Microamperometric Measurements of Photosynthetic Activity in a Single Algal Protoplast

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ABSTRACT The effects of p-benzoquinone (BQ) on photosynthetic and respiratory electron transport in a single algal protoplast (radius, 100 μm) was investigated quantitatively by amperometric measurements using microelectrodes. Under light irradiation (25 kLx) in the presence of 1.00 mM BQ, a single protoplast consumed BQ by $(2.9 \pm 0.2) \times 10^{-13}$ mol/s and generated p-hydroquinone (QH_2) by $(2.7 \pm 0.3) \times 10^{-13}$ mol/s, suggesting that BQ was quantitatively reduced to QH_2 via the intracellular photosynthetic electron-transport chain. The generation of QH_2 increased with light intensity and with concentration of BQ added to the outside solution but became saturated when the light intensity was above 15 kLx or the BQ concentration was higher than 0.75 mM. The addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, a photosynthetic electron-transport inhibitor, decreased the generation of QH_2 upon light irradiation, suggesting that BQ accepts electrons from a site in the photosynthetic electron-transport chain after the photosystem II site. The presence of 1.00 mM BQ increased the generation of photosynthetic oxygen by $\sim(2.6 \pm 1.0) \times 10^{-13}$ mol/s, which was ~1.5–2 times larger than that expected from the consumption of BQ. The electrons produced by the additional generation of oxygen is used to reduce intracellular species as well as to reduce BQ.

INTRODUCTION

The quantitative detection and monitoring of redox species inflowing or outflowing from a cell at the single, living cell level is extremely important to elucidate biological functions because cellular energy production by respiration and photosynthesis are based on biological redox reactions. The effects of electron-accepting redox species on biological electron-transport chains have been studied conventionally by oxygen electrodes (Barr et al., 1975; Inoue and Nishimura, 1971; Saha et al., 1971) or by spectrophotometric measurements (Vernon and Shaw, 1969; Katoh and Pietro, 1967) in solutions suspended with chloroplasts and mitochondria. It is, however, difficult to quantitatively determine the fluxes of oxygen and electron mediators to or from a single, living cell. We have recently determined the evolution of oxygen from a single protoplast by using a microelectrode (Matsue et al., 1992). Microelectrodes have been proven as an effective tool to probe the concentration of redox species at the single-cell level. Amperometric measurements using microelectrodes allow for refined data in localized space on the scale of the tip size and have been used for the imaging of living cells (Tsionsky et al., 1997) and for the study of cellular processes such as catecholamine release (Wightman et al., 1996, 1988), NO release (Malinski and Taha, 1992), photosynthesis (Matsue et al., 1993, 1992), respiration (Lau et al., 1992), oxidative stress (Arbault et al., 1995), and membrane permeability (Yasukawa et al., 1998a,b). Many laboratories, including ours, have done intracellular electrochemical measurements using microelectrodes to monitor intracellular reactions; however, the insertion of microelectrodes physically injures cells and might trigger undesired intracellular reactions. It is, therefore, desirable to characterize a cell by extracellular measurement to obtain data on the intact, living cells.

We report here the quantitative investigation of the effects of p-benzoquinone (BQ) on photosynthesis in a single, living protoplast by extracellular measurements. We placed a carbon-disk or an Au-disk microelectrode very close to a single cell and monitored the localized concentrations of BQ and hydroquinone (QH_2) to determine the consumption rate of BQ and the generation rate of QH_2 at the single-cell level. We also investigated the influence of light intensity, BQ concentration, and electron-transport inhibitors, such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), on the generation rate of QH_2 from a single, living cell. In addition, photosynthetic oxygen evolution from a single protoplast was determined by microamperometric measurement. The oxygen evolution is markedly accelerated in the presence of BQ in a solution.

MATERIALS AND METHODS

The carbon-disk electrode and the Au-disk electrode used in this study were prepared as follows. The carbon fiber (Union Carbide, Danbury, CT) pitch fiber; 10 μm in radius) was slightly etched electrochemically (60 Hz, AC voltage with 5–10 $V_{p\text{-}p}$) in a solution containing 0.5 mM $K_2Cr_2O_7$ and 5.0 M H_2SO_4. An Au wire (10 μm in radius) was also etched electrochemically (300 Hz, AC voltage with 5–10 $V_{p\text{-}p}$) in a $NaNO_3$ saturated aqueous solution. The etched carbon fiber or Au wire was inserted into a soft-glass capillary that was tapered with a micropipet puller (Narishige, Tokyo, type PN-3). The carbon tip was sealed by dipping into an epoxy resin (Oken Co., Tokyo). The epoxy resin in the capillary was hardened at 60°C for 12 h, and the Au tip region was thermally fused in vacuo. The tips were

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then carefully polished with a diamond grinder (number 5000) on a turntable (Narishige model EG-6) to give disk-shaped carbon and Au electrodes. The radii of the carbon-disk and the Au-disk microelectrode were determined from steady-state voltammograms of 1.00 mM BQ and 4.00 mM $Fe(CN)_6^{4-}$ and found to be 7.7 μm for the carbon disk and 8.0 μm for the Au disk. From microscopic measurements, the radius of the carbon tip, including an insulating glass sheath, was 10 μm, and the radius of the Au tip was 13 μm. The other end of the carbon fiber was connected to a Cu wire with conductive paste (DOTITE, Fujikura Kasei) and used for the lead to an external amplifier. The Au wire was spot welded with Cu wire for the external lead.

Reagent grade p-benzoquinone (BQ), 2,5-dichloro-1,4-benzoquinone, $K_4Fe(CN)_6$, $K_3Fe(CN)_6$, and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were purchased from Wako Pure Chemicals (Tokyo, Japan) and used without further purification. A protoplast with a radius of 100 μm was made from marine alga *Bryopsis plumosa* by a method described in detail elsewhere (Tatewaki and Nagata, 1970) in synthetic artificial sea water containing 480.2 mM NaCl, 2.3 mM $NaHCO_3$, 11.1 mM $CaCl_2$, and 83.8 mM $MgCl_2$. The single protoplast was isolated and transferred to a measurement solution of synthetic artificial sea water containing 1.00 mM BQ. The measurements were begun ~20 min after the protoplast was prepared in artificial sea water containing 1.00 mM BQ. All aqueous solutions were prepared with distilled and deionized water. The position of the microelectrode was controlled by a three-dimensional manipulator system (Shimazu, MMS-77) under an inverted microscope (Nikon, DIAPHOT 300). The image was monitored on a CRT (NEC, PC-TV 455) through a CCD color video camera (Sony, DXC-107A). Redox current was amplified with a current amplifier (Nihon Kohden, CZE-2300). The electrode potential and data acquisition were controlled with a personal computer (NEC, Tokyo, 98Note, SX/E) equipped with a 12-bit AD/DA board (AB 98–57B, Adtec, Tokyo). The reduction of BQ near the protoplast was monitored by amperometry at −0.20 V versus Ag/AgCl and the oxidation of QH_2 at 0.80 V or 0.50 V versus Ag/AgCl. The reduction current of the oxygen was measured by differential pulse amperometry (DPA, 0.10 → −0.60 → −0.90 V, 0.50 → 3.00 → 0.50 s) using a carbon-disk electrode to avoid the negative effects of BQ in solution. All measurements were performed at 25°C in a shielded box to remove electrical noises from external sources. Light irradiation was performed with a built-in light source in the microscope. These amperometric responses were converted into localized concentrations of BQ, QH_2, and oxygen by using calibration lines. The diffusion coefficients of BQ and QH_2 were determined by potential-step amperometry, and both were found to be 6.2×10^{-6} cm^2/s. The diffusion coefficient of oxygen is 2.1×10^{-5} cm^2/s (Ikeuchi et al., 1995; Tsushima et al., 1994). These values were used to determine the concentrations at the surface of the protoplast.

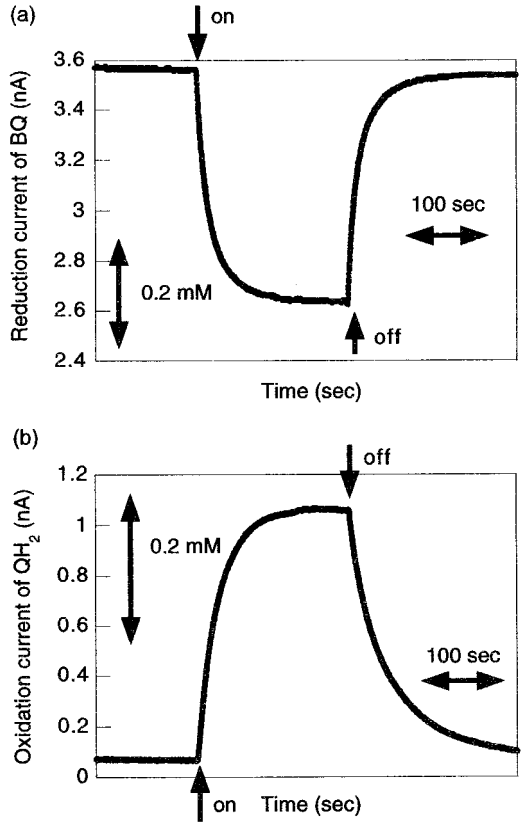

FIGURE 1 Responses of reduction current for BQ (*a*) and the oxidation current for QH_2 (*b*) upon light irradiation (25 kLx). A carbon microelectrode was placed approximately 1 μm away from a protoplast membrane. The potential was held at −0.20 V versus Ag/AgCl for BQ and 0.80 V versus Ag/AgCl for QH_2. The reduction current of BQ immediately decreases upon light irradiation whereas the oxidation current of QH_2 increases. This phenomenon indicates that BQ is reduced to QH_2 by a photosynthetic electron-transport chain in the protoplast.

RESULTS AND DISCUSSION

We investigated the Hill reaction (Hill, 1937), the electron transfer from the photosynthetic electron-transport chain to an electron acceptor, by microelectrochemical measurement at a single-cell level. Fig. 1 shows responses of the reduction current for BQ and the oxidation current for QH_2 upon light irradiation at a carbon-disk microelectrode that was placed close to a single protoplast (distance, ~1 μm) in the presence of 1.00 mM BQ. The reduction current of BQ immediately decreased after light irradiation and reached a steady state in 120 s, indicating that the concentration of BQ at the protoplast surface decreased due to the consumption of BQ by the Hill reaction. The reduction current of BQ returned to its original level when the light was turned off. The oxidation current for QH_2 showed the opposite response; the concentration of QH_2 rapidly increased upon light irradiation and decreased to its original level when the light was turned off. The above results suggest that the BQ that was added to the outside solution permeated through the cell membrane to accept two electrons from the photosynthetic electron-transport chain to yield QH_2, which diffuses to the extracellular medium. The transient responses when the light was turned on and off show that the release of QH_2 lags behind the consumption of BQ. This transient capture of BQ without the instantaneous release of QH_2 can be attributed to a cellular pool of the BQ/QH_2 redox couple under light irradiation. The cellular pool could affect the photosynthetic activity and other intracellular reactions. A similar response was also observed when 2,5-dichloro-1,4-benzoquinone was used as the redox mediator, but an impermeable mediator such as $Fe(CN)_6^{3-}$ (Yasukawa et al., 1998a) showed no response. High permeability for redox species is necessary to observe these kinds of light-induced responses in the redox current.

From the amperometric responses, the consumption of BQ and generation of QH_2 by the photosynthetic Hill reaction at the single-cell level were determined. If it is assumed that diffusion is spherical and the concentration of redox

species inside the protoplast is uniform in a steady state, the generation rate of redox species (f) can be expressed by (Cussler, 1984)

$$f = 4\pi r_s D(C_s - C^*), \quad (1)$$

where r_s (cm) is the radius of the protoplast, C^* (mol/cm^3) is the concentration of redox species at the bulk solution, C_s (mol/cm^3) is the concentration of redox species at the surface of the protoplast, and D is the diffusion coefficient. We measured the steady-state reduction current of BQ and the steady-state oxidation current of QH_2 at various electrode-protoplast distances to determine the surface concentration of BQ and QH_2 in a steady state. Fig. 2 shows the responses for the redox current as the carbon microelectrode moved stepwise to the surface of a single protoplast. The reduction current of BQ and the oxidation current of QH_2 changed stepwise and were synchronized with the step-by-step movement of the microelectrode. It should be noted that each step in the redox response is very flat; thus, the redox reaction at the microelectrode should be in a steady state. The steady-state redox current at each step was converted into a localized concentration using a calibration line. The variations in the concentrations of BQ and QH_2 as a function of distance from the surface of the protoplast are shown in Fig. 3. Under light irradiation, the concentration of BQ decreases significantly whereas the concentration of QH_2 increases in the region close to the protoplast due to the photosynthetic Hill reaction. The decrease in the BQ concentration and increases in the QH_2 concentration in the dark is a consequence of the consumption of BQ and release of QH_2 by respiratory electron transport (Ikeda et al., 1996; Rabinowitz et al., 1998).

Under spherical diffusion conditions, concentration (C) of the redox species in the steady state can be expressed by (Cussler, 1984)

$$C = (C_s - C^*)r_s/(r + r_s) + C^*, \quad (2)$$

where r is the distance from the protoplast surface. In Fig. 3 *c*, the concentrations of BQ as a function of $r_s/(r + r_s)$ were plotted to determine the surface concentrations of BQ. From the intercepts at $r_s/(r + r_s) = 1$, the surface concentrations of BQ were found to be 0.65 mM under light irradiation (25 kLx) and 0.90 mM in the dark. The substitution of these values into Eq. 1 gives the consumption rates of BQ by a single protoplast. We determined the consumption rates of seven different protoplasts and found the value to be $(2.9 \pm 0.2) \times 10^{-13}$ mol/s under light irradiation (25 kLx) and $(0.8 \pm 0.3) \times 10^{-13}$ mol/s in the dark; thus, photosynthesis under 25 kLx light consumed BQ ~3–4 times more than respiration in the dark. Similarly, the surface concentrations and generation rates of QH_2 from single protoplasts were also determined. The results are summarized in Table 1. The generation rates of QH_2 from a single protoplast under light irradiation and in the dark are balanced with the consumption rates of BQ, indicating that BQ is almost quantitatively reduced to QH_2 by the photosynthetic and respiratory electron-transport chains in the protoplast.

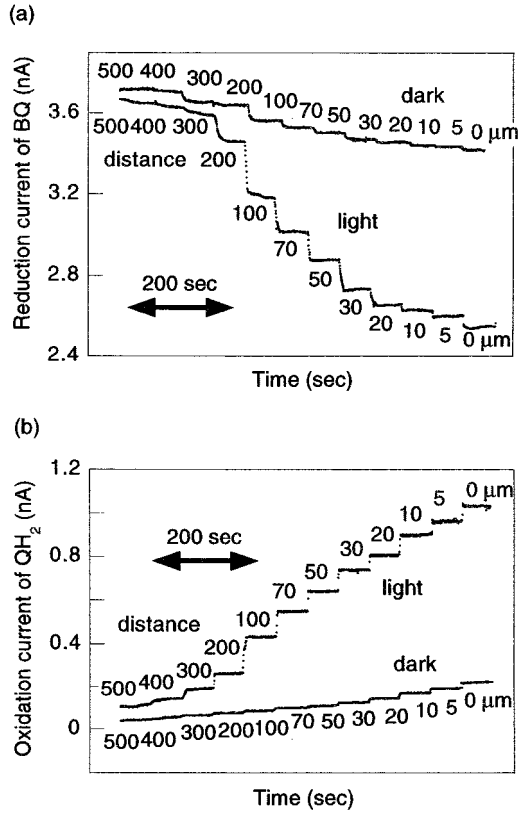

FIGURE 2 Responses of the reduction currents for BQ (*a*) and the oxidation current for QH_2 (*b*) when a carbon microelectrode moved stepwise to the surface of the protoplast under continuous light irradiation (25 kLx) and in the dark. The potentials were set at −0.20 V versus Ag/AgCl for BQ reduction and at 0.80 V versus Ag/AgCl for QH_2 oxidation. The redox currents show staircase responses that are synchronized with the movement of the microelectrode. The localized concentrations of BQ and QH_2 were determined from the step heights using calibration lines.

The amperometric response is, in general, affected by the shielding effect of the cell membrane (Schroeder et al. 1996) and by the cellular regeneration or consumption of redox species (Fosset et al., 1991) when the microelectrode-protoplast distance is within the size of electrode diameter. In the present case, these undesired effects were small as evidenced by the fact that the changes in concentrations of BQ and QH_2 with distance follow the theoretical relationship (Fig. 3, *c* and *d*). As BQ and QH_2 easily permeate the protoplast membrane (Yasukawa et al., 1998a), interference in the diffusion region by the membrane does not significantly affect the amperometric response.

The light intensity affects the generation of QH_2 from the protoplast (Fig. 4). The magnitude of the response for the oxidation current of QH_2 to light irradiation increased with an increase in the light intensity, although the pattern of the responses was not significantly affected by the light intensity. The inset in Fig. 4 is a plot of the response current against the light intensity. The generation of QH_2 is almost proportional to light intensity up to 15 kLx. The response becomes saturated when the light intensity is larger than 15

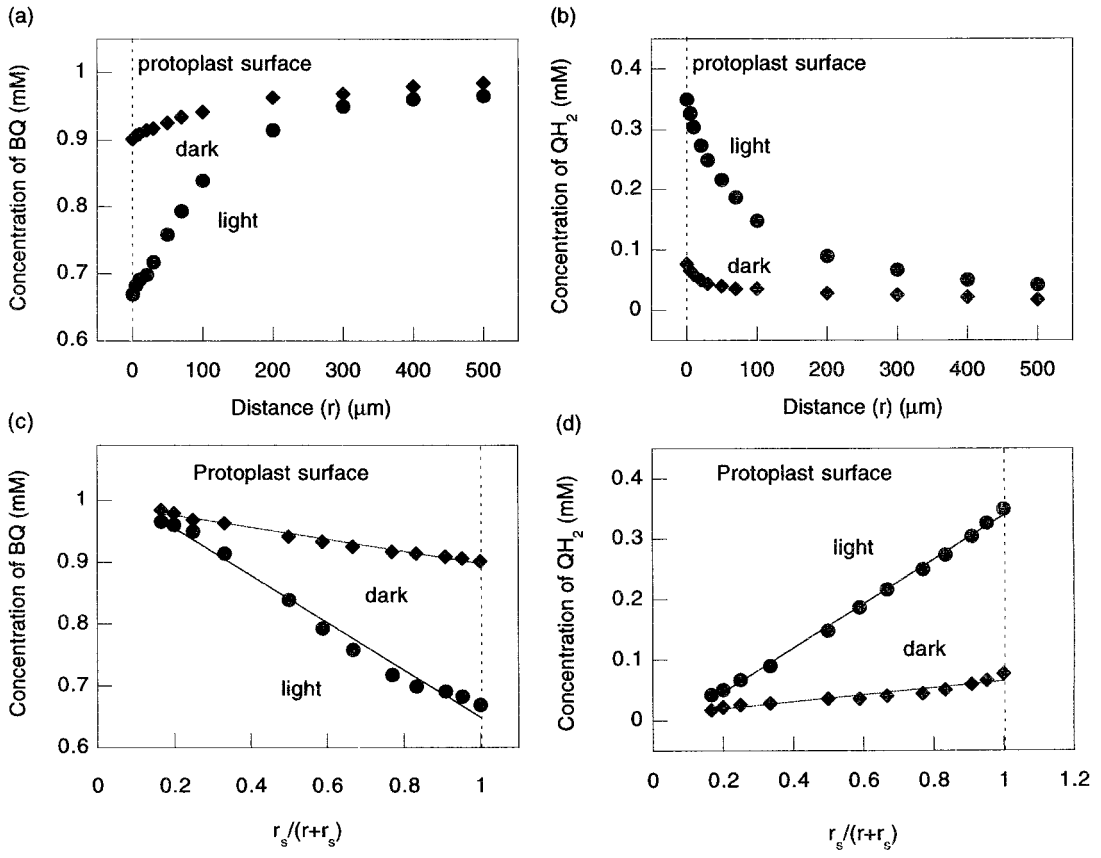

FIGURE 3 The variation of the localized concentration of BQ (*a*) and QH_2 (*b*) as a function of distance (*r*) from the surface of the protoplast. The concentration of BQ decreases rapidly in the vicinity of the surface of the protoplast whereas the concentration of QH_2 increases. (*c* and *d*) Plots of the concentration versus $r_s/(r + r_s)$ (r_s, protoplast radius). The surface concentrations of BQ and QH_2 at the protoplast membrane are determined from the intercepts at $r_s/(r + r_s) = 1$ of these plots.

kLx. This saturation intensity is similar to the value reported in a recent study (Yasukawa et al., 1998a) in which oxygen generation from the algal protoplast by photosynthesis shows saturation at light intensity higher than 15 kLx. The response of the QH_2 oxidation current is also affected by the initial concentration of BQ in the solution (Fig. 5). The response to the light irradiation (25 kLx) increases with the BQ concentration but tends to be saturated when the BQ concentration is above 0.75 mM.

The addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a photosynthetic electron-transfer inhibitor, drastically reduces the response. The response of the oxidation current for QH_2 to light irradiation is ~10 times smaller in the presence of 5.00 μM DCMU than that without DCMU (Fig. 6). This concentration of DCMU can sufficiently inhibit the photosynthetic activity of algal cells as the DCMU concentration that gives 50% inhibition of electron transport

TABLE 1 Generation rate (mol/s) of BQ, QH_2, and O_2 from a single algal protoplast in the presence of 1.00 mM BQ

Species	In the dark	Under light irradiation (25 kLx)
BQ	$-(0.8 \pm 0.3) \times 10^{-13}$	$-(2.9 \pm 0.2) \times 10^{-13}$
QH_2	$(0.4 \pm 0.2) \times 10^{-13}$	$(2.7 \pm 0.3) \times 10^{-13}$
O_2		$(3.6 \pm 0.7) \times 10^{-13}$
O_2		$(1.0 \pm 0.3) \times 10^{-13}$*

The protoplast radius was 100 μm.
*Without BQ in solution.

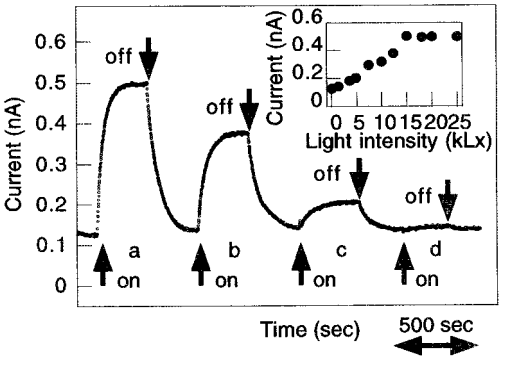

FIGURE 4 Response of oxidation current for QH_2 upon light irradiation at an Au microelectrode (0.50 V versus Ag/AgCl) that had been placed approximately 1 μm away from the protoplast membrane. Light intensity: (*a*) 18.0 kLx; (*b*) 12.3 kLx; (*c*) 4.8 kLx; (*d*) 1.3 kLx. The magnitude of the response increases with the light intensity. The inset shows the variation of the oxidation current as a function of light intensity. The response is saturated when the intensity is larger than 15 kLx.

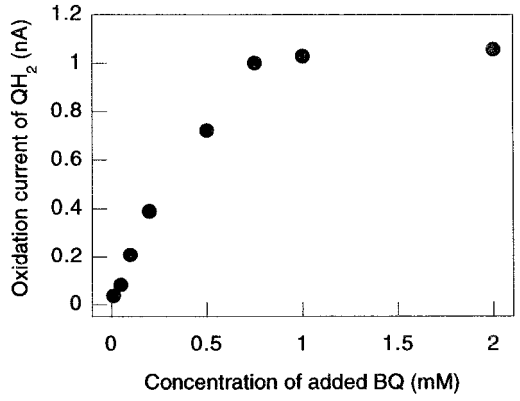

FIGURE 5 Relationship between the oxidation current of the released QH_2 and the concentration of BQ that was added to the outside solution under light irradiation (25 kLx). A carbon microelectrode (0.80 V versus Ag/AgCl) was placed approximately 1 μm away from the protoplast membrane. The concentration of QH_2 become saturated when the concentration of BQ is above 0.75 mM.

in intact algal cells is 1.0×10^{-7} to 5.0×10^{-7} M (Izawa and Good, 1972; Matsue et al., 1993). DCMU selectively blocks the electron transfer to plastquinone in photosystem II (Mets and Thiel, 1989; Izawa and Good, 1972). The fact that DCMU lowers the photo-induced intracellular reduction of BQ to QH_2 indicates that BQ accepts electrons from a site in the photosynthetic electron-transport chain after the photosystem II site. In the dark, however, the addition of 5.00 μM DCMU did not obviously affect the oxidation current of QH_2 that was generated from the protoplast. DCMU does not inhibit the respiratory electron-transport chain of the algal protoplast at this concentration level.

We also determined the variation of reduction current of oxygen upon light irradiation to investigate the interaction of BQ with the intracellular electron-transport process. Fig. 7 shows the responses of oxygen reduction current to light irradiation (25 kLx) in the presence of 1.00 mM BQ. The reduction current rapidly increased upon light irradiation and then decreased rapidly to give a steady-state value. Switching the light off returns the reduction current to the original level. As the reduction current is directly proportional to the localized concentration of oxygen (Matsue et al., 1992) the current response reflects the change in the oxygen concentration at the surface region of the protoplast. The response without BQ was studied recently (Matsue et al., 1992); the peak of the oxygen concentration appearing immediately after light irradiation is related to the rate of photosynthetic electron transport (i.e., light reaction), and the oxygen concentration in the steady-state appearing later is limited by the generation of photosynthesis-related chemicals such as $NADP^+$ and ADP (i.e., dark reaction). The response pattern was significantly affected by adding BQ into the culture medium. With 1.00 mM BQ in the medium, the response of the oxygen reduction current showed no peak and remained constant at a level of the peak height that was observed without BQ. This indicates that the oxygen generation in the presence of 1.00 mM BQ is controlled by the photosynthetic electron-transport process throughout the light irradiation period. As BQ can efficiently accept two electrons from the electron-transport chain, the generation of oxygen proceeds efficiently without the regeneration of photosynthesis-associated chemicals by the dark reaction.

Steady-state oxygen generation from a single protoplast under continuous light irradiation (25 kLx) was determined by the same method as used for BQ and QH_2 and was found to be $(1.0 \pm 0.3) \times 10^{-13}$ mol/s without BQ and $(3.6 \pm 0.7) \times 10^{-13}$ mol/s with 1.00 mM BQ in the solution (see Table 1). It appears that the additional electron flow from the photosynthetic electron-transport chain to BQ generates ex-

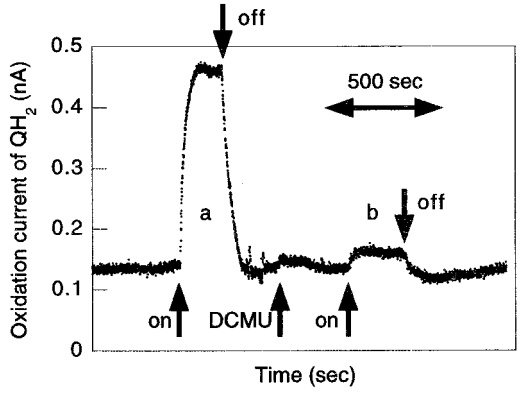

FIGURE 6 Responses of oxidation current for QH_2 upon light irradiation (18 kLx) without (*a*) and with (*b*) 5.0 μM DCMU in solution. An Au microelectrode (0.50 V versus Ag/AgCl) was placed approximately 1 μm away from the protoplast membrane. The addition of DCMU drastically reduces the response of the oxidation current. DCMU inhibits the photosystem-II-driven electron-transport to block the reduction of BQ via the photosynthetic Hill reaction. The site from which BQ accepts electrons is in the photosynthetic electron-transport chain after photosystem II.

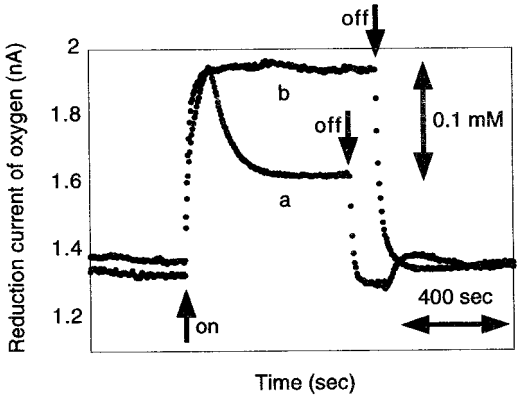

FIGURE 7 Responses of oxygen reduction current upon light irradiation (25 kLx) without (*a*) and with (*b*) 1.00 mM BQ in solution. A carbon microelectrode was placed approximately 1 μm away from a protoplast membrane. The reduction current for the oxygen was measured by differential pulse amperometry (DPA, $0.10 \rightarrow -0.60 \rightarrow -0.90$ V vs. Ag/AgCl, $0.50 \rightarrow 3.00 \rightarrow 0.50$ sec). The generation oxygen rate in the presence of 1.00 mM BQ is large compared with that without BQ. BQ functions efficiently as an electron-acceptor to accelerate the photosynthetic electron transfer to increase the oxygen generation.

tra oxygen from the protoplast; however, an analysis of material balance discloses a different aspect. The presence of 1.00 mM BQ increased the oxygen generation rate from a single protoplast by 2×10^{-13} to 3×10^{-13} mol/s. If we assume a stoichiometric redox reaction, this additional oxygen generation requires the consumption of 4×10^{-13} to 6×10^{-13} mol/s BQ as the oxidation of water to oxygen is a four-electron process and the reduction of BQ to QH_2 is a two-electron one. However, the consumption of BQ by a single protoplast under light irradiation (25 kLx) is only $(2.9 \pm 0.2) \times 10^{-13}$ mol/s (see Table 1). Thus, the electrons produced via additional oxygen generation should be used to reduce some intracellular species as well as to reduce BQ. It is well known that the BQ/QH_2 redox couple acts as an effective electron mediator to facilitate the reduction of biological molecules (Ikeda et al., 1996). In addition, an analysis of transient response (Fig. 1) suggests that the BQ/QH_2 couple is pooled under light irradiation. This increase in oxygen generation could be a consequence of the intracellular reduction that is mediated by the BQ/QH_2 couple. Another possible explanation for the additional oxygen generation in the presence of BQ is that BQ activates the intrinsic photosynthetic activity. A detailed and quantitative tracing of intracellular species will clarify the mechanism for unexpected oxygen generation upon light irradiation in the presence of BQ.

CONCLUSION

A quantitative analysis of the effects of BQ on photosynthetic activity was done using microamperometric measurements. A microelectrode was placed close to an algal protoplast, and the redox responses of BQ, QH_2, and oxygen were measured to trace the localized concentrations of these species around a single, intact algal protoplast (radius, 100 μm). The results showed that BQ permeated through the cell membrane and accepted two electrons from the photosynthetic and respiratory electron-transport chain to form QH_2, which was released from the protoplast. The BQ inflowing into the cell is almost quantitatively reduced to QH_2 by photosynthetic and respiratory electron-transport chains in the protoplast. The consumption of BQ by photosynthesis in a single protoplast under 25 kLx light irradiation was $(2.9 \pm 0.2) \times 10^{-13}$ mol/s, which was three to four times larger than that by respiration. These effects of BQ were blocked in the presence of 5.00 μM DCMU. The addition of 1.00 mM BQ to the culture medium increased photosynthetic oxygen generation from $(1.0 \pm 0.3) \times 10^{-13}$ mol/s to $(3.6 \pm 0.7) \times 10^{-13}$ mol/s. The increase in oxygen generation was 1.5–2 times larger than that expected from the consumption of BQ by the protoplast. The reducing power produced by the additional oxygen generation is consumed for the reduction of BQ to QH_2 as well as for the reduction of intracellular species, which is probably mediated by the BQ/QH_2 redox couple.

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