Interaction of Lipophilic Quinones with Membrane Fragments of *Paracoccus denitrificans* and *Staphylococcus epidermidis*

By VLADIMÍR MIKEŠ and VLADIMÍR DADÁK

Department of Biochemistry, University J. E. Purkyně, 61137 Brno, Czechoslovakia

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In quinone-depleted mitochondrial and *Paracoccus denitrificans* membranes the quantum yield of fluorescence of ostruthin (6-geranyl-7-hydroxycoumarin) was maintained, whereas an increase in the quantum yield took place after extraction of *Staphylococcus epidermidis* membrane. A marked quenching effect of ubiquinone and menaquinone each with two isoprene units in the side chain on the ostruthin fluorescence was found with all types of quinone-depleted particles. When the homologues of menaquinone and ubiquinone with six isoprene units in the side chain were re-incorporated, a quenching of the ostruthin fluorescence was observed in the *S. epidermidis* membranes but not in those of *P. denitrificans*. The different behaviour of both bacterial preparations is attributable to the more specific binding of ubiquinone in the particles of *P. denitrificans*.

The membrane fraction of the bacterium *Paracoccus denitrificans* contains the same ubiquinone homologue Q-10 as the mitochondrial membrane (Schles & Smith, 1968), whereas the membrane fraction of the bacterium *Staphylococcus epidermidis* contains the menaquinone homologues MK-7 and MK-8 (Jeffries *et al.*, 1969). It was shown that both quinones are involved in bacterial electron transport (Kröger *et al.*, 1971), but further functions which are important for bacteria cannot be excluded.

Ostruthin (6-geranyl-7-hydroxycoumarin) was found to be inhibitory for growth and respiration of *Staphylococci* and other Gram-positive bacteria (Dadák & Hođák, 1966). The effect of ostruthin on respiration could be neutralized by vitamin K and other lipophilic quinones (Dadák, 1965). In the water phase, ostruthin as a phenolic coumarin exhibits a fluorescence with an emission maximum at 455 nm. This maximum corresponds to the fluorescence of the ostruthin anion. In organic solvents or on penetration of ostruthin into the membrane, its dissociation is suppressed and a new emission maximum at 395 nm appears. This is the maximum of undissociated ostruthin (Zbořil & Dadák, 1973; Yakatan *et al.*, 1972). The present paper reports a study of the interaction of Q and MK with bacterial membranes by making use of this fluorescent probe.

Materials and Methods

Ubiquinone and menaquinone homologues were generously provided by F. Hoffman-La Roche, Basel, Switzerland. Ostruthin was a gift from the

Abbreviations used: Q-*n*, ubiquinone, MK-*n*, menaquinone (homologue of vitamin K₂), each with *n* isoprene units in the side chain.

Institute of Organic Chemistry, University of Vienna, Vienna, Austria. Phospholipid micelles were prepared from Asolectin (American Lecithin Co., Woodside, NY, U.S.A.) as described by Kagawa *et al.* (1973).

Staphylococcus epidermidis 104-7 HIM was grown aerobically for 17 h at 37°C in a medium containing 12 g of Bacto beef extract, 12 g of Bactopeptone (both from Imuna n.p., Šarišské Michalany, Czechoslovakia) and 6 g of NaCl/litre, pH 7.2. *Paracoccus denitrificans* N.C.I.B. 8944 was grown to reach a stationary phase, requiring 48 h at 37°C in the synthetic medium of Chang & Morris (1962) with 0.05 M-glucose and 0.2% KNO₃. The membrane fraction from *S. epidermidis* was prepared from harvested cells suspended in 0.1 M-sodium/potassium phosphate buffer, pH 7.2, by using a Braun MSK homogenizer (B. Braun, Melsungen, Germany) with glass beads (diameter 0.17–0.18 mm). The unbroken cells and cell walls were removed by centrifugation at 10000g for 15 min and the membrane fraction was collected by centrifugation of the supernatant at 70000g for 30 min. Plasma-membrane fraction from *P. denitrificans* was prepared by sonicating the cells for 9 × 10 s at 0–5°C in 5-ml batches containing 20 mg bacterial dry wt./ml by using the M.S.E. 500 W Ultrasonic disintegrator. After 4-fold dilution with 0.1 M-sodium/potassium phosphate buffer, pH 7.2, the unbroken cells were removed and the membrane fraction was sedimented at 105000g for 30 min. The upper red-brown layer of fragments was separated for further use. The membrane fractions from both bacterial strains were washed once with phosphate buffer and suspended in 0.1 M-KCl. Extraction of freeze-dried particles and re-incorporation of lipophilic quinones were performed as described by Norling *et al.* (1974).

Mitochondrial fragments were prepared from pig heart muscle (Zbořil & Dadák, 1973).

Fluorescence measurements

The titration of a certain concentration of the dye with particles can be expressed by eqn. (1), modified from that published by Lenaz *et al.* (1976):

$$\frac{1}{F - F_0} = \frac{1}{(F_1 - F_0)P} \cdot \frac{W_w}{W_p} + \frac{1}{F_1 - F_0} \quad (1)$$

where F is the measured fluorescence, F_0 the fluorescence of the dye without particles, F_1 the fluorescence at infinite concentration of particles, W_w the weight of water phase, W_p the weight of membrane phase and P the partition coefficient. This equation gives the binding of the dye as a simple transition into the hydrophobic phase. It can be compared with eqn. (2), which expresses this process as a macromolecule–ligand interaction:

$$\frac{1}{F - F_0} = \frac{1}{K \cdot N(F_1 - F_0)} \cdot \frac{1}{M} + \frac{1}{F_1 - F_0} \quad (2)$$

where N is the binding capacity, K the association binding constant and M the concentration of membrane phase in mg/ml. The other symbols have the same meaning as in eqn. (1). A comparison of these two equations shows that P in eqn. (1) corresponds to $K \cdot N$ in eqn. (2). From these equations the F_1 values were determined. The binding capacity was determined as described by Scatchard (1949).

Results

Ostruthin could be incorporated into the mitochondrial and the bacterial membranes. A new emission maximum at 395 nm appeared as a result of this incorporation. The position of the ostruthin fluorescence maximum indicated that the environment of the chromophore was very similar to that in Asolectin dispersions, in which the probe was at least partly located in the hydrocarbon phase of the phospholipids. As shown in Fig. 1, there was a difference in the binding capacity of ostruthin between bacterial and submitochondrial particles. Evaluation by a Scatchard (1949) plot gave a binding capacity of 20 ± 5 nmol of ostruthin/mg of protein for bacterial membranes, compared with that of 45 ± 5 nmol/mg of protein for mitochondrial membranes found previously (Zbořil & Dadák, 1973). The same low binding capacity of bacterial membranes was also observed in freeze-dried preparations. Variations of the membrane binding capacity, the binding constant or the partition coefficient were reflected in the change of the slope in Fig. 1, whereas the intercept on the ordinate was unchanged. The quantum yield of fluorescence as reflected in the value of F_1 seemed to be very similar for both mitochondrial and bacterial

Fig. 1. Double-reciprocal plots of the ostruthin fluorescence in membrane fragments against protein concentration. To the membrane fragments in 3 ml of 0.1 M-potassium phosphate, pH 7.4, an ethanolic solution of ostruthin was added to a final concentration of $3 \mu\text{M}$, and fluorescence was measured at 395 nm in a 1 cm-light-path quartz cuvette excited at 350 nm. The final concentration of ethanol was lower than 2%. Fluorescence intensity is given in relative units. For definition of symbols see the Materials and Methods section. Δ , Submitochondrial particles; $\square$, *S. epidermidis* particles; $\circ$, *P. denitrificans* particles.

membranes. The binding constant varied with the concentration of the membrane used for titration, between 0.1 and $1 \mu\text{M}$ -ostruthin, although the linearity of the Scatchard plot was satisfying.

Fig. 2 shows the effect of pentane extraction of bacterial membranes on ostruthin fluorescence. After the extraction of the *P. denitrificans* membrane, the quantum yield of fluorescence was maintained, but a small decrease in the binding capacity to 17 nmol of ostruthin/mg of protein was observed. This behaviour resembled very closely the results obtained with submitochondrial particles after pentane treatment (Zbořil & Dadák, 1973). On the other hand the pentane extraction of *S. epidermidis* membrane resulted in an appreciable enhancement of the quantum yield of ostruthin fluorescence. Obviously the pentane extraction results in a qualitative change in the binding of ostruthin to the *S. epidermidis* membrane. Similar results with both bacterial strains were obtained when neutral lipids were extracted with 96% (v/v) acetone. Also in this case the quantum yield of ostruthin fluorescence with *P. denitrificans* particles remained unaltered, whereas a marked increase in the quantum yield took place after extraction of *S. epidermidis* particles. The lipophilic quinones ubiquinone and menaquinone are two main constituents that are extracted with pentane and 96% acetone

Fig. 2. Double-reciprocal plots of ostruthin fluorescence against bacterial membrane fragments

For assay procedures see Fig. 1. Concentration of ostruthin was $2\mu\text{M}$. (a) Membranes of *P. denitrificans*: $\circ$, freeze-dried; $\bullet$, pentane-extracted. (b) Membranes of *S. epidermidis*: $\square$, freeze-dried; $\blacksquare$, pentane-extracted.

(Norling *et al.*, 1974; Lester & Fleischer, 1961). As there is no overlap in the spectra of ostruthin and menaquinone or ostruthin and ubiquinone, the quenching of fluorescence by energy transfer does not take place. A marked quenching effect of lipophilic quinones was, however, found when homologues with short side chains were bound to phospholipid vesicles prepared from Asolectin.

Fig. 3 illustrates the effect of Q-2 and MK-2 on the fluorescence of ostruthin incorporated into quinone-depleted particles. In this case ubiquinone and menaquinone also quenched the ostruthin fluorescence. When ostruthin and Q-2 interacted with the *P. denitrificans* membrane, the quenching effect was practically the same as that when ostruthin and MK-2 were bound to the *S. epidermidis* membrane. On the basis of our results it is not possible to express this quenching effect by an appropriate equation, since it depends on the as-yet unknown concentration of the quenching substance in the hydrophobic phase. Moreover, the relation between the amount of lipo-

Fig. 3. Quinone quenching of ostruthin fluorescence in pentane-extracted membrane fragments (Stern-Volmer plots)

To the membrane fragments (0.083 mg of membrane protein/ml) in 0.02M-potassium phosphate, pH 7.4, an ethanolic solution of ostruthin was added and fluorescence I_0 was measured. After addition of the quinone solution, fluorescence I was measured. Other details are as in Fig. 1. $\bullet$, *P. denitrificans* membrane, quenching with Q-2; $\blacksquare$, *S. epidermidis* membrane, quenching with MK-2.

Table 1. Quenching of ostruthin fluorescence with menaquinone in *S. epidermidis* membranes

The membrane concentration was 0.083 mg of protein/ml; ostruthin was $5\mu\text{M}$. For assay procedures see the legend to Fig. 1.

Membrane fraction	Fluorescence (arbitrary units)
Freeze-dried	61
Native extract re-incorporated	62
MK-depleted	81
MK re-incorporated (1.5 nmol of MK-6/mg of protein)	75
MK re-incorporated (3.3 nmol of MK-6/mg of protein)	70

philic quinone added to the solution and its concentration in the hydrophobic phase is not linear.

Different results were obtained when higher homologues MK-6, Q-6 or Q-10 were re-incorporated into the membrane after quinone depletion. These results are summarized in Tables 1 and 2. We observed a quenching of the ostruthin fluorescence with MK-6 in *S. epidermidis* particles. No quenching was found when the ubiquinone homologue Q-6 was re-incorporated into *P. denitrificans* particles. Even when MK-6 was used for re-incorporation into the *P. denitrificans* membrane no quenching was detected. Re-incorporation of Q-10 led to an increase in the fluorescence intensity. Thus two important factors influence the effect of lipophilic quinones on the ostruthin fluorescence in the membrane, namely the length of the isoprenoid side chain and the specific response of bacterial material.

Table 2. Quenching of ostruthin fluorescence with ubiquinone and menaquinone in *P. denitrificans* membranes
For conditions see Table 1.

Membrane fraction	Fluorescence (arbitrary units)
Freeze-dried	83
Q-depleted	85
Q re-incorporated (10nmol of Q-6/mg of protein)	86
MK re-incorporated (10nmol of MK-6/mg of protein)	84
Q re-incorporated (10nmol of Q-10/mg of protein)	96

Discussion

Ostruthin, the natural geranyl derivative of 7-hydroxycoumarin (umbelliferone), interacts with mitochondrial fragments (Zbořil & Dadák, 1973), bacterial membranes and phospholipid micelles. The same change in ostruthin fluorescence indicates a similarity of the environment of the chromophore in preparations which differ markedly in lipid composition. This suggests that ostruthin binding may be a simple transition into the hydrophobic phase. The quinone homologues with short isoprenoid chains are able to quench the ostruthin fluorescence, probably owing to their greater mobility in the particles and phospholipid dispersions. To elucidate the nature of the quenching of the fluorescence of 12-(9-anthroyl)-stearic acid by ubiquinone, Chance *et al.* (1975) assumed a different mobility of the ubiquinone in the oxidized and the reduced mitochondrial membrane. Owing to the relatively short side chain of the ostruthin molecule, its mobility within the membrane is greater than that of the 12-(9-anthroyl)stearic acid, whose chromophore is located in the hydrocarbon region. In our case the polar ring of the ostruthin molecule may occupy a similar position, as was found with 4-pentadecylumbelliferone, where the chromophore should be located in the region between the glycerol portion and the polar head groups on the outside of the membrane (Pohl, 1976). The benzoquinone and the naphthoquinone rings are effective in the quenching of the ostruthin fluorescence. Since there is no overlap in the spectra of quinone and ostruthin rings, the quenching by energy transfer cannot be considered. The dynamic quenching is described by the hyperbolic relation similar to that of the quenching corresponding to the formation of a non-fluorescent ground-state complex (classical Stern-Volmer relation; Brand & Withold, 1967). The other possibility, i.e. quenching within the active sphere, is given by the exponential relation. As shown in Fig. 3, at least in the low concentration range the results obtained follow the Stern-Volmer equation

valid for dynamic quenching (Chance *et al.*, 1975). It could be expected that higher quinone homologues will be less effective in the quenching than the short ones, owing to their deeper location and limited diffusion mobility in the membrane. The results given in Tables 1 and 2 show that MK-6 exerts a similar quenching effect to that of MK-2 in the pentane-extracted *S. epidermidis*. The replenishment of *P. denitrificans* particles with Q-6 has, however, no influence on the ostruthin fluorescence, in contrast with Q-2. If Q-6 is replaced by MK-6 in the *P. denitrificans* membrane, no quenching takes place. These findings are consistent with the results shown in Fig. 2, implying different effects of pentane treatment on both membranes.

Maggio *et al.* (1977) found that ubiquinones are practically immiscible with the phospholipids in monolayers. It would seem from our results that the different behaviour of both bacterial preparations is attributable to the specific binding of ubiquinone in the *P. denitrificans* membrane. In this case Q-10 might be bound to some specific region or to some specific protein and has consequently a much lower diffusion mobility than the MK-6 in *S. epidermidis*. The relatively firm binding of Q-6 prevents the quenching of ostruthin fluorescence. We have actually observed that the extraction of MK homologues from *S. epidermidis* proceeds much more readily than the extraction of Q-10 from *P. denitrificans*. The extraction of Q-10 may result in such a change in the particles, which causes an alteration in the binding parameters as given in eqn. (2). On the other hand the extraction of MK may simply modify the micro-environment of the fluorescent probe.

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