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An ultramicroelectrode for determination of intracellular oxygen. Light-irradiation-induced change in oxygen concentration in an algal protoplast

Tomokazu Matsue, Shinji Koike, Takayuki Abe, Takeyuki Itabashi and Isamu Uchida

Department of Molecular Chemistry and Engineering, Faculty of Engineering, Tohoku University, Sendai (Japan)

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An oxygen ultramicroelectrode was fabricated by pyrolysis of butane inside a glass capillary, followed by electrodeposition of Pt. The intracellular determination of oxygen in an algal protoplast (diameter, approx. 80 μm) with the ultramicroelectrode indicated that at a light intensity of 25 klx, the oxygen efflux from a single protoplast caused by the light reactions is approx. $5 \cdot 10^{-13}$ mol/s and that corresponding to the dark reactions approx. $1 \cdot 10^{-13}$ mol/s.

Introduction

A living cell possesses various electroactive chemicals which function to maintain its biological activities. Thus, amperometric determination, based on electron transfer reactions at the electrode surfaces in extremely small biological environments is important to clarify the reactions occurring in living materials. Since the pioneering works done by Adams and coworkers [1], many in vivo voltammetric studies to determine the neurotransmitters [2], have been extensively carried out. Recently, Ewing and co-workers [3] fabricated ultramicroring electrodes with tip diameters of a few micrometers and used them for the determination of catecholamine concentration in a cell. Among the redoxactive chemicals, oxygen seems to be especially important since it is intrinsically involved in biological energy production, chemical conversion, etc. So far, oxygen electrodes have usually been employed for determining the concentration of oxygen in biological fluids. It is, however, difficult to apply the conventional oxygen electrode for the determination of oxygen in microenvironments such as single cells.

We report here the amperometric determination of oxygen in a living protoplast using a novel oxygen ultramicroelectrode, which can be inserted into a single cell, and can demonstrate the effect of light irradiation at the single cell level. The present system provides information on the changes in oxygen concentration upon external chemical or physical stimulations of the order of a second in duration.

Materials and Methods

The ultramicroelectrode for the determination of oxygen was fabricated by deposition of carbon from pyrolyzed butane inside quartz capillaries [3]. A scanning electron microscope measurement indicated that the carbon layer had the thickness of approx. 50 nm. The cavity of the tip was filled with a low-viscosity epoxy resin. The tip was then cut and polished on a turntable using 50 nm alumina fine powder, resulting in the formation of a carbon ultramicroring at the cross-section. Since the electrochemical reduction of oxygen at the bare electrode proceeds only slowly, we electrodeposited Pt particles [4], catalysts for the reduction of oxygen to water, by a controlled current electrolysis at 0.1 nA for 1 s in 5.0 mM K_4PtCl_6/0.1 M KNO_3 aqueous solution. The ring radius (r) at the tips of the electrodes were determined from the steady-state oxidation current (i_{ss}) of $Fe(CN)_6^{4-}$ in 0.1 M KCl solution in the linear sweep voltammogram using the following equation [3]:

$$i_{ss} = 5.8FC^*Dr \quad (1)$$

where F is Faraday constant, C^* the bulk concentra-

Correspondence to: T. Matsue, Department of Molecular Chemistry and Engineering, Faculty of Engineering, Tohoku University, Sendai 980, Japan.

tion, D the diffusion coefficient. The microelectrodes with ring radii less than 2 μm were used for intracellular measurements.

The protoplast of the marine alga *Bryopsis plumosa* was prepared according to Takewaki and Nagata [5]. We selected the protoplasts with a diameter of approx. 80 μm for the intracellular amperometric measurments. The protoplast was placed in deoxygenated sea water. The insertion of the oxygen ultramicroelectrode was carried out with a three-dimensional micromanipulator system (Narishige MO-188) under an inverted microscope (Nikon TMD). A two-electrode configuration in an aluminum sealed box was used in the amperometric measurement to avoid system noise. The small current was amplified with an amplifier (Nihon Koden CZE-2300). The light irradiation was carried out with the built-in light source of the microscope. The light intensity was controlled by using light filters and determined by a lux meter (Sibata Scientific ANA-999).

Results and Discussion

Fig. 1 shows the linear sweep voltammograms of oxygen at a Pt/carbon microring electrode (ring radius, 0.65 μm) placed outside and inside a protoplast. The reduction for oxygen starts at approx. 0.0 V vs. Ag/AgCl. The freshly prepared electrode showed a sigmoidal wave [6] (curve a); however, the electrode was gradualy deactivated with successive potential scans and finally gave a steady-state response (curve b). The

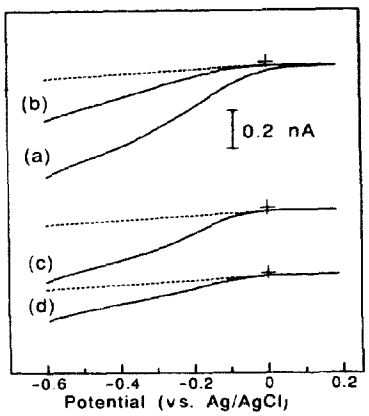

Fig. 1. Linear sweep voltammograms for oxygen at Pt/carbon ultramicroelectrode in sea water (a and b) and in a single protoplast (c and d). Ring radius: 0.65 μm. Scan rate: 50 mV/s. (- - - - -), background voltammogram. The microelectrode showed a large reduction current on the first scan (curve a); however, the electrode was deactivated gradually and finally gave a steady-state voltammogram (curve b). The electrode showing the steady-state response was inserted into the protoplast. Curves (c) and (d) are, respectively, the voltammograms in the protoplast under light irradiation and in the dark.

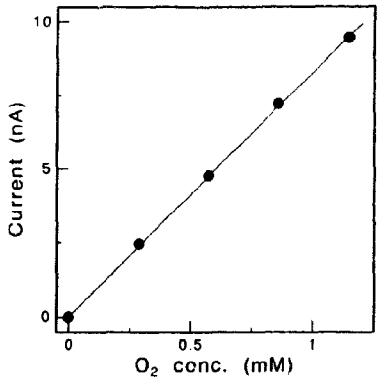

Fig. 2. Relationship between the reduction current at -0.55 V vs. Ag/AgCl and the concentration of oxygen in sea water. Ring radius, 1.9 μm. We used this relationship for the determination of the intracellular concentration of oxygen, assuming that the reduction process and diffusion coefficient of oxygen are the same as those in sea water.

electrode showing the steady-state behavior was used for the intracellular measurements. The insertion of the microring electrode into the protoplast caused no obvious distortion in the voltammetric shape. Adsorption of hydrophobic biomolecules such as lipids and proteins sometimes blocks the approach of hydrophilic redox species to the electrode surface. Oxygen is relatively hydrophobic and can easily penetrate hydrophobic layers [7]. Therefore, the oxygen inside the protoplast is reduced smoothly at the microring surface. The voltammetric behavior also suggests that there are no major electroactive species in the cell in the potential region investigated. Light irradiation increases the reduction current for oxygen (curve c) because of photosynthetic production of oxygen inside the protoplast. On the other hand, the reduction current was small in the dark (curve d) due to the consumption of oxygen by respiration.

Since the voltammetric response contains the undesired contribution from the charging current for the double layer at the electrode–solution interface, we used amperometric measurements at a fixed electrode potential, -0.55 V vs. Ag/AgCl, which is negative enough to reduce oxygen. The reduction current at -0.55 V vs. Ag/AgCl increases linearly with the concentration of oxygen in sea water (Fig. 2). Since the intracellular fluid of the protoplasts is equivalent to sea water by nature, in the following discussion we have assumed that the reduction process and diffusion coefficient ($2 \cdot 10^{-5}$ cm^2 s^{-1} [8]) of oxygen are the same as those in sea water.

Fig. 3 shows the variation of the reduction current for oxygen inside the protoplast upon light irradiation of various intensities at an oxygen ultramicroelectrode (ring radius, 1.9 μm). The concentration increased

rapidly upon light irradiation and returned to the original level in a few seconds after turning the light off. The response was rapid and depended on the light intensity. Under strong light irradiation, a peak appears immediately after the light irradiation, then the current decreases finally to give a steady-state value. The peak height decreases with decreasing light intensity and below 2.5 klx no peak response is observed. Another interesting fact, which can be seen from the figure, is that at the light intensities higher than 2.5 klx the response is independent of light intensity. Under low light intensities, the response had no peak and the steady-state current decreased with decreasing light intensity.

These changes in the reduction current for oxygen (i.e., oxygen concentration) are closely related to the activity of photosynthesis, consisting of the 'light' and 'dark' reactions. At the initial stage, after strong light irradiation (approx. 1 min), the light reaction is the major rate-determining process for oxygen evolution. The irradiation initiates the light reaction in chloroplasts to produce oxygen by photolysis of water with consumption of the photosynthesis-associated chemicals, such as $NADP^+$ and ADP. However, the depletion of these chemicals slows down the light reaction and after 1 min the oxygen evolution rate is controlled by the regeneration rate for these chemicals, i.e., the rate of the 'dark' reactions. The dark reactions are basically light-independent. Therefore, the oxygen concentration in the steady-state region at light intensities higher than 2.5 klx is almost constant (Fig. 3). Under low light irradiation, the rate of the light reaction is slower than that of the dark reactions; therefore, the oxygen evolution is controlled by the light reaction

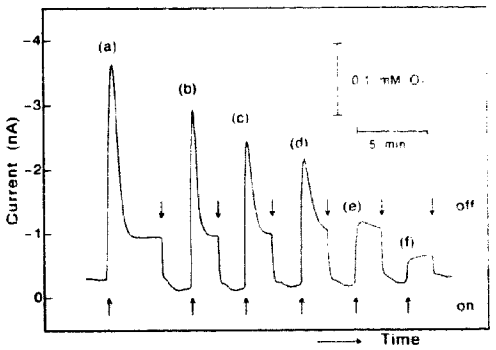

Fig. 3. Intracellular response of the reduction current for oxygen in a protoplast (diameter, approx. 80 μm) upon light irradiation. Light intensity: (a), 25 klx; (b), 18 klx; (c), 10 klx; (d), 7 klx; (e), 2.5 klx; (f), 0.7 klx. Under strong light irradiation, a peak was observed immediately after the light irradiation and a steady-state current after approx. 1 min. The peak reflects the rate of the light reactions triggered by light irradiation. The steady-state value is a consequence of the oxygen evolution being controlled by the dark reactions which are light-independent.

throughout the period of light irradiation. From the dependence of peak and steady-state concentrations on the light intensity, it can be concluded that the rate of the light reaction is almost equal to that of the dark reactions at the light intensity of 2.5 klx.

The intracellular determination of oxygen using the ultramicroelectrodes gives the rates of the light and dark reactions at a single-cell level. Since protoplasts used in the present study contain many, randomly distributed chloroplasts, the concentration of oxygen inside the protoplast can be assumed to be uniform. In this case, the efflux (f) of oxygen from the protoplast, which is equivalent to the rate of photosynthesis, can be formulated as follows [9]:

$$f = 4\pi a^2 DC^*[1/(\pi Dt)^{1/2} + 1/a] \quad (2)$$

where a is the radius of the protoplast ($4 \cdot 10^{-3}$ cm), D the diffusion coefficient of oxygen, C^* the concentration of oxygen inside the protoplast, t the time. When the time domain for measuring the oxygen concentration is longer than 5 s (i.e., the oxygen concentration does not change greatly in 5 s), Eqn. 2 is simplified to:

$$f = 4\pi aDC^* \quad (3)$$

If we adopt this equation, the oxygen evolution rate from the single protoplast at the peak concentration time under the irradiation of 25 klx (Fig. 3) is calculated to be approx. $5 \cdot 10^{-13}$ mol/s. The rate of the dark reaction corresponds to an oxygen evolution rate of $1 \cdot 10^{-13}$ mol/s which, in turn, would be the rate for CO_2 fixation.

Since the intrinsic response of the ultramicroelectrode is rapid, the electrode can be used for monitoring the transient change in oxygen concentration in less than 1 s. The present procedure for determining the concentration of oxygen in extremely small environments will provide a new way to explore biological reactions.

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