Coupling between the respiratory chain and the luminescent system of *Vibrio harveyi*

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(Received 30 December 1991; revised 18 March 1992; accepted 15 April 1992)

Effects of monovalent cations on luminescence and respiratory activity were studied in the marine luminous bacterium *Vibrio harveyi*. Maximum oxygen uptake was observed in the presence of Na⁺ over the pH range tested (6.5–8.5). At alkaline pH, effects of monovalent cation on luminescence were similar to those on the oxygen uptake. Although KCN addition caused a marked increase in luminescence, the enhanced luminescence with Na⁺ was still greater than that with Li⁺. However, at acidic pH, K⁺ increases luminescence more than Na⁺ does. These results indicate that there is not only a competitive but also a cooperative relationship between luminescence and respiration. The respiratory NADH oxidase in the membrane fraction of *V. harveyi* showed some distinctive characters which are unique to the respiratory-dependent primary Na⁺ pump, suggesting the possibility of coupling between the Na⁺ pump and the luciferase system. This was also supported by the results from CCCP-resistant growth and luminescence at alkaline pH. The coupling mechanisms between luminescence and respiration in *V. harveyi* are discussed.

Introduction

Luminescent bacteria are isolated mainly from marine environments, i.e. seawater and animals in the ocean. Most of them are classified into two major genera: *Vibrio* and *Photobacterium* (Nealson & Hastings, 1979; Baumann *et al.*, 1983).

Detailed biochemical studies have been conducted on the luminescent systems in *Vibrio fischeri*, *V. harveyi* and *Photobacterium phosphoreum* (Hastings & Nealson, 1977; Nealson & Hastings, 1979; Meighen, 1988, 1991). The luminescent reaction which is catalysed by luciferase involves the oxidation of FMNH₂ and a long-chain aldehyde. Since NADH is necessary for reduction of flavin mononucleotide, the luciferase pathway can be viewed as a branch of the electron transport system in which electrons are shunted to oxygen. Although several studies (Nealson *et al.*, 1970; Watanabe *et al.*, 1975; Hastings *et al.*, 1977; Ulitzur *et al.*, 1981; Grogan, 1984) have suggested that the luminescent system is a competitive bypass of the respiratory chain, precise

coupling mechanisms between luminescent and respiratory systems are still unknown.

The occurrence of a novel respiratory chain was recently found in the moderately halophilic bacterium *Vibrio alginolyticus* (Tokuda & Unemoto, 1981, 1982). This respiratory chain translocates sodium ions from inside to outside the cells at alkaline pH, as a direct result of electron transport. Instead of a proton-motive force, this bacterium is able to use the sodium-motive force for various cellular functions. A similar respiratory chain has also been found in other marine bacteria (Kogure & Tokuda, 1986, 1989; Tokuda & Unemoto, 1983; Tokuda & Kogure, 1989). However, to our knowledge, the coupling between luminescence and the Na⁺-dependent respiratory chain has not yet been investigated. The purpose of this work is to clarify the possible relationship between the two.

Methods

Bacterial strain and growth conditions. *Vibrio harveyi* (ATCC 14126) was grown aerobically at 20 °C in PYG medium, which contained 0.3 M-NaCl, 50 mM-MgSO₄·7H₂O, 10 mM-CaCl₂·2H₂O, 10 mM-KCl, 0.5% Polypepton (Nihon Pharmaceutical), 0.1% Yeast extract (Difco) and 0.3% glycerol in deionized water. The pH was adjusted to 8.5 with 50 mM-Tricine/NaOH. The growth of *V. harveyi* was monitored as OD₆₀₀ with a Hitachi Model 101 spectrophotometer.

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Abbreviations: HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; CCCP, carbonylcyanide-*m*-chlorophenyl hydrazine.

Preparation of cell suspension. The culture of *V. harveyi* was harvested at late-exponential phase ($OD = 1.0$) by centrifugation and washed twice with salt buffer at pH 6.5–8.5. The buffer contained 25 mM-Tris/HCl, 30 mM-MgSO₄·7H₂O, and 0.3 M-monovalent cation (Li⁺, Na⁺, K⁺, Rb⁺), added as chlorides. The cell suspension, which contained about 0.3 mg protein per ml, was kept on ice until the measurement of luminescence and rate of oxygen consumption.

Measurement of luminescence. Luminescence of the cell suspension was measured with an ATP photometer (SAI Technology Model 3000). The suspension (100 or 500 µl) was taken into a glass vial and kept at 25 °C. After 5 min measurement of luminescent intensity was initiated. The suspension was rapidly mixed just before each measurement. Luminescence was measured for 6 s and the peak value was monitored. Light intensity was expressed in count units of the photometer per mg cell protein or optical cell density. Luminescence was determined from duplicate assays.

Measurement of oxygen consumption rate. The rate of oxygen consumption was determined at 25 °C with an oxygen electrode (Rank Brothers) attached to a CU-228 recorder (Tokyo Riko). The measurement was started by the addition of 20 µl cell suspension to 2 ml air-saturated buffer. The final cell concentration was the same as those for luminescence measurement. The oxygen consumption rate was determined from duplicate assays.

Preparation of cell membrane fraction. Cells were harvested at late-exponential growth phase. Membrane fractions were prepared by the osmotic lysis method (Tokuda, 1986). The membrane fraction samples were kept below –70 °C until NADH oxidase assay.

Assay for NADH oxidase. NADH oxidase activity in the cell membrane fraction was determined at 25 °C from the decrease in A_{340} (Tokuda & Unemoto, 1984). The standard assay mixture contained 20 mM-Tris/HCl, 0.3 M-NaCl, 0.2 mM-NADH and about 20 µg membrane protein in a final volume of 1 ml. The assay was started by addition of the membrane to the buffer. The rate of decrease of A_{340} was followed using a Hitachi U-3200 spectrophotometer. The effect of monovalent cations (Li⁺, Na⁺, K⁺, Rb⁺), pH and HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) on the oxidase activity was observed. For calculation of the NADH oxidase activity, a value of 6.22 was used as the millimolar absorption coefficient. Activity of the oxidase was determined from duplicate assays. Deviations for most data shown were <0.01 µmol NADH min⁻¹ (mg protein)⁻¹.

Growth and luminescence in the presence of a proton conductor. Bacterial cells were aerobically precultured in the PYG medium (pH 6.5 and 8.5) at 20 °C. Cells in late-exponential phase were transferred into 5 ml fresh medium and incubated under the same conditions until the OD_{600} reached about 0.1. Then the proton-conductor CCCP (carbonyl cyanide-*m*-chlorophenyl hydrazone), was added to give a final concentration of 10 µM. Growth and luminescence of cells were monitored as was described above. The medium pH was constant during the cultivation.

Protein concentration. Protein was determined by the Lowry method, using bovine serum albumin as a standard.

Results

Effect of monovalent cations on luminescence and respiratory activity

Both luminescent intensity and rate of oxygen consumption varied with the monovalent cation present. As

Table 1. Effect of monovalent cations on oxygen consumption rate and luminescence of *V. harveyi*

Data shown are means ± standard deviations.

Monovalent cation (0.3 M)	O ₂ uptake rate* [nmol O ₂ min ⁻¹ (mg protein) ⁻¹]	Luminescence† [count units (mg protein) ⁻¹]
Li ⁺	49.7 ± 5.2	19193 ± 1577
Na ⁺	69.7 ± 10.4	32148 ± 4400
K ⁺	29.2 ± 9.3	8115 ± 1100
Rb ⁺	7.1 ± 3.0	697 ± 198

* Oxygen consumption rate at pH 6.5.

† Luminescence at pH 8.5 measured after 5 min pre-incubation.

shown in Table 1, maximal oxygen consumption occurred in the presence of Na⁺. The order of the monovalent cations was the same within the pH range (6.5–8.5) tested (data not shown).

The effects of monovalent cations on the luminescence in alkaline conditions were similar to those exerted on the oxygen consumption (Table 1). *V. harveyi* showed maximum luminescence in the presence of Na⁺ at pH 8.5. However, at acidic pH, K⁺ increased luminescence more (data not shown). While Na⁺ had a fairly constant effect on luminescence regardless of pH, the effect of K⁺ was pH-sensitive; luminescent intensity at pH 6.5 was usually more than 5 times greater than that at pH 8.5.

The effects of cyanide addition on luminescence at pH 8.5 are shown in Fig. 1. Increased luminescence was observed in the presence of Na⁺ or Li⁺, and decreased luminescence in the presence of K⁺ or Rb⁺. The enhanced luminescence with Na⁺ was about two times greater than that with Li⁺. The difference of enhanced luminescence between them was almost similar to that before the addition of cyanide.

NADH oxidase in membrane fractions of *V. harveyi*

Fig. 2 shows the effect of monovalent cation concentration on NADH oxidase activity of *V. harveyi*. It is clear that this bacterium requires Na⁺ for maximal activity. The activity with other cations (K⁺, Li⁺, Rb⁺) was much smaller than with Na⁺. The pH dependency of the NADH oxidase with 0.3 M-monovalent cations is shown in Fig. 3. The optimum pH was about 7.5 to 8.0. The NADH oxidase was very sensitive to HQNO, which is a specific inhibitor of the Na⁺-driven respiratory chain in *V. alginolyticus* (Tokuda & Unemoto, 1984). Less than 1 µM-HQNO almost completely suppressed the NADH oxidase activity (Fig. 4). These characteristics of the NADH oxidase of *V. harveyi* are quite similar to those of *V. alginolyticus* (Tokuda, 1984).

Fig. 1. Effect of KCN on luminescence of *V. harveyi* at pH 8.5. Luminescence was measured in the buffer containing 0.3 M-monovalent cation. Just after the determination of luminescence at zero time, KCN was added to the suspension to a final concentration of 2 mM (dotted line). Symbols: ■, Li⁺; ●, Na⁺; ○, K⁺; □, Rb⁺.

Fig. 2. Effect of monovalent cation on NADH oxidase of *V. harveyi*. NADH oxidase activity was measured in the assay buffer at pH 7.5. Symbols: ■, Li⁺; ●, Na⁺; ○, K⁺; □, Rb⁺.

Fig. 3. Effect of pH on NADH oxidase of *V. harveyi*. NADH oxidase activity was measured in the assay buffer containing 0.3 M monovalent cation. Symbols: ■, Li⁺; ●, Na⁺; ○, K⁺; □, Rb⁺.

Fig. 4. Effect of HQNO on NADH oxidase of *V. harveyi*. NADH oxidase activity was measured in the assay buffer containing 0.3 M-Na⁺ at pH 7.5.

Growth and luminescence in the presence of proton conductor

Fig. 5 shows the growth and luminescence of *V. harveyi* in the presence of CCCP. At pH 6.5, 10 μM-CCCP almost completely inhibited the growth (Fig. 5a), whereas at pH 8.5, only a slight inhibitory effect was observed (Fig. 5b). Just after CCCP addition, the luminescence at pH 6.5 started to decrease rapidly and

became undetectable within 1 h. However, at pH 8.5, 10 μM-CCCP did not inhibit luminescence.

Discussion

The oxygen uptake rate of *Vibrio harveyi* was influenced by the presence of monovalent cations (Table 1). The maximum oxygen uptake was observed in the presence

Fig. 5. Growth and luminescence of *V. harveyi* in the presence of CCCP. The medium contained either (a) 50 mM-MES/NaOH (pH 6.5) or (b) 50 mM-Tricine/NaOH (pH 8.5). When the OD_{600} reached about 0.1, CCCP was added. Symbols: ○, 10 mM-CCCP (final concentration); ●, control (0 mM-CCCP).

of sodium. Na^+ was also required for maximum luminescence at pH 8.5. Moreover, at alkaline pH, the effects of other monovalent cations on luminescence and on oxygen consumption showed similar patterns (Table 1). Since the luciferase pathway would account for at most about 20% of the total oxygen uptake (Hastings & Nealson, 1977; Makemson, 1986), we considered that the rate of oxygen consumption by the cells almost corresponds to the respiratory activity. Thus, these results indicate a cooperative relationship between the luminescent and respiratory systems.

Past experiments using respiratory inhibitors have suggested the bacterial luminescent system to be an alternative electron transport pathway that competes with the cytochrome system for both NADH and oxygen as a main electron donor and acceptor (Nealson *et al.*, 1970; Watanabe *et al.*, 1975; Hastings & Nealson, 1977; Ulitzur *et al.*, 1981; Grogan, 1984). However, the simple competitive model can not fully explain the results described above. The existence of both cooperative and competitive relations between the two systems requires an alternative explanation.

It seems possible to explain these phenomena when we hypothesize the following. (1) Luminescence and oxygen

uptake rate reflect the rate of electron flow through each system i.e., changes in luminescence and oxygen uptake rate are directly caused by those in the electron transport activity in the luciferase pathway and the respiratory chain, respectively. (2) The respiratory electron flow is primarily determined by the monovalent cations. (3) Some of the electrons which flow through the respiratory system are shunted to the luciferase system. (4) All of the electron supply to luciferase occurs in connection with respiration. The results shown in Fig. 1 support our hypothesis. Although cyanide clearly increased the luminescence in the presence of Na^+ and Li^+ ions, the enhanced luminescence with Na^+ was still greater than that with Li^+ . The increase in luminescence by cyanide has been previously explained as the blockage of electron flow through cytochrome to oxygen by KCN, which increases the electron flow rate through the luciferase system, leading to an increase in luminescence if electron supply is rate-limiting in the luciferase system. However, the above results (Fig. 1) strongly suggest that the increase in luminescence primarily depends on the respiratory activity, which is determined by the monovalent cation. In other words, an apparent increase in luminescence caused by KCN also depends on the

respiratory activity. Thus it seems very reasonable to conclude that monovalent cations affect bacterial luminescence by altering respiratory activity.

However, at acidic pH, the effects of monovalent cations on luminescence were not in accord with those on respiratory activity. It was noticeable that K^+ increases luminescence more than Na^+ . Watanabe *et al.* (1977) also observed the stimulatory effect of K^+ in *Photobacterium phosphoreum*. At present, the reason for the brighter luminescence with K^+ than with Na^+ at pH 6.5 is unknown.

The requirement of Na^+ for maximum oxygen uptake is probably attributable to the respiratory NADH oxidase, which also required Na^+ for maximum activity. Figs 2 and 3 show the monovalent cation and alkaline pH characteristics of the NADH oxidase of *V. harveyi*. These are in good agreement with the Na^+ -dependent NADH oxidase of *V. alginolyticus* (Tokuda & Unemoto, 1984). Moreover, marked sensitivity to HQNO (Fig. 4), which specifically inhibits the Na^+ -driven NADH:quinone oxidoreductase of *V. alginolyticus*, was also observed. These results strongly indicate that *V. harveyi* possesses a respiratory chain quite similar to that of *V. alginolyticus*. This is also supported by the growth and luminescence in the presence of CCCP (Fig. 5). Since CCCP increases the proton permeability of the cell membrane, the CCCP resistance suggests that growth, at alkaline pH, luminescence or other cellular functions do not depend on the proton-driven respiratory system (H^+ pump), but on the sodium-driven one (Na^+ pump). Similar growth patterns have also been observed in *V. alginolyticus* and other marine bacteria which possess the primary Na^+ pump (Tokuda & Unemoto, 1983; Kogure & Tokuda 1986). Although inhibition of bacterial bioluminescence by CCCP has been reported (Grogan, 1984; Guerrero & Makemson, 1989), this is the first observation on the CCCP resistant growth and luminescence of *V. harveyi*, particularly at alkaline pH.

We conclude that the luminescent system couples to this Na^+ -driven respiratory chain, particularly at alkaline pH. This also raises the possibility that the mode of coupling between luminescent and respiratory system could change with external pH. The correlation between luminescence and respiratory activity shown at alkaline pH suggests that the Na^+ pump may play an important role in coupling to the luciferase system.

We wish to acknowledge valuable discussions with Dr H. Tokuda, Institute of Applied Microbiology, University of Tokyo.

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