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Korormicin, an Antibiotic Specific for Gram-negative Marine Bacteria, Strongly Inhibits the Respiratory Chain-linked Na^+-Translocating NADH: Quinone Reductase from the Marine *Vibrio alginolyticus*

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Marine bacteria require a significant amount of NaCl for optimal growth and have quite different physiological character from terrestrial counterparts. For example, marine bacteria possess a unique type of NADH:quinone reductase, a membrane-bound enzyme which takes part in the respiratory chain[1]. This enzyme requires Na^+ for its activity and with its Na^+-pumping activity generates sodium-motive force which enables these bacteria to live easily in the marine environment. This energy-acquisition system was found only in Gram-negative marine and moderately halophilic bacteria but not in halo-tolerant bacteria or archaea which also live in the environment with high salinity[2]. In this regard it is probable that marine bacteria produce unique metabolites by their unique metabolic pathways which do not occur in other microorganisms including extensively studied terrestrial microorganisms.

In the course of our searching project for useful compound in the metabolites of marine bacteria, we have found and reported several unique compounds[3,4]. Among them a marine bacterial strain *Pseudoalteromonas* sp. F-420 was found to produce an antibiotic compound named korormicin (Fig. 1)[5]. This compound showed very interesting bioactivity, that is, it inhibited specifically the growth of Gram-negative marine and halophilic bacteria, whereas Gram-positive strains and non-halophilic bacteria were insensitive to this compound. The data suggests that korormicin has some specific targets solely found in Gram-negative marine bacteria.

In this study Na^+-translocating NADH:quinone reductase (NQR) widely distributed in the Gram-negative marine and halophilic bacteria was selected as the first candidate target molecule of korormicin. This paper describes the result of the inhibition experiment.

Results and Discussion

The Na^+-translocating NADH:quinone reductase (NQR) from the marine *Vibrio alginolyticus* is composed of 6 different subunits[6,7] and catalyzes two successive reactions[8]. First, the FAD-containing subunit reacts with NADH and reduces the quinone substrate by a one-electron transfer pathway to produce semiquinone radicals. Then, semiquinone radicals are quickly converted to quinol by the action of other subunits. The latter, but not the former, reaction specifically requires Na^+ for the activity and is directly linked to the extrusion of Na^+ from the cells. The former NADH dehydrogenase (NDH) activity could be measured from the decrease in NADH using 2-methyl-1,4-naphthoquinone (menadione) as the substrate. The total NADH:quinone reductase (NQR) activity could be measured from the reduction of ubiquinone-1 (Q-1) to ubiquinol-1. Thus, the effects of korormicin on the NDH and NQR activities were examined as shown in Fig. 2a. Korormicin, up to 100 nM, did not affect the NDH activity at all. On the other hand 1 nM korormicin effectively inhibited NQR activity. This means that korormicin strongly inhibits the latter part of the NQR reaction that is directly linked to the Na^+-pumping activity.

Using the membrane fraction and the Na^+-loaded cells of *V. alginolyticus* 138-2, TOKUDA and UNEMOTO reported that 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) also inhibits NQR, but not NDH, activity[9]. In our experiment using the purified enzyme preparation (Fig. 2b) more than 100 nM HQNO was needed to inhibit 50% of the NQR activity, so the inhibitory activity of korormicin on NQR activity was much more significant compared to that of HQNO.

Fig. 1. Structure of korormicin.

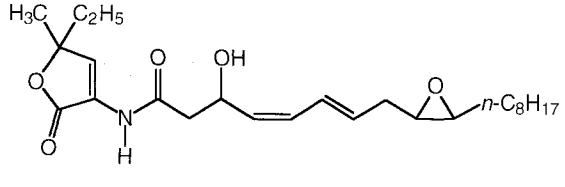
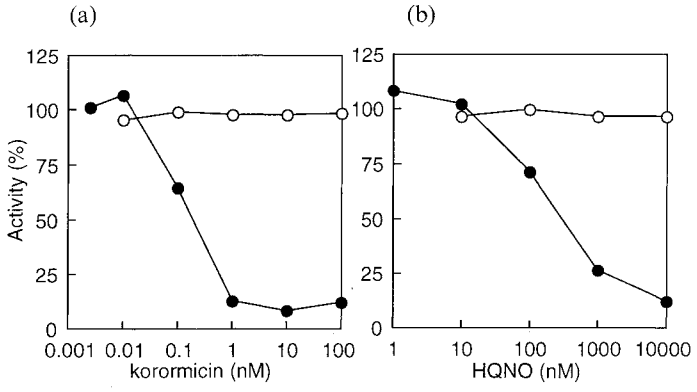
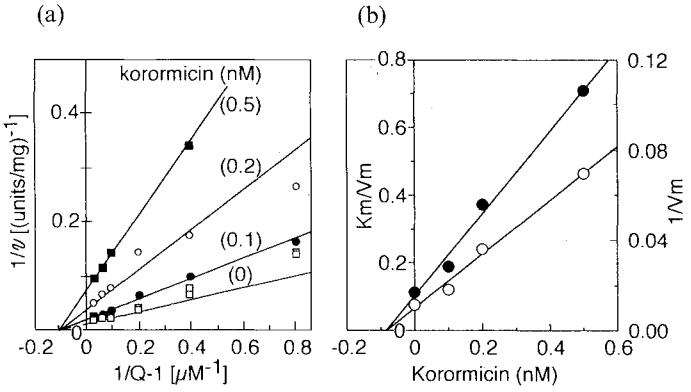


Fig. 2. Effects of korormicin (a) and HQNO (b) on the NDH (○) and NQR (●) activities of the NQR complex.



The NQR complex purified from *V. alginolyticus* had NDH and NQR activities of 120 and 53 units/mg protein, respectively, and 0.12 μg protein of the enzyme was used for each assay.

Fig. 3. Analysis of the mode of action of korormicin to the NQR activity.



(a) Double-reciprocal plots of initial velocity *vs.* Q-1 concentration at fixed levels of korormicin indicated in the figure. The initial velocity (v) was expressed in units/mg protein and 0.12 μg protein of the enzyme was used for each assay. (b) Secondary plots of slope (Km/Vm) *vs.* inhibitor (●) and intercept (1/Vm) *vs.* inhibitor (○). Vm and Km are usual meanings in enzyme kinetics.

The mode of action of korormicin to the NQR activity was investigated. As shown in Fig. 3a which provides the double-reciprocal plots, korormicin acted as a noncompetitive inhibitor for Q-1. From the secondary plots (Fig. 3b), the inhibitor constants for the free enzyme (Ki) and the enzyme-substrate complex (Kii) were estimated to be 83.7 and 81.1 pM, respectively. Since Ki is almost identical to Kii, korormicin acts as a fully noncompetitive inhibitor for Q-1.

It is reported that wide variety of Gram-negative marine bacteria possess NQR[10], but Gram-positive bacteria seem to lack this enzyme[2]. In the paper-disk method korormicin was a specific growth inhibitor against Gram-negative marine bacteria and Gram-positive strains were resistant to this drug. On the other hand HQNO inhibited some Gram-positive bacteria as well as non-halophilic bacteria (Table 1). Furthermore, some Gram-negative marine bacteria were sensitive to HQNO while others were not from the result of the paper-disk method. The wide antibacterial spectrum of HQNO was described by MACHAN *et al.*[11]. Considering that HQNO is known to inhibit several enzymes including 5-lipoxygenase from rat[12], mitochondrial complex I and III from beef heart[12], and cytochrome b/c_1 complex from *Rhodospirillum rubrum*[13], this compound may attack some other target molecule insensitive to korormicin on the Gram-positive bacteria. It is interesting that some Gram-negative marine bacteria

Table 1. Antibacterial spectrum of korormicin and HQNO.

Strains	Halophilicity	Korormicin	HQNO
Gram-positive strains			
Marinococcus halophilus IAM12844^T	+	−	++
Rhodococcus rhodochrous ATCC12674	−	−	+
Brevibacterium linens JCM6894	−	−	+
Staphylococcus aureus IFO12732	−	−	++
Bacillus substilis IFO3134	−	−	+
Gram-negative strains			
Alteromonas macleodii ATCC27126^T	+	++	−
Pseudoalteromonas haloplanktis ATCC14393^T	+	++	−
Deleya aquamarina ATCC33127	+	+	−
Shewanella putrefaciens ATCC8071^T	+	++	−
Vibrio alginolyticus 138-2	+	++	−
Salinivibrio costicola ATCC33508^T	+	++	++
Photobacterium phosphorum IAM12085	+	++	++
Escherichia coli IFO15034	−	−	−
Edwardsiella tarda ATCC15947^T	−	−	−
Xanthomonas campestris IFO13551	−	−	−
Acinetobacter sp. ATCC31012	−	−	−

Inhibitor, 10 μg/disk (8 mm i.d.), was used to examine the antibacterial activity on Marine Agar plate. Strength of inhibition was estimated by the diameter of inhibition circle; ++, 15 mm and larger; +, 10~15 mm; −, no inhibition circle observed.

were found insensitive to HQNO by the paper-disk method. Although korormicin and HQNO may diffuse differently in the agar plate, it could be said that these two antibiotics act on the intact cells with different manner or that some living cells are able to degradate HQNO in the medium.

In conclusion we demonstrated that korormicin, a specific inhibitor against Gram-negative marine bacteria produced by the marine bacterium *Pseudoalteromonas* sp. F-420, strongly inhibits the respiration-coupled NQR complex purified from *V. alginolyticus* 138-2. It inhibits the NQR activity of the complex in the fully noncompetitive mode, but does not affect the NDH activity. The inhibition constant was around 80 pM using Q-1 as quinone substrate. In comparison with the known NQR inhibitor HQNO, inhibition activity of korormicin against NQR was much higher, although both compounds inhibited the quinol formation reaction. As HQNO is known to inhibit bacteria other than Gram-negative marine species and enzymes other than NQR, korormicin can be said to be a more selective inhibitor. Recently, the existence of NQR in non-halophilic bacterium *Haemophilus influenzae* was reported[14]. The action of korormicin on NQR from this strain is to be investigated.

Experimental

Chemicals and Enzyme Preparation

Q-1 and HQNO were purchased from Sigma Chemical Co. (St. Louis, MO). Korormicin from *Pseudoalteromonas* sp. F-420 was prepared as reported[5]. Purification of NQR complex from *V. alginolyticus* 138-2 was described elsewhere[7]. Purified NQR complex was resuspended in 10 mM Tris-HCl (pH 7.6) containing 10 mM NaCl, 0.1 mM EDTA, 10% (w/v) glycerol, 0.2 mg/ml L-phosphatidylcholine from soybean, and 0.1% (w/v) Liponox DCH[8].

Enzyme Assays and Kinetic Analyses

The NDH and NQR activities were measured by the method described by NAKAYAMA *et al.*[7]. One unit of enzyme activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADH or the reduction of 1 μmol Q-1 in 1 minute at 30°C.

For kinetic analyses, the line of best fit for each set of double-reciprocal plots and kinetic constants were calculated using the program Enzyme Kinetics (Trinity Software, Campton, NH).

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