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The fluorescence intensity of the lipophilic probe *N*-phenyl-1-naphthylamine responds to the oxidation-reduction state of purified *Escherichia coli* cytochrome *o* incorporated into phospholipid vesicles

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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 the redox state of purified cytochrome *o* incorporated into lipid vesicles formed from purified or *E. coli* phospholipids. NPN was bound to the proteoliposomes to produce a steady-state level of fluorescence intensity. Addition of the substrate ascorbate, in the presence of phenazine methosulfate as an electron donor, did not alter the fluorescence. However, following complete removal of oxygen from the medium by oxidation of the substrate by molecular oxygen catalyzed by cytochrome *o*, there was an increase in the fluorescence of NPN. This coincided with the reduction of cytochrome *o*. Reoxidation of the cytochrome by addition of oxygen decreased the fluorescence to steady-state levels until the oxidant had been completely reduced. The fluorescence changes were dependent on the incorporation of cytochrome *o* into phospholipid vesicles but were insensitive to the state of energization of the vesicle membrane.

N-Phenyl-1-naphthylamine; Lipophilic probe; Cytochrome *o*; Proteoliposome; Oxidation-reduction state

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. This change is sensitive to the state of membrane energization of the cell. Thus, energization decreases fluorescence intensity and deenergization reverses this effect [2–5]. Recently, we have shown that the alteration in fluorescence intensity in *Escherichia coli* has two components [6]. The first, which occurs in intact cells, involves the response of the probe to changes in the outer membrane coupled to inner membrane energization. It is sensitive to uncouplers, and responds to membrane energization by ATP hydrolysis or substrate oxidation through the respiratory chain. The second component, which can also be demonstrated in inverted inner membrane vesicles, is insensitive to uncouplers but 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 [7]. Since membrane vesicles contain all of the components of the respiratory chain, i.e. flavoproteins, quinones and cytochromes, it was not possible to determine whether NPN was responding to the redox state of a specific component of the chain. Here, we show using purified cytochrome *o*, a major oxidase of the respiratory chain of *E. coli* [8], incorporated into

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phospholipid vesicles that the fluorescence intensity of NPN will respond to the redox state of this cytochrome.

2. MATERIALS AND METHODS

2.1. *Preparation of cytochrome* o

Cytochrome *o* was obtained from membranes of *E. coli* RG167 pRG110 (*F⁻ rpsL thi gal nadA cydA2 lon100 cyo sr1300::Tn10 recA*) grown on DL-lactate as carbon source. pRG110 contains a 5.7 kb *Sph*I-*Sal*I fragment of the *E. coli* genome carrying the *cyo* gene cloned into pBR322. This strain was generously provided by Dr R.B. Gennis (University of Illinois). The cytochrome was purified as in [9] with the modification that the elution buffer for the DEAE-BioGel A column contained 1% (w/v) octyl glucoside instead of Triton X-100.

2.2. *Isolation of* E. coli *phospholipids*

E. coli phospholipids were prepared as described [10] from *E. coli* B (Grain Processing Co.) and stored at −70°C at a final concentration of 100 mg (dry wt) per ml in 2 mM 2-mercaptoethanol.

2.3. *Reconstitution of proteoliposomes containing cytochrome* o

Bath-sonicated phospholipids for use in reconstitution experiments were prepared at a concentration of 50 mg/ml phospholipid in 50 mM potassium phosphate buffer, pH 7.5, as in [10]. Phospholipids (1.0 ml) were mixed with octyl glucoside (0.16 ml of 12.5% octyl glucoside dissolved in buffer), purified cytochrome *o* (7.7 mg protein) and 50 mM potassium phosphate (pH 7.5) to a total volume of 4.4 ml. The sample was mixed briefly, incubated at 0°C for 20 min, and then diluted rapidly into 120 ml phosphate buffer containing 1 mM dithiothreitol at room temperature. After stirring for 10 min, proteoliposomes were collected from the diluted solution by centrifugation at 120000 × *g* for 2 h. The supernatant was discarded and the proteoliposomes were resuspended in fluorescence assay buffer (10 mM Hepes, pH 7.4, containing 0.3 M KCl and 5 mM $MgCl_2$) to a final volume of 0.38 ml to give a concentration of 20 mg/ml protein. The suspension was then rapidly frozen in liquid N_2, thawed at room temperature, and sonicated for 10–20 s with a bath-type sonicator (80 W, 50/60 Hz Bransonic 12, Branson Cleaning Equipment Co., Shelton, CT). Samples (0.1 ml) of this proteoliposome preparation were added directly to fluorescence or cytochrome reduction assay mixtures.

Proteoliposomes were also prepared from commercially available purified phospholipids. Phosphatidylcholine (egg yolk) (45 mg) and phosphatidylethanolamine (*E. coli*) (5 mg) were combined and dried in a stream of N_2 gas to remove solvents. The dried phospholipids were then resuspended in 1.0 ml of 50 mM potassium phosphate buffer, pH 7.5, to be used for the preparation of cytochrome *o* proteoliposomes as described above.

In some experiments, *E. coli* phospholipids (50 mg/ml in 50 mM phosphate buffer, pH 7.5) were sonicated and subsequently combined with cytochrome *o*-containing column fractions in fluorescence and cytochrome reduction assays. The phospholipid/protein ratio was varied in these experiments by altering the amount of phospholipid added to a constant protein concentration of 0.5 mg/ml.

2.4. *Measurement of NPN fluorescence and cytochrome reduction*

NPN fluorescence and cytochrome reduction assays were performed as described in [7].

2.5. *Determination of protein*

Protein was determined by the method of Lowry et al. [11] using bovine serum albumin as a standard.

2.6. *Chemicals*

Commercially available phospholipids, phenazine methosulfate (PMS) and NPN were obtained from Sigma. 3,3',4',5-Tetrachlorosalicylanilide (TCS) was purchased from Eastman.

3. RESULTS AND DISCUSSION

Cytochrome *o* was solubilized by Triton X-114 from membranes of a strain in which the cytochrome *o* gene was carried on a plasmid, and subsequently purified by chromatography. The purified cytochrome *o* contained 0.1 mg phospholipid/mg protein. Its dithionite-reduced minus oxidized difference spectrum measured at 77 K is shown in fig.1. Absorption maxima at 553.75, 558.25 and 563.75 nm were present in the fourth-derivative spectrum.

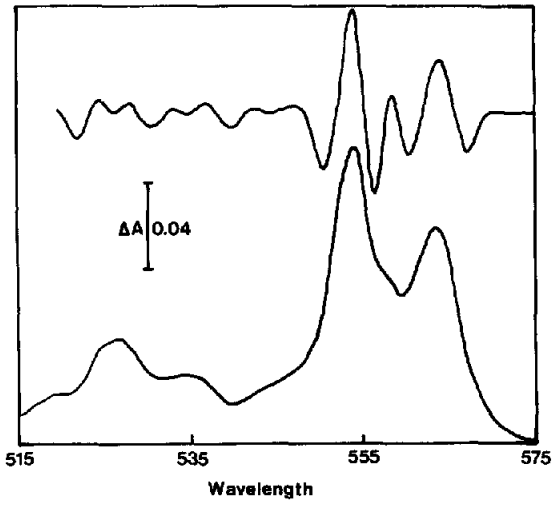

Fig.1. Dithionite-reduced minus H_2O_2-oxidized difference spectra of purified cytochrome *o* measured at 77 K. Upper curve: fourth-order finite difference spectrum calculated from the lower curve.

Cytochrome *o* was incorporated into proteoliposomes with *E. coli* phospholipids as described by Viitanen et al. [10]. Addition of NPN to the proteoliposome suspension gave a rapid increase in fluorescence of the probe as it entered a hydrophobic environment (fig.2, curve 1). Addition of PMS-ascorbate, as a substrate to reduce cytochrome *o* [12], produced little initial change in the fluorescence of NPN. However, after about 3.5 min there was a sudden increase in fluorescence of the probe. This coincided with the medium in the cuvette becoming anaerobic and was accompanied by reduction of cytochrome *o* to its anaerobic steady-state level (fig.2, curve 1A). Addition of H_2O_2 resulted in immediate reoxidation of cytochrome *o* and loss of the NPN fluorescence increase observed on anaerobiosis. The return to anaerobic conditions resulted in rereduction of cytochrome *o* and restoration of increased NPN fluorescence (fig.2, curves 1,1A). Addition of KCN prior to substrate resulted in an immediate increase in the fluorescence of NPN when ascorbate was added (fig.2, curve 2). This

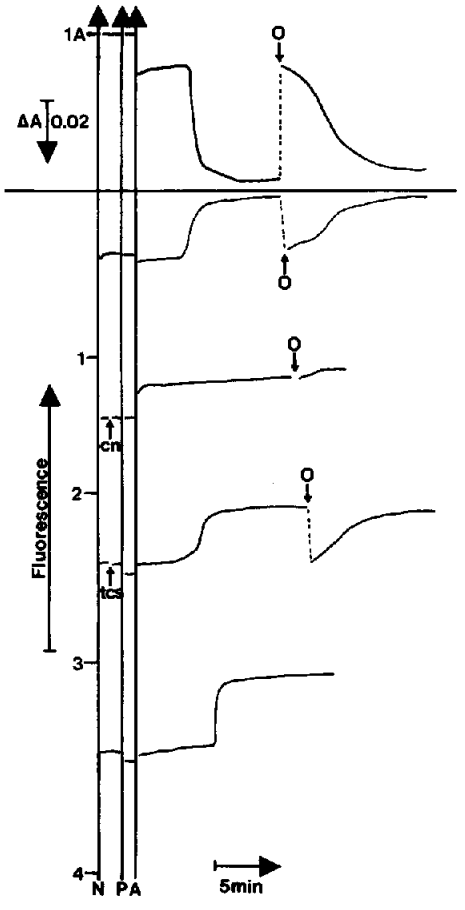

Fig.2. Changes in fluorescence of NPN (1–4) and reduction level of cytochrome *o* (1A) following addition of substrates to cytochrome *o* proteoliposomes. The measuring systems are described in section 2. NPN fluorescence intensity increases vertically. It was excited with light at 340 nm and the emission was measured at 420 nm. Cytochrome *o* reduction is shown by a decrease vertically. The absorption of the sample at 559 nm was compared with that at the reference wavelength of 580 nm. Protein concentration was 1 mg/ml. Phospholipid concentration [*E. coli* phospholipids, curves 1,1A,2,3; purified phosphatidylcholine : purified phosphatidylethanolamine (9 : 1), curve 4] was 4.5 mg/ml. N, addition of NPN to 3.7 μM; P, 2.5 μM PMS; A, 2.5 mM ascorbate; cn, 2 mM KCN; tcs, 1.5 μM TCS; O, 1 μl 3% H_2O_2.

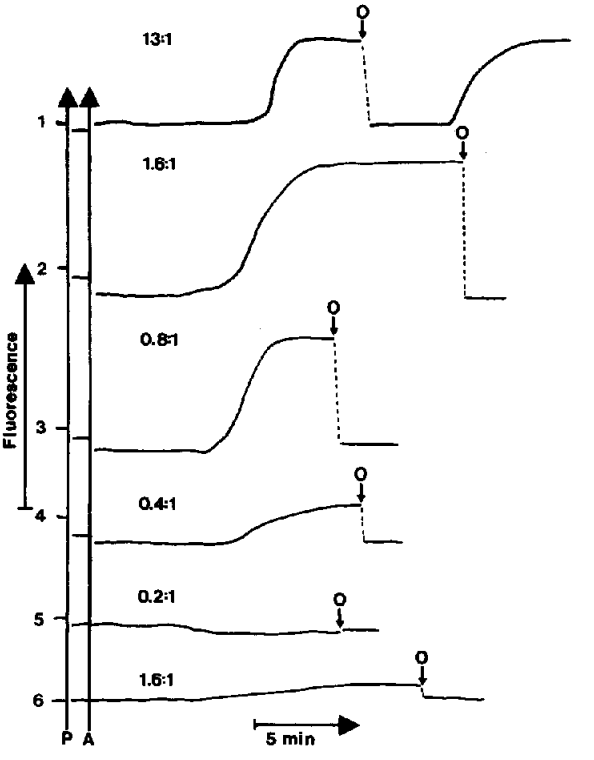

Fig.3. Changes in fluorescence of NPN in proteoliposomes formed by the addition of cytochrome *o* to preformed *E. coli* phospholipid vesicles. Various amounts of phospholipid vesicles were added to a constant amount (0.5 mg protein/ml) of cytochrome *o* in the fluorescence cuvette to give the phospholipid/protein ratios shown beside each curve. NPN was present at 1.25 μM except in the experiment giving curve 6 in which it was omitted; P, 2.5 μM PMS; A, 2 mM ascorbate; O, 1 μl 3% H_2O_2 added.

was accompanied by an increase in the aerobic steady-state level of reduction of cytochrome *o* (not shown but see fig.4 of [7]). The inhibitor blocked reoxidation of cytochrome *o* by H_2O_2 and prevented quenching of NPN fluorescence by H_2O_2. The fluorescence behaviour of NPN was unaffected by the presence of the uncoupler TCS (fig.2, curve 3). The fluorescence behaviour of NPN could also be seen in proteoliposomes of cytochrome *o* in which *E. coli* phospholipids had been replaced by a 9:1 ratio of purified egg yolk phosphatidylcholine and *E. coli* phosphatidylethanolamine (fig.2, curve 4).

The results described above show that NPN responds to the redox state of cytochrome *o* incorporated into phospholipid vesicles. The uncoupler TCS has no effect on this behaviour. Thus, it is likely that NPN is responding to the redox state of the cytochrome directly and not via alterations in the electrochemical gradient of protons [12].

The role of lipid vesicles in the NPN fluorescence response was examined more fully. It was observed that addition of cytochrome *o* to preformed vesicles of *E. coli* phospholipid gave a system in which NPN responded as satisfactorily to the redox state of the cytochrome as when the cytochrome was pre-incorporated into the vesicles. As shown in fig.3, the optimal response was obtained at a phospholipid/protein ratio of 1.6:1 by weight. A nearly optimal response was observed at a ratio of 0.8:1. However, the NPN response was severely diminished at lower phospholipid/protein ratios although the oxidase activity of cytochrome *o* was unimpaired (not shown). These results indicate that the NPN response depends on the incorporation of cytochrome *o* into a phospholipid environment. Since the response was severely affected at phospholipid/protein ratios of 0.4:1 or less, it is likely that the cytochrome *o* needs to be incorporated into vesicles. Even at the lowest phospholipid/protein ratios there would be enough phospholipid available to saturate binding sites on cytochrome *o* if the need for phospholipid in the NPN response was to fill binding sites on the cytochrome. Fig.3, curve 6, confirms that the fluorescence response depends on the presence of NPN.

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REFERENCES

[1] Träuble, H. and Overath, P. (1973) Biochim. Biophys. Acta 307, 491–521.
[2] Helgerson, S.L. and Cramer, W.A. (1976) J. Supramol. Struct. 5, 291–308.
[3] Helgerson, S.L. and Cramer, W.A. (1977) Biochemistry 16, 4109–4177.
[4] Nieva-Gomez, D. and Gennis, R.B. (1977) Proc. Natl. Acad. Sci. USA 74, 1811–1815.
[5] Nieva-Gomez, D., Konisky, J. and Gennis, R.B. (1976) Biochemistry 15, 2747–2753.
[6] Sedgwick, E.G. and Bragg, P.D. (1987) Biochim. Biophys. Acta, in press.
[7] Sedgwick, E.G. and Bragg, P.D. (1987) FEBS Lett. 218, 22–26.
[8] Ingledew, W.J. and Poole, R.K. (1984) Microbiol. Rev. 48, 222–271.
[9] Withers, H.K. and Bragg, P.K. (1987) Biochim. Biophys. Acta 892, 10–22.
[10] Viitanen, P., Newman, M.J., Forster, D.L., Wilson, T.H. and Kaback, H.R. (1986) Methods Enzymol. 125, 429–452.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Matsushita, K., Patel, L. and Kaback, H.R. (1984) Biochemistry 23, 4703–4714.