bulk aqueous phase at all, perhaps remaining closely associated with the negative charges in the polar head group region of the phospholipid bilayer. *E. coli* are known to have a negative surface charge (6, 18). Alternatively, protons may accumulate in an unstirred layer of structured water at a membrane-water interface. This possibility is intriguing since the lateral mobility of protons within the structured water layer would be expected to be quite high (17). At the moment, however, there is little direct evidence to indicate exactly what happens to those protons translocated across the cytoplasmic membrane by the respiratory chain which do not appear in the external medium.

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