S19-009

EPR study on the photo-reduction of spin probe in the green alga *Chlorella ellipsoidea*

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Keywords : EPR , spin-label , Chlorella , mutant , photoreduction

1. Introduction

The spin-label-electron-paramagnetic resonance (EPR) method with 2,2,6,6-tetramethyl-4-oxopiperidinonxy free radical (TEMPO) as a spin probe has been used to determine cell volume (Blumwald et al., 1983). This method utilizes a characteristic of TEMPO that this compound possesses NO radical when oxidized and is highly permeable against biological membranes. Cells treated with TEMPO thus contain NO radical in the presence of an appropriate oxidant such as potassium ferricyanide and the radical concentration can be readily determined by EPR. Preliminary study in this laboratory showed that EPR signals from intracellular TEMPO diminished in several min under illumination. It was thus assumed that TEMPO is reduced by photo-induced electron flow in the photosystems.

This study describes detailed EPR analysis of biophysical reduction of TEMPO in the cells of the green alga, *Chlorella ellipsoidea*.

2. Materials and Methods

2.1 Cells and culture condition

Cells of *C. ellipsoidea* (UTEX 20) was grown on 5% CO_2 or air in the Bold's Basal medium under continuous illumination at a photon flux density of 100 μmol m^{-2} s^{-1} at 25°C.

2.2 Measurement of photosynthetic rate

The rate of photosynthesis was measured with a Clark-type oxygen electrode at pH 7.8 and 25°C under illumination of three different wavelength regions, i.e., white, blue, and red at 1100, 700, and 600μE $m^{-2}s^{-1}$, respectively.

2.3 EPR measurement of intracellular spin probe

Cells were harvested and resuspended at 1.0~1.1 mg Chl mL^{-1} in a solution of 2.0 mM TEMPO and 40 mM $K_3[Fe(CN)_6]_3$. The cells were loaded into a sealed-grass capillary (i.d., 1.0 mm) in a final volume of 20 μL and illuminated with white, blue and red light. EPR measurement was carried out with Varian E-109 system X-band spectrometer. TEMPO is oxidized by $K_3[Fe(CN)_6]_3$ and penetrates the plasma membrane rapidly and reaches an equilibrium. The addition of the paramagnetic quencher Na_2Mn-EDTA, which is not able to permeate the plasma membrane, broadens the EPR signal of extracellular TEMPO and this in turn visualizes intracellular EPR signal.

3. Results and Discussion

3.1 Photosynthetic parameters at saturating light of three different colors.

Photosynthesis was saturated with light at intensities of 1100, 600 and 700 μE $m^{-2}s^{-1}$ with white, blue, and red lights, respectively. 5%-CO_2 grown cells exhibited $K_{1/2}$[DIC] values of 773-900 μM (Table 1) whereas those of air grown cells were at 179-225 μM (Table 1). This difference in the affinity for DIC has been ascribed to the operation of inorganic-carbon concentrating mechanisms in *C. ellipsoidea* (Matsuda and Colman, 1995) and the results showed clearly that wavelength of light does not change affinity of cells significantly. Although the reason is not clear, P_{max} values tended to be low when illuminated with red light (Table 1).

Table. 1 Photosynthetic parameters of *C. ellipsoidea* grown in 5% CO_2 and air at pH 7.8. The light intensities were 1100 (white light), 600 (blue light), and 700 (red light) μE $m^{-2}s^{-1}$.

Growth condition	Color of light	P_{max}[a] (μmol O_2 mg Chl^{-1} h^{-1})	$K_{1/2}$[DIC][b] (μM)
5% CO_2	white	67	773
	blue	64	900
	red	51	897
Air (0.034 %)	white	95	225
	blue	91	191
	red	71	179

P_{max}[a] is the maximum rate photosynthesis, $K_{1/2}$[DIC] is the DIC concentration at half maximum photosynthesis

3.2 Measurement of EPR signal of intracellular TEMPO

EPR signal from intracellular TEMPO was measured at 0, 2.5 and 5 min after starting illumination (Fig. 1), then light was turned off and measurement was carried out continuously at 2.5 and 5 min after turning off the light (Fig. 1). This assay was carried out in the presence and the absence of DCMU. The intensities of spectra were decreased rapidly during illumination without DCMU addition irrespective of colors of light (Fig. 1), but recovered by turning off the light (Fig. 1). This reduction of signal intensity would be probably due to a rapid photoreduction of TEMPO even in the presence of $K_3[Fe(CN)_6]_3$ in the cells and the dark incubation causes the re-oxidization of TEMPO by $K_3[Fe(CN)_6]_3$. The addition of 2 μM DCMU drastically inhibited the photoreduction (Fig. 1) and such inhibition was more obvious with 20 μM of DCMU (Fig. 1). These results indicate that photoreduction of spin probe such as TEMPO probably occurs at the acceptor site of PSII. TEMPO probably accept electron from Q_B or PQ pool. The addition of Phenazinemetholsulphate (PMS) did not enhance such reduction (data not shown), which indicates that cyclic electron flow did not donate electron to TEMPO.

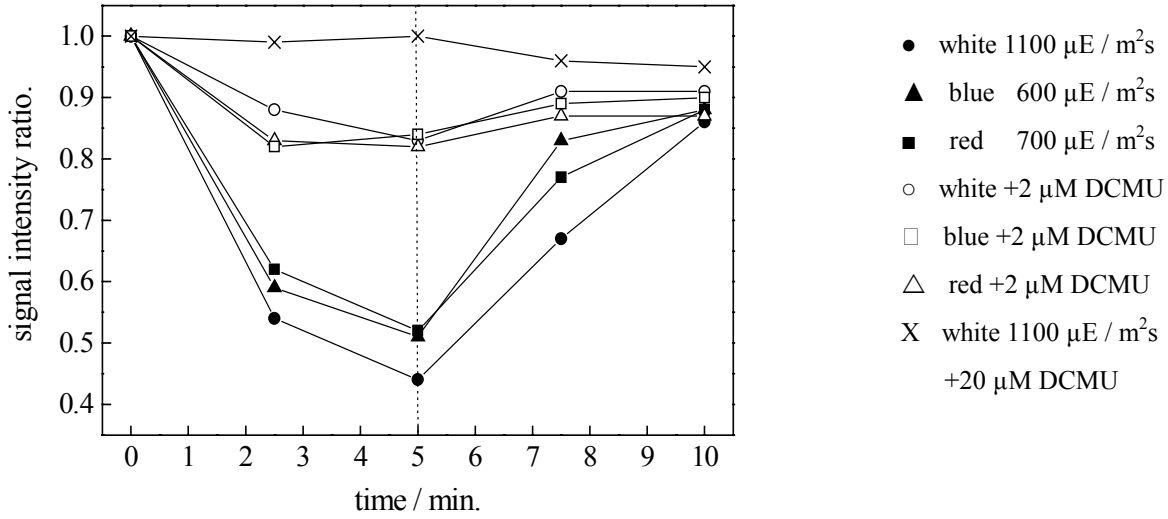

Fig. 1 White light illumination to air grown cells of wild type. See text for details.

3.3 Photoreduction of TEMPO in the dark

Mitochondorial respiration proceeds in the presence of DCMU. This implies that reduction of TEMPO under illumination is largely due to light-driven electron flow. Interestingly, photoreduction of TEMPO in the dark immediately after illumination was more rapid than that in the light with 20 μM DCMU (Fig. 2). Cells boiled for 10 min

showed significantly slow reduction rate of TEMPO over 30 min (Fig. 2) indicating that reductions of TEMPO both under the light and the dark certainly relates to biological electron flows in the cells. Given these, it is suggested that respiratory electron flow might be more active than that in the light.

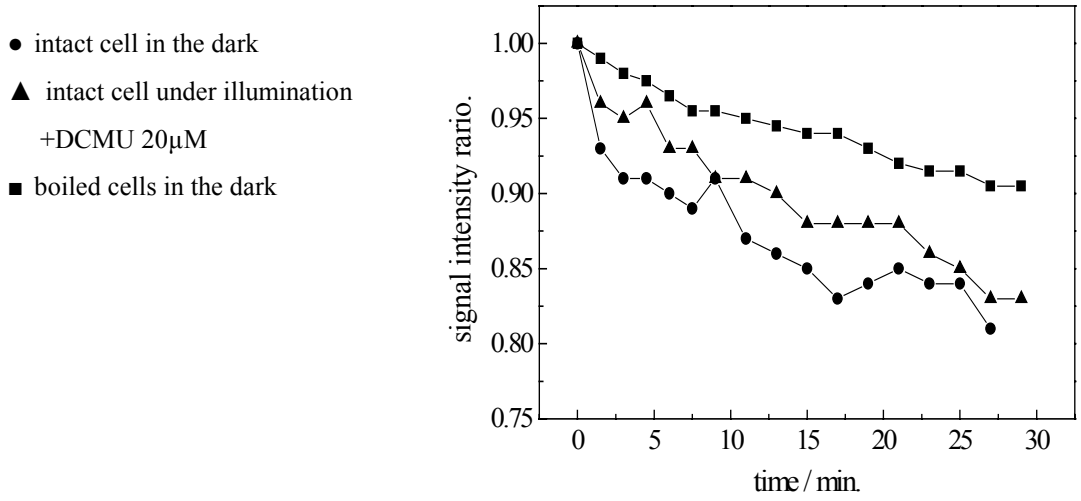

Fig. 2 Dark respiration of high CO_2-grown cells

Reference

Blumwald E, Mehlhorn RJ, Packer L (1983) *Proc. Natl. Acad. Sci. USA* **80**: 2599-2602
Matsuda Y and Colman B (1995) *Plant Physiol* **108**: 247-252