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STIMULATION OF RESPIRATION-LINKED PROTON EFFLUX IN ESCHERICHIA COLI BY CARBONYLCYANIDE-p-TRIFLUOROMETHOXYPHENYLHYDRAZONE (FCCP)

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SUMMARY

The proton efflux from intact, anaerobic Escherichia coli cells following a small oxygen pulse is both slow ($t_{1/2}\simeq 10s$) and inefficient ($H^+/O\simeq 0.5$). Very low levels (<80 nM) of the proton ionophore carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP), which have no detectable effect upon active transport, cause a 3-5 fold stimulation in the extent of proton efflux without affecting the efflux rate. At slightly higher concentrations of FCCP (80 nM to 0.5 μM), a sharp inhibition of this increased proton efflux occurs, with the H^+/O ratio obtained in the presence of 0.5 μM FCCP approximately equal to that obtained in the absence of FCCP. Still higher concentrations of FCCP (> 1 μM), which inhibit active transport, cause a further gradual decrease in the H^+/O ratio. The unusual increase in the apparent efficiency of H^+ efflux by <80 nM FCCP is not accompanied by an increase in the rate of membrane deenergization following an O_2 pulse, although such an increase is seen with the higher (uncoupling) FCCP concentrations.

INTRODUCTION

There is a great deal of evidence which suggests that in bacteria the vectorial transfer of hydrogen ions across the cytoplasmic membrane during oxidative electron transport is a primary coupling mechanism between respiration and processes such as active transport and ATP synthesis (see e.g. 1,2). However, for intact cells under normal conditions the efficiency of proton efflux into the medium after an oxygen pulse is variable and rather low, with an H^+/O ratio for log phase Escherichia coli grown on succinate of ∿0.5 (3,4). Furthermore, the rate at which protons appear in the medium after a small oxygen pulse ($t_{1/2}\simeq 10s$) is very much slower than both the rate of oxygen consumption ($t_{1/2}\leq 1s$) and the rate of membrane

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energization ($t_{1/2}$≤1s)(4-6). Treatment of E. coli cells with a mobile counterion such as SCN^-, or with colicin E1 plus K^+ leads to an increase in the apparent H^+/O ratio to values >2.0, an increase in the rate of proton efflux ($t_{1/2}$≤1s), and an increase in the rate of membrane deenergization following the oxygen pulse (4-7). These findings have led us to consider the possibility that the initial site of deposition for protons translocated across the cytoplasmic membrane in intact cells is a compartment which is not in rapid equilibrium with the bulk medium, possibly the periplasm (4-6).

We wish to report here that at exceedingly low concentrations (≤80 nM), the proton ionophore FCCP[2] apparently removes, at least partially, the equilibration barrier between protons translocated across the cytoplasmic membrane after an oxygen pulse and the external medium.

EXPERIMENTAL PROCEDURES

Cells of Escherichia coli strains B/1,5 and ML 308-225 were grown on a minimal medium as described earlier (4,7). The carbon source was 1% succinic acid (w/v). Sterile 250 ml Erlenmeyer flasks containing 50-60 ml of medium were innoculated from overnight cultures and incubated at 37° with vigorous shaking for 4.5-5.5 hours (mid-logarithmic phase growth). Cells were harvested by centrifugation at 4°, washed twice in 150 mM KCl, 0.5 mM 3-(N-morpholino)propanesulfonic acid-KOH (pH 7.0), and resuspended in this medium to a final concentration of about $3x10^9$ cells/ml.

Oxygen pulse induced changes in the pH of an anaerobic cell suspension were monitored as described elsewhere. Changes in the fluorescence intensity of N-phenyl-1-naphthylamine (NPN) were detected as described in (8).

FCCP was kindly supplied by Dr. P. G. Heytler. The cell strains used in this study were the generous gifts of Drs. H. R. Kaback (ML 308-225) and S. Silver (B/1,5).

RESULTS

In midlogarithmic phase cells grown with succinate as carbon source the rate of proton extrusion was found to be quite slow ($t_{1/2}$≃10 sec for a 5.5 ng atom O pulse) and the efficiency was low - the number of protons appearing in the medium for each oxygen atom added (H^+/O ratio) typically falling around 0.5 (refs. 3-7, Fig. 1a). This slow rate of H^+ efflux

[2]Abbreviations: FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; NPN, N-phenyl-1-naphthylamine.

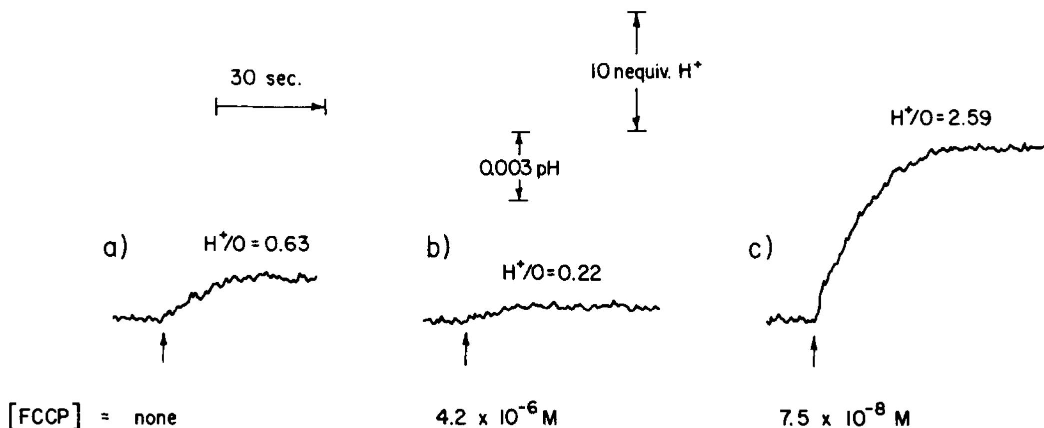

Figure 1. Stimulation and inhibition of oxygen-pulse induced proton efflux in an anaerobic E. coli suspension. Midlogarithmic phase cells of E. coli strain ML 308-225 were washed twice and suspended to a final concentration of 1.5×10^9 cells/ml as described in Methods. FCCP was added in 5 µl of ethanol. Samples were incubated for 2 min. after addition of FCCP before the addition of the oxygen pulse (arrow), which contained 5.5 ng atoms O.

and low H^+/O ratio has been found to be independent of the presence of substrate for small O_2 pulses (<11 ng atoms O/pulse) such as those used in this study (4,5). Concentrations of FCCP >1 µM, which inhibit proline transport in intact cells (8), decreased the maximum extent of the net proton extrusion following an oxygen pulse, and the H^+/O ratio declined (Fig. 1b). However, very low concentrations of FCCP (≤0.1 µM) caused a substantial increase in the extent of the net proton efflux, resulting in a 3-5 fold increase in the observed H^+/O ratio to values >2.0 (Figs. 1c, 2). This increase in the extent of proton efflux into the medium was not accompanied by any detectable change in either the half-time for proton efflux ($t_{1/2} \simeq 10$ sec., Fig. 1) or the rate of the subsequent very slow ($t_{1/2} > 10$ min.) leakage of the extruded protons back into the cells (not shown).

The effect of varying FCCP concentrations on proton efflux and the H^+/O ratio for the B/1,5 cell strain is shown in Fig. 2. Nearly identical results were obtained with the ML 308-225 strain as well (not shown). In both strains the half-time for proton efflux was unaffected by the

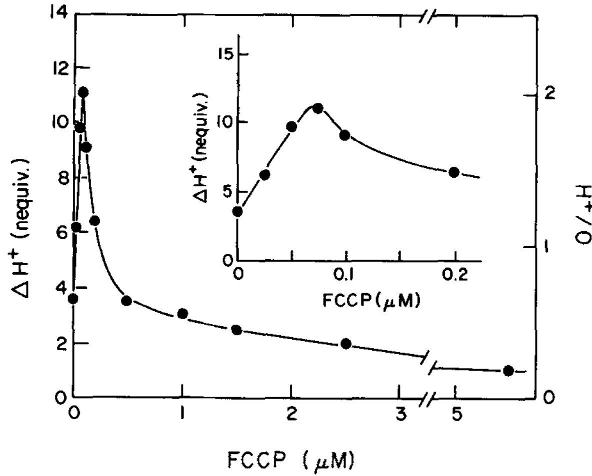

Figure 2. Concentration dependence of the FCCP-dependent stimulation and inhibition of net proton efflux (ΔH^+) in E. coli cells. Reaction conditions were as described in Methods and the legend to Figure 1. The concentration of E. coli B/1,5 cells was 3×10^9 cells/ml. The oxygen pulses used contained 5.5 ng atoms O.

presence of FCCP (typical $t_{1/2} \simeq 10$ sec.), although a 3-5 fold increase in the H^+/O ratio was observed with 75-80 nM FCCP. Half-maximal stimulation of the H^+/O ratio occurred at about 40 nM FCCP.

In experiments with intact cells of dark-grown Rhodopseudomonas spheroides, Scholes et al. (9) noted earlier that 0.5 μM FCCP caused an approximately two-fold increase in the extent of H^+ efflux following a 23.5 ng atom O pulse at pH 6, but had the opposite effect at pH 7, causing ~50% inhibition of H^+ efflux. Experiments with intact E. coli ML 308-225 cells indicated that the H^+/O ratio, both in the presence and absence of 75 nM FCCP, tended to be slightly (~20%) larger in cells incubated at pH 6-6.5 than at pH 7 or 7.5. However, at all pH's tested (pH 6-7.5) 75 nM FCCP caused at least a 3-fold increase in the apparent H^+/O ratio.

Increasing the FCCP concentration above 75 nM caused a sharp decrease in the H^+/O ratio, so that the ratio observed in the presence of 0.5 μM FCCP was approximately equal to the initial value observed in the absence of FCCP. FCCP concentrations of 2-3 μM were required to further decrease

the H^+/O ratio to a value equal to one-half that observed in the absence of FCCP.

In previous papers we have demonstrated that the fluorescence intensity of the lipophilic probe NPN in an anaerobic cell suspension could be partially and transiently decreased by the addition of a small pulse of oxygen, and that the maximum extent of this oxygen pulse induced fluorescence decrease ($\Delta F\downarrow_{max}$) was linearly related to the amount of oxygen added in the pulse (and hence to the membrane energy level) over the range 0-11 ng atoms O/pulse (5,6). On the other hand, the first-order relaxation ($\Delta F\uparrow$) of the oxygen pulse-induced fluorescence decrease was independent of the magnitude of the energizing events. Rather, the rate of this relaxation was found to be correlated with deenergizing processes, being very much faster in cells treated with FCCP (4 μM) or colicin E1 (5,6).

In the presence of a very low concentration of FCCP (80 nM) the maximum extent of the transient fluorescence decrease induced by a 5.5 ng atom O pulse was lowered by 25-30% (Fig. 3), but there was no significant effect on the rate of the subsequent relaxation ($\Delta F\uparrow$) (Fig. 3). Higher concentrations of FCCP led to little further decrease in $\Delta F\downarrow_{max}$ (not shown). However, higher FCCP concentrations (>0.1 μM) did cause an increase in the rate of $\Delta F\uparrow$, indicating that the membrane's ability to maintain the energization generated after the oxygen pulse was diminished.

DISCUSSION

The function of FCCP and its chlorinated analog CCCP as proton carriers across bilayer membranes has been well studied in both artifical membrane and biomembrane systems (10-12). In mitochondrial and chloroplast membranes the presence of FCCP invariably leads to the loss of the membrane's ability to maintain a gradient of protons, and membrane enzymatic functions such as active transport and ATP formation, which utilize the energy available in transmembrane H^+ gradients, are inhibited by FCCP (1,2). In intact

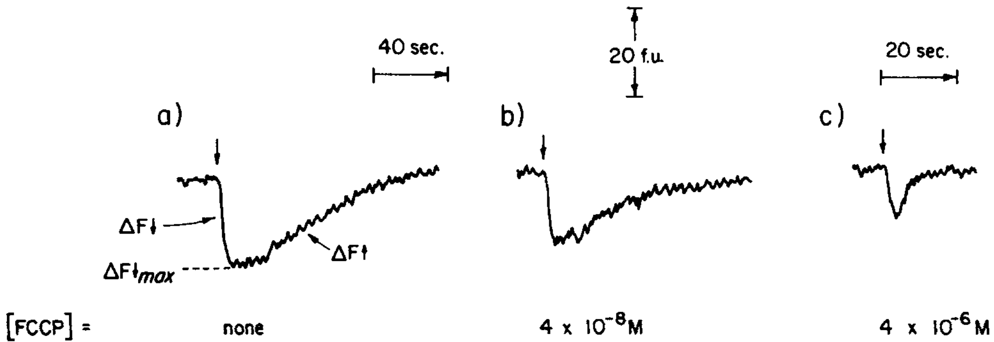

Figure 3. Effect of FCCP on the oxygen-pulse induced changes in N-phenyl-1-naphthylamine (NPN) fluorescence intensity in an anaerobic E. coli suspension. Reaction conditions are given in Methods. The final concentration of the B/1,5 cells used in this experiment was $4x10^9$ cells/ml. Cells were incubated with stirring under nitrogen for ~30 min. to establish anaerobiosis. During this time the fluorescence intensity of the sample rose from the aerobic level of 56 arbitrary fluorescence units (f.u.) to a steady-state value of 130 f.u.. The oxygen pulses (downward arrows) contained 5.5 ng atoms O. A downward deflection represents a decrease in fluorescence intensity. Note the faster time scale for trace c only.

E. coli cells the same general effects of FCCP can be observed, but, as shown in this paper, the situation is somewhat more complex.

The source of this complexity can most likely be traced, at least in large part, to the complex nature of the E. coli cell envelope. For instance, Helgerson and Cramer (8) have shown that the presence of lipopolysaccharide in the intact E. coli outer membrane restricts the movement of large hydrophobic molecules such as FCCP to the inner (cytoplasmic) membrane. We have shown in earlier studies that the cell envelope also contains a barrier which restricts the movement of respiratory protons translocated across the cytoplasmic membrane from entering the external medium (4-6). As a result, the rate of proton extrusion appears slow and the number of protons entering the external medium represents only a fraction of those actually transported across the cytoplasmic membrane.

The data obtained with low (<0.1 μM) FCCP levels can most easily be interpreted as resulting from an effect of FCCP on a site other than the cytoplasmic membrane. This seems likely since these concentrations actually

increase the apparent efficiency of proton efflux and do not increase the rate of membrane deenergization following an oxygen pulse. FCCP may be interacting with the cell envelope in a manner which relieves the envelope barrier to extruded protons, so that most if not all of the protons translocated across the cytoplasmic membrane appear in the external medium. Interestingly, the half-time for proton efflux is not significantly altered by 75 nM FCCP, although the H^+/O ratio is greatly increased.

At higher levels (>0.1 μM), FCCP behaves in a manner consistent with its classical function as a membrane proton conductor, deenergizing the cytoplasmic membrane. Thus, the H^+/O ratio is inhibited and the rate of the membrane deenergization indicating fluorescence change is greatly increased.

The mechanism by which low concentrations of FCCP relieve the envelope H^+ permeability barrier is not known. It is possible, for example, that the effect is unrelated to the function of FCCP as a proton conductor, perhaps involving instead a conformational transition in the envelope upon FCCP binding. Carbonylcyanide phenylhydrazones such as FCCP and CCCP are known to react readily with sulfhydryl residues, for instance (13-15).

Finally, it should be pointed out that the FCCP induced increase in the apparent H^+/O ratio is clearly distinct from the increase in the H^+/O ratio caused by SCN^-, colicin E1 plus K^+, or valinomycin plus K^+. Unlike the effects of 80 nM FCCP, these latter treatments, which result in changes in counterion permeability, also lead to a much faster rate of H^+ efflux and subsequent influx (3,4,7), as well as a faster relaxation of the fluorescence decrease (ΔF↑) (5,6), indicating that these treatments cause the depolarization of the cytoplasmic membrane.

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