ANILINONAPHTHALENESULFONATE AS A FLUORESCENT PROBE OF THE ENERGIZED MEMBRANE STATE IN ESCHERICHIA COLI CELLS AND SONICATED MEMBRANE PARTICLES

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SUMMARY. The enhancement of 1-anilino-8-naphthalenesulfonate fluorescence was observed followed by the binding of the probe to the E.coli membrane. The fluorescence intensity of the probe is quenched upon energization of intact cells. The experiments with sonicated membrane particles, in which the orientation of the membrane is "inside-out", showed an energy linked enhancement of the fluorescence intensity of the probe. The changes in the fluorescence intensity of fluorochrome-stained membranes can also be induced by generation of K^+ ion diffusion potential.

The use of the fluorescent probe ANS^- have been recently extended to the study of the function of biological energy-conserving membranes (1-8). The present report describes changes in the fluorescence intensity of ANS^- that accompany the energization of intact E. coli cells and sonicated membrane particles.

MATERIALS AND METHODS. The bacterial strain used was E. coli ML 308-225 (i^-, z^-, y^+, a^+), a generous gift of Dr H.R.Kaback, Roche Institute of Molecular Biology, Nutley, N.J.,U.S.A. Cultivation of bacteria, preparation of suspensions of cells and sonicated particles were done as described earlier (9). For the preparation of K^+-loaded cells the same procedure was employed with additional incubation of

Abbreviations: ANS^- (1-anilino-8-naphthalenesulfonate), CCCP (carbonylcyanide p-trichloromethoxyphenylhydrazone), DCCD (N, N-dicyclohexylcarbodiimide), $TPMP^+$ (triphenyl methylphosphonium cation), PCB^- (phenyl dicarbaundecaborane anion).

cells after EDTA treatment in the 0.1 M K_2HPO_4 buffer at pH 7 for 15 min at 37°. The fluorescence was measured in the range of 500-1000 nm with an EF-3M fluorimeter connected with amplifier pH 340 and automatic recorder KSP-4. A 120 W quartz-Hg lamp and a filter with maximum transmission at 360 nm were used to provide a source of exciting light. The changes in the intrinsic fluorescence of cells constitute less than 10 % of those of ANS^--stained cell fluorescence. Exposure of the cell suspension to the air was minimized by a stream of nitrogen at the mouth of cuvette. Measurements were carried out at room temperature.

RESULTS AND DISCUSSION. Fig. 1 depicts the time-course of the changes in the fluorescence intensity of ANS^- upon addition of E.coli cells. It could be seen (Expt. A) that the interaction between the cells and ANS^- is followed by a biphasic increase in the probe fluorescence. At first, a rapid phase is observed which constitutes approximately 50 % of the total fluorescence increment and is complete

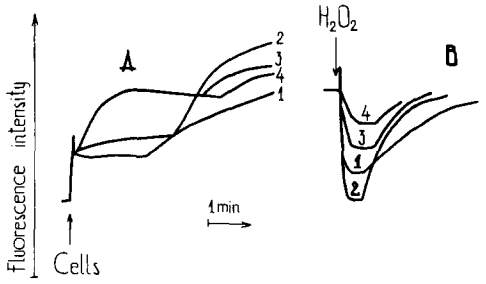

Fig. 1. The time-course of the changes in the fluorescence intensity of ANS^- upon addition of E. coli cells and hydrogen peroxide. The incubation medium contained 0.25 M sucrose, 25 mM Tris-H_2SO_4 (pH 7.5), $5 \cdot 10^{-5}$ M ANS^-, 2 mM succinate, catalase (0.1 mg/ml), $1 \cdot 10^{-4}$ M DCCD and $1 \cdot 10^{-6}$ M CCCP (curve 2), $2 \cdot 10^{-6}$ M CCCP (curve 3), $3 \cdot 10^{-6}$ M CCCP (curve 4). Additions: E. coli ML 308-225 cells at concentration of 2 mg dry wt/ml and 1300 nmoles of hydrogen peroxide.

within 10 s. The incubation of cell suspension under conditions of limited exhibition to the air atmosphere results in the exhaustion of oxygen in the medium and in an increase in the fluorescence intensity (Fig. 1A, curve 1). The injection of oxygen induces (Fig. 1B, curve 1) quenching of ANS^- fluorescence which is followed by an exponential return of intensity to the initial level. The oxygen-induced quenching was blocked by respiration inhibitor cyanide at concentration of 2-3 mM (not shown). Fig. 1A, curve 2 demonstrates that the uncoupler CCCP at concentration of $1 \cdot 10^{-6}$ M stimulates the ANS^- fluorescence increase when incubation medium becomes anaerobic. At concentrations of 2 and $3 \cdot 10^{-6}$ M (curves 3 and 4) CCCP causes an increase in the fluorescence intensity of ANS^- immediately after the addition of cells in spite of the presence of oxygen in medium. The increase in ANS^- fluorescence after the exhaustion of oxygen in the medium is less pronounced (see curves 3 and 4). It could be seen that at high concentrations CCCP lowers the final level of fluorescence intensity to some extent. At any rate this could be due to non-energy-linked quenching of ANS^- fluorescence by CCCP (4). The next set of experiments (see Fig. 1B and Table 1) was undertaken to reveal the energy-linked nature of the respiration-induced quenching of the ANS^- fluorescence. Fig. 1B depicts the CCCP effect on the respiration-induced quenching of probe fluorescence. The effect of CCCP on the rate of exponential return of fluorescence to the initial intensity after exhaustion of oxygen is given in Table 1. It could be seen that CCCP at concentration of $1 \cdot 10^{-6}$ M stimulates to some extent the quenching (curve 2, Fig. 1B) and accelerates the return of intensity ($t_{0.5}$ = 66 s without CCCP and $t_{0.5}$ = 26 s in the presence of CCCP, see Table 1). At higher concentrations of CCCP the decrease in the respiration-induced rate and the amplitude of fluorescence quenching occurs (see curves 3 and 4, Fig. 1B). These CCCP

Table I. The effect of CCCP on the rate of exponential return of ANS^- fluorescence intensity to initial level after the exhaustion of oxygen in medium.

Incubation mixture contained 0.25 M sucrose, 25 mM Tris-H_2SO_4 (pH 7.5), $5 \cdot 10^{-5}$ M ANS^-, 2 mM succinate, $1 \cdot 10^{-4}$ M DCCD, 0.1 mg/ml of catalase and 2 mg dry wt of cells per ml. Concentration of CCCP is shown in the table. Transitional quenching of ANS^- fluorescence was induced by addition of 1300 nmoles of H_2O_2.

No. of expt.	Concentration of CCCP in the incubation mixture	Half-time ($t_{0.5}$) of the return in seconds
1	None	66
2	$1 \cdot 10^{-6}$ M	26
3	$2 \cdot 10^{-6}$ M	18
4	$3 \cdot 10^{-6}$ M	21

concentrations also cause the stimulation of the rate of the return (see Expts. 3 and 4, Table 1).

The energy-linked quenching of ANS^- fluorescence on addition of respiratory substrates has been demonstrated by Reeves et al (4) in experiments with membrane vesicles prepared by the osmotic lysis of E. coli cells. This fact indicates that these vesicles have the same membrane polarity as intact cells. Evidence validating the dependence of the sign of the energy-linked changes in the ANS^- fluorescence on the membrane orientation is shown in Fig. 2. It is seen that energization of sonicated membrane particles by the addition of succinate or ATP leads to the ANS^- fluorescence enhancement. Cessation of respiration by the addition of cyanide (Expt. A) or deenergization of particles by the addition of uncoupler (Expt. B) causes the exponential quenching of fluorescence. In the suspension of ANS^--stained cells opposite changes in the fluorescence intensity are observed (Expt. C). The addition of succinate to aerobic cell suspension results in the decrease of the fluorescence intensity. The addition of cyanide induces an exponential increase in the fluo-

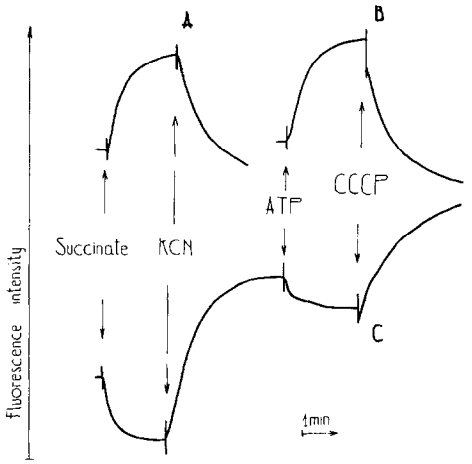

Fig. 2. The time-course of the energy-linked ANS^- fluorescence changes in sonicted membrane particles and intact E. coli cells. The incubation medium contained 50 mM Tris-H_2SO_4 (pH 7.5), 5 mM $MgSO_4$, $1 \cdot 10^{-4}$ M ANS^-, sonicated particles (Expts. A and B) at concentration of 2.5 mg of protein/ml or E. coli ML 308-225 cells (Expt. C) at concentration of 2 mg dry wt/ml. Additions: 4 mM succinate, 5 mM KCN, 1.2 mM ATP and $2 \cdot 10^{-6}$ M CCCP.

rescence intensity. The addition of ATP causes a small non-specific quenching of fluorescence. Treatment of cells with the uncoupler leads to the further increase in the fluorescence intensity. This fact indicates that in spite of the inhibition of respiratory chain by cyanide there exists an energized state in cells.

The data of Figs. 1 and 2 show that the energy input from either the oxidation of respiratory substrates or ATP hydrolysis is coupled with the generation of the high-energy state of membrane, the fact being indicated by the energy-linked changes of ANS^- fluorescence. Measurements of the energy-linked quenching amplitude and the rate of the exponential return provide the quantitive parametres of the efficiency of energy conservation in bacterial cells. The decrease in the quenching amplitude and increase in the rate of return indicates the dissipation of high-energy state of cell membrane. In-

version of the membrane polarity by sonication leads to the inversion of the sign of the energy-linked changes in the ANS^- fluorescence.

The mechanism of the energy-linked changes in the fluorescence intensity of ANS^--stained E. coli membrane was examined in the next set of experiments. The electrostatic potential difference across the cell membrane was generated by the valinomycin-induced diffusion of K^+ ions down their concentration gradient. Cells, previously loaded with K^+, were incubated in K^+-free acidic medium. It can be seen (Fig. 3A) that generation of diffusion potential (negative inside the cells) by the addition of valinomycin results in the transitional decrease of the ANS^- fluorescence intensity. This effect is followed by an exponential return of intensity up to the initial level. The addition of CCCP induces a small increase of the fluorescence intensity. If CCCP is added before valinomycin (Fig. 3B), the effect of valinomycin is abolished and an increase in the fluorescence intensity occurs. Electroneutral K^+/H^+ exchange catalyzed by nigericin causes (Fig. 3C) an increase in the fluorescence intensity and abo-

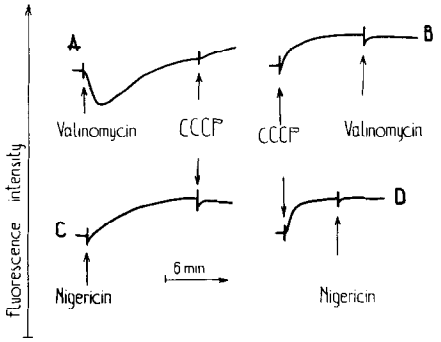

Fig. 3. The effect of nigericin, valinomycin and CCCP on the fluorescence intensity of ANS -stained K^+-loaded E. coli cells. The incubation medium contained 0.4 M sucrose, 3 mM glycil-glycine (pH 3.9), 5 mM NaCN and K^+-loaded E. coli ML 308-225 cells at concentration of 1.2 mg dry wt/ml. Additions: 1 mg of nigericin or valinomycin, $1 \cdot 10^{-6}$ M CCCP.

lishes the effect of CCCP. Fig. 3D demonstrates the abolition of the nigericin-induced effect on the fluorescence by CCCP. Thus, the experiments of Fig. 3 show that the non-enzymatic generation of negative electrostatic potential inside the cells leads to the decrease in the ANS^- fluorescence intensity which resembles the energy-linked quenching of ANS^- fluorescence. Therefore, our results can be used to support a conception of the electrogenic nature of the energy-linked changes in the fluorescence intensity of ANS^--stained bacterial membranes. It may be concluded from the data presented above that energy-linked ANS^- fluorescence changes may be used for the sensitive estimation of the high-energy state in bacteria.

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