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The fluorescence intensity of the lipophilic probe *N*-phenyl-1-naphthylamine responds to the oxidation-reduction state of the respiratory chain in everted membrane vesicles of *Escherichia coli*

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N-Phenyl-1-naphthylamine (NPN), a reagent which has been used previously to probe the fluidity or microviscosity of the membrane lipids of intact cells of *Escherichia coli*, was found to respond to metabolic changes in everted inner membrane vesicles from this organism. NPN was bound to the vesicles to produce a steady-state level of fluorescence intensity. Addition of substrate or ATP did not alter the fluorescence. However, following complete removal of oxygen from the medium by oxidation of substrate through the respiratory chain, there was an increase in the fluorescence of NPN. Reoxidation of the components of the respiratory chain by the addition of oxygen, ferricyanide, fumarate or nitrate decreased fluorescence to the steady-state level until the oxidant had been completely reduced. The fluorescence changes were insensitive to the state of energization of the membrane. It is proposed that NPN responds to the state of reduction of components of the respiratory chain either directly by reacting with a component of the chain or indirectly through an effect transmitted to the membrane by a change in the conformation of respiratory chain components.

N-Phenyl-1-naphthylamine; Respiratory chain; Lipophilic probe; Membrane energization

1. INTRODUCTION

Uncharged lipophilic molecules such as *N*-phenyl-1-naphthylamine (NPN) have been used as probes of the fluidity or microviscosity of the lipid bilayer of biological membranes [1]. NPN fluoresces weakly in aqueous environments but becomes strongly fluorescent in nonpolar environments. The increase in fluorescence intensity is accompanied by changes in fluorescence polarization, lifetime and rotational relaxation time [2,3]. These changes are sensitive to the state of membrane energization of the cell. Thus, energization decreases fluorescence intensity and deenergization reverses this effect. All previous work has used intact cells, and the explanations of the fluorescence behaviour of the probe have involved alterations in the binding of NPN to the outer membrane [4,5] or changes in the environment of the probe in the outer membrane [2,3].

Here, we show that NPN can bind to everted inner membrane vesicles, and that its fluorescence behaviour is dependent on the activity or the state of reduction of the respiratory chain. Thus, NPN may be a probe of conformational events in the membrane dependent on the respiratory chain.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains*

The bacterial strains used in this study were *E. coli* AN180 (*thi argE mtl xyl rspL*) and *E. coli* GR19N (F^- *thi1 rha4 lacZ82 gal33 cydA1*).

2.2. *Growth of cultures*

All cultures were grown at 37°C to stationary phase (16 h). GR19N was grown with vigorous aeration on a medium containing (per l): 7 g K_2HPO_4, 3 g KH_2PO_4, 0.5 g Na citrate, 0.2 g $MgSO_4$, 1.0 g $(NH_4)_2SO_4$, 5 g L-proline, 2.27 mg thiamine HCl, 12 μmol ferric citrate, 1 μmol ammonium molybdate, 1 μmol selenous acid and 6.6 g casein amino acids (Difco). The medium was inoculated with a 10% (v/v) aliquot of a culture that had been preadapted to the same medium.

AN180 was grown aerobically on penassay broth medium (Difco). For anaerobic growth on nitrate, the cells were grown as nonstirred standing cultures in 1-l flasks completely filled with a medium containing (per l): 10.1 g K_2HPO_4, 3.3 g KH_2PO_4, 0.22 g $MgSO_4$, 1.1 g $(NH_4)_2SO_4$, 0.55 g Na citrate, 12 μmol ferric citrate, 1 μmol ammonium molybdate, 1 μmol selenous acid, 1 mg thiamine HCl, 50 mg L-arginine, 5.5 g Bactopeptone (Difco), 5.5 g KNO_3, 11 g glucose and 4.6 g $NaHCO_3$.

Cells were harvested by centrifugation, washed once in 50 mM Tris-HCl, pH 8.0, and stored at −20°C.

2.3. *Preparation of everted membrane vesicles*

2.5 g (wet wt) of cells were resuspended in 20 ml of 50 mM Tris-HCl, pH 8.0, containing 5 mM $MgCl_2$. The cell suspension was disrupted by two passages through a French press (Aminco) at 1400 kg/cm². Unbroken cells were removed by centrifugation at $1000 \times g$ for 20 min, and the membrane vesicles were then pelleted from the supernatant by centrifugation at $200\,000 \times g$ for 1.75 h. The vesicles were washed once in 50 mM Hepes-KOH, pH 7.4, containing 5 mM $MgCl_2$, and resuspended in 1.5 ml of the same buffer to give a protein concentration of approx. 40 mg/ml.

2.4. *Quinacrine and NPN fluorescence assays*

The fluorescence of quinacrine or NPN was measured at 22°C with a Turner model 420 spectrofluorometer connected to a Linear chart recorder. The reaction mixture, in a cuvette of 1 cm light path and in a final volume of 2 ml, contained 10 mM Hepes-KOH, pH 7.4, 0.3 M KCl and 5 mM $MgCl_2$. The assays were started by addition of 5 μM quinacrine or 0.5–2.0 μM NPN.

2.5. *Measurement of cytochrome reduction*

The reduction of the *b* cytochromes at room temperature was measured with a Perkin-Elmer 356 spectrophotometer operating in the dual-wavelength mode, connected to a Perkin-Elmer model 56 chart recorder. The absorption of the sample at 559 nm was compared with the reference wavelength at 580 nm. The contents of the cuvette and order of additions, including addition of the fluorescent dye, were identical to those used in parallel fluorescence experiments.

2.6. *Determination of protein*

Protein was measured by the method of Lowry et al. [6], using bovine serum albumin as a standard.

2.7. *Chemicals*

Carbonyl cyanide *m*-chlorophenylhydrazone and *N*-phenyl-1-naphthylamine were obtained from Sigma.

3. RESULTS AND DISCUSSION

Addition of NPN to a suspension of everted inner membrane vesicles gave a rapid increase in the fluorescence of the probe as it entered a hydrophobic environment in the vesicles (fig.1). Addition of D-lactate did not immediately alter the fluorescence intensity. However, after about 1 min there was an increase in fluorescence which coincided with the medium becoming anaerobic; oxygen had been removed by oxidation of the D-lactate through the respiratory chain. Addition of H_2O_2 (which yielded oxygen with the catalase associated with the vesicle preparation) quenched the fluorescence which had arisen on anaerobiosis. The fluorescence increased once more on complete utilization of H_2O_2 (not shown). Succinate (and formate) oxidation caused a similar change in the fluorescence of NPN (fig.1, curve 2). The smaller change in fluorescence on anaerobiosis with succinate compared with D-lactate resembles the

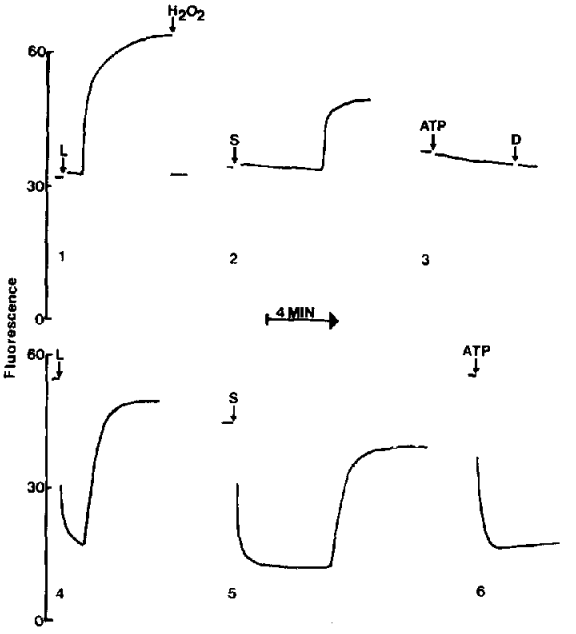

Fig.1. Changes in the fluorescence of NPN (upper panel) and of quinacrine (lower panel) following addition of substrates. The everted membrane vesicles (protein, 0.5 mg/ml) were prepared from *E. coli* GR19N grown on L-proline. The measuring systems are described in section 2. L, 10 mM D-lactate; H_2O_2, 5 μl of 3% H_2O_2; S, 10 mM succinate; ATP, 10 mM ATP; D, 0.5 mM *N,N'*-dicyclohexylcarbodiimide.

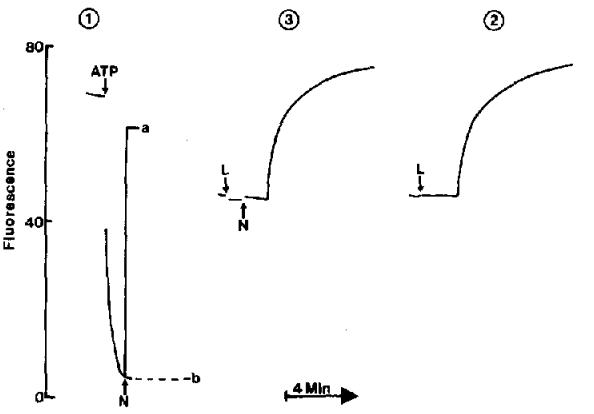

Fig.2. Changes in the fluorescence of quinacrine (1) and NPN (2,3) on addition of nigericin. The measuring systems are described in section 2. The everted membrane vesicles (protein, 0.5 mg/ml) were prepared from *E. coli* AN180 grown on penassay broth. ATP, 10 mM ATP; N, 2.5 μg/ml nigericin; L, 10 mM D-lactate. In 1, nigericin was added only to the sample giving the curve 1a.

relative amounts of cytochrome *b* reduced by the two substrates (not shown). There was no relationship between the NPN fluorescence changes and the ability of the substrate to cause transmembrane proton translocation. This was measured by the substrate-dependent quenching of the fluorescence of quinacrine. As seen in fig.2 (curves 3,6) ATP hydrolysis energized proton translocation but was without effect on the fluorescence of NPN. Addition of ATP after a system oxidizing D-lactate had become anaerobic had no effect on the fluorescence of NPN (not shown).

These results suggested that in contrast to the effect with intact cells there was no relationship between membrane energization and the NPN fluorescence changes observed in everted inner membrane vesicles. This view was supported by the following evidence. Addition of nigericin to membrane vesicles energized by ATP discharged the proton gradient by K^+/H^+ exchange [7] (fig.2, curve 1). However, nigericin (fig.2, curves 2,3) and the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (not shown) had no effect on the increase in the fluorescence of NPN which occurred after substrate oxidation had removed all the oxygen from the medium.

The results above suggest that when the respiratory chain becomes reduced by substrate on anaerobiosis there is an increase in the fluorescence of the inner membrane-bound NPN. Reoxidation of the respiratory chain components should therefore result in fluorescence quenching, as was observed following the addition of H_2O_2 (fig.1). In fig.3 the changes in fluorescence of NPN and in the reduction of *b*-type cytochromes were followed in parallel experiments. *E. coli* GR19N, a strain lacking *d*-type cytochrome, was used [8]. Fig.3 (curve 1) shows that the increase in fluorescence of NPN which occurred at anaerobiosis was paralleled by reduction of the *b*-type cytochromes. Addition of ferricyanide gave transient oxidation of cytochrome and quenching of the fluorescence of NPN.

Growth of *E. coli* on L-proline induces the fumarate reductase system [9]. Vesicles prepared from proline-grown cells showed the usual increase in the fluorescence on NPN on anaerobiosis. Addi-

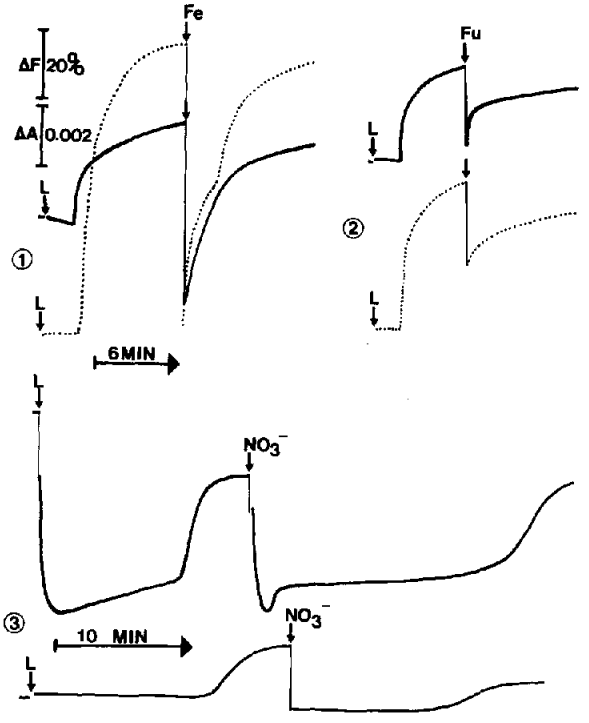

Fig.3. Changes in the fluorescence of NPN (1,2, solid line; 3, lower trace) and quinacrine (3, upper trace), and in the reduction level of the *b*-type cytochromes (1,2, broken lines) following addition of substrates and oxidants. The measuring systems are described in section 2. The everted membrane vesicles (protein, 0.5 mg/ml) used in 1 and 2 were prepared from *E. coli* GR19N grown on L-proline. For 3 the vesicles (protein, 0.5 mg/ml) were prepared from *E. coli* AN180 grown anaerobically on nitrate. L, 10 mM D-lactate; Fe, 0.1 mM ferricyanide; Fu, 0.1 mM fumarate; NO_3^-, 1 mM nitrate.

tion of fumarate oxidized a portion of the cytochrome. There was simultaneously a decrease in the fluorescence of NPN (fig.3, curve 2).

Growth on nitrate under anaerobic conditions was used to induce the nitrate reductase system [10]. Addition of D-lactate to everted inner membrane vesicles established a transmembrane proton gradient, as shown by the quenching of the fluorescence of quinacrine [11,12] (fig.3, curve 3). The gradient collapsed at anaerobiosis but could be reestablished following addition of a limiting amount of nitrate. The gradient collapsed following complete reduction of the added nitrate. The fluorescence behaviour of NPN was somewhat

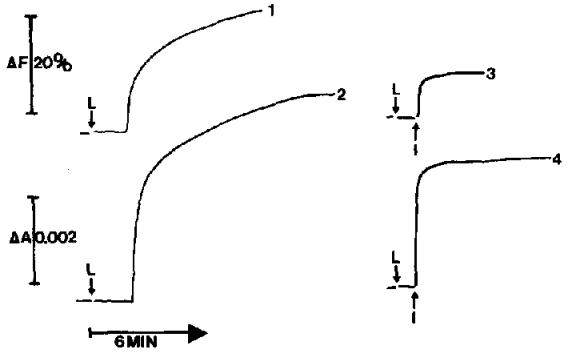

Fig.4. Effect of cyanide on the fluorescence of NPN (1,3) and the reduction level of the *b*-type cytochromes (2,4). The measuring systems are described in section 2. The everted membrane vesicles (protein, 0.5 mg/ml) were prepared from *E. coli* GR19N grown on L-proline. L, 10 mM D-lactate; I, 4 mM KCN.

similar. That is, an increase in fluorescence occurred on depletion of oxygen and nitrate, and quenching was produced by oxidation of the nitrate reductase respiratory chain following addition of nitrate.

The close parallel between the fluorescence response of NPN and the reduction of the *b*-type cytochromes was also supported by the effect of KCN on these processes. Addition of KCN to a suspension of everted inner membrane vesicles oxidizing D-lactate caused an immediate increase in the aerobic steady-state level of reduction of the *b*-type cytochromes (fig.4, curves 2,4). The fluorescence of NPN behaved similarly. Thus, there was an increase in fluorescence on anaerobiosis (fig.4, curve 1) or following the addition of KCN (fig.4, curve 3). The proportional changes observed were similar to those shown by the cytochromes.

The results described above suggest that NPN does not respond to the level of energization of everted inner membrane vesicles but responds to the state of reduction of the components of the respiratory chain. Although there is a close relationship between the level of reduction of the *b*-type cytochromes and the behaviour of NPN, it is also possible that the NPN is responding to the redox state of the quinones of the respiratory chain. This possibility is being examined at present. The mechanism for the increase in fluorescence has not been determined. It could involve a direct interaction with the *b*-type cytochromes or

other compounds of the respiratory chain, or an indirect effect transmitted to the membrane by a change in the conformation of respiratory chain components.

ACKNOWLEDGEMENT

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