Artificial Control of Enzyme Reactions by the Photoexcitation of Hematoporphyrin Adsorbed on Living Yeast Cells

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The objective of our research was to establish control of biological functions by the illumination of a photosensitizer of hematoporphyrin that was externally provided in a biological system. We found using an ESR method that the reduction of 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPOL), as a reduction probe, caused by enzyme reactions can be controlled through the photoexcitation of hematoporphyrin adsorbed on the living yeast cells (HP/Yeast system). On the basis of the effects of some electron-transfer blockers through its reduction rate, it has been postulated that the reduction of TEMPOL is mainly conducted through ferredoxin (ferredoxin-NAD(P)$^+$ reductase), which could be controlled through the illumination of HP connected with a mitochondrial electron-transfer system. A possible mechanism for controlling the reduction of TEMPOL is discussed in connection with the energy levels of HP and the redox potentials of the components in yeast cell participating in this phenomenon.

The industrial use of biological systems has the advantages that enzyme reactions occur with high reaction selectivity and high efficiency under mild conditions. However, there are some demerits such as low stability, difficulty of control, and slow reaction rate of the systems. For effective utilization of biological systems, therefore, it is important to control biological functions from the outside of the systems. Hamachi et al.[1—4] recently reported that the adsorption of oxygen to myoglobin could be controlled by illumination. Such myoglobin was produced by the reconstitution of the synthesized ruthenium complex pendent iron(III) porphyrin and apomyoglobin. This result indicates that biological functions can artificially be controlled from the outside of systems.

We previously reported the reactivity of photosensitizers in heterogeneous media.[5,6] The formation of heterogeneous systems such as chlorophyll on MgO and hematoporphyrin on the separated cell wall of yeast led to a remarkable increase of their reactivity with oxygen. These results suggest that the formation of heterogeneous systems plays a crucial role for their efficient charge-separation during illumination. In photosynthesis, the reaction centers of chlorophyll drive various functions of enzymes after their charge-separation. Therefore, biological functions are expected to be controlled through the illumination of a photosensitizer that is externally provided in a biological system.

During our attempt to establish control of biological functions using a photosensitizer of hematoporphyrin (HP), we found using an ESR method that the reduction of 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPOL) caused by enzyme reactions can be controlled through the illumination of HP provided in the living yeast cells (HP/Yeast system). Based on the reduction rates of TEMPOL in the presence of various electron-transfer blockers under illumination, it was found that some electron-transfer systems in yeast cell are related to the reduction of TEMPOL. In this paper we will present the details of the experimental results and discuss a possible mechanism for controlling the TEMPOL reduction in relation to the redox potentials of the components in yeast cell.

Experimental

Materials Hematoporphyrin (HP) dihydrochloride, malonic acid (a blocker of succinic acid-coenzyme quinone reductase), diphosphoric acid (a blocker of ferredoxin), *N*-ethylmaleimide (NEM, an SH-blocker), and iodoacetic acid (an SH-blocker) were obtained from Wako Pure Chemical Industries, Ltd. β-Nicotinamide adenin dinucleotide reduced form (NADH) and its oxidized form (NAD$^+$) were purchased from Oriental Yeast Co., Ltd. 4-Hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPOL) and sodium azide (NaN_3, a blocker of cytochrome *c* oxidase) were purchased from Aldrich Chem. Co., Inc. (USA). Antimycin A (a blocker of coenzyme quinol-cytochrome *c* reductase) and *p*-chloromercuriobenzensulfonic acid monosodium salt (PCMS, a blocker of NADH-coenzyme quinone reductase) were obtained from Sigma Chemical Co. (USA). Commercially available dry yeast was used for preparing the sample. HP adsorbed on yeast cells (HP/Yeast system) was prepared as follows. Dry yeast cells were centrifugally washed by pure water several times. One mmol dm^{-3} HP ethanol solution was added to the cell suspension. The sample was used for experiments after centrifugal washing by pure water several times.

Apparatus. The fluorescence spectra were recorded using a Hitachi Co., Ltd., F-2000 spectrophotometer. The fluorescence image of the HP/Yeast system was observed using an Olympus Optical Co., Ltd., FLUOVIEW laser scanning microscope with 488 nm Ar$^+$ laser and 510 nm cut off filter. The oxygen consumption measurements of the HP/Yeast system were conducted using a Clark-

type oxygen electrode (Central Kagaku Co.). Before ESR measurements, the HP/Yeast system (yeast, 1×10^9 cm^{-3}) was mixed with 0.1 mmol dm^{-3} TEMPOL and various solutions, such as mitochondrial electron transfer blockers, SH-blockers, ferredoxin blocker, or coenzymes. This mixture was fed into a flat quartz ESR cell, which was inserted into the cavity of an ESR spectrometer (JEOL RE-2X). The sample after 60-s mixing was irradiated by a 500-W xenon lamp with a 500 nm low-pass filter and an IR cut filter (Toshiba, Y-50 and IRA-20). All ESR measurements were conducted during irradiation at room temperature.

Results

The mitochondrial oxygen consumption for the HP/Yeast system was confirmed to be complete, using a Clark-type oxygen electrode. The oxygen consumption rate observed was 0.34 mmol dm^{-3} min^{-1} per 1×10^9 cells. From this result, we can regard the HP/Yeast system under closed condition as an anaerobic system Figs. 3 and 4 and Table 1).

In order to verify the electronic states of the HP/Yeast system, we conducted the measurements of the fluorescence and fluorescence excitation spectra, as shown in Fig. 1. In Fig. 1A, the fluorescence of the excitation at 400 nm for the HP/Yeast system had the maxima at 580 (weak) and 623 nm (strong). On the other hand, the fluorescence of the excitation at 400 nm for yeast cells had no maximum (Fig. 1B). Therefore, the above two maxima originated in HP which existed in yeast cells. In Fig. 1C, the excitation of

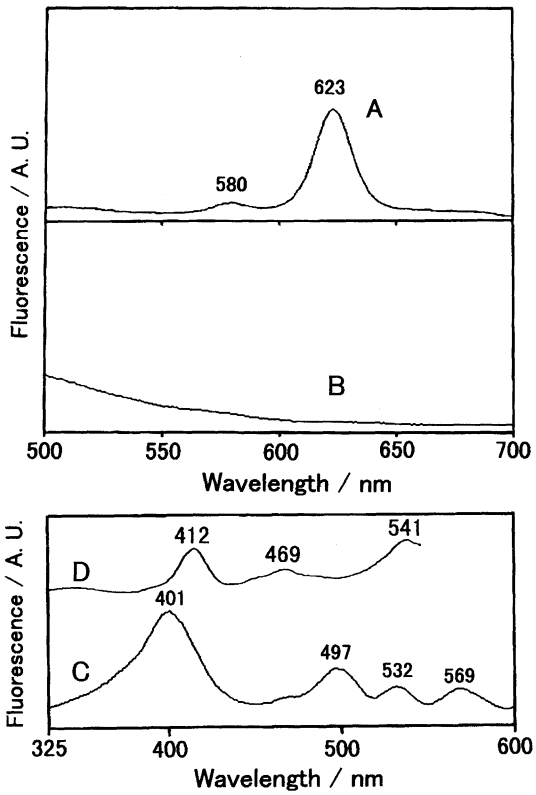

Fig. 1. Fluorescence (A) and fluorescence excitation spectra (C and D) of the HP/Yeast system and fluorescence spectrum of yeast cells (B). Excitation wavelength: 400 nm (A and B), emission wavelength: 623 nm (C), 580 nm (D), content of HP in yeast cells: 0.09 mmol dm^{-3}.

the fluorescence emitted at 623 nm had the maxima at 401, 497, 532, and 569 nm. These values are similar to those of HP in heterogeneous media.[6] The excitation of the fluorescence emitted at 580 nm had the maxima at 412, 469, and 541 nm, as shown in Fig. 1D. The maxima at 412 and 541 nm are nearly at the absorption maxima of cytochromes.[7] Therefore, an energy transfer from HP to cytochromes (in cell organelles?) might have occurred.

In order to clarify the distribution of HP in yeast cell, we attempted the observation for the fluorescence image of the HP/Yeast system using a laser scanning microscope, as shown in Fig. 2. The fluorescence emission from HP was distributed in the whole or a part of yeast cells.

Figure 3A shows the characteristics of a light dependent decay of TEMPOL found on the HP/Yeast system. This curve was traced by fixing the field at the position of the central signal of TEMPOL, as shown in Fig. 3D. An addition of 1 mmol dm^{-3} hexacyanoferrate(III) ion, as an oxidant, in the reaction mixture led to about 70% recovery of the original signal intensity of TEMPOL. Therefore, this phenomenon can be regarded as a light dependent reduction of TEMPOL brought about by the HP/Yeast system. A small decay was observed in the HP/Yeast system in the dark (Fig. 3B). The light dependent reduction of TEMPOL was not observed in the cases for yeast cells without HP adsorption, 0.05 mmol dm^{-3} HP aqueous solution (O_2 presence), and the suspension of the separated cell wall of yeast adsorbing ca. 0.02 mmol dm^{-3} HP (O_2 presence, Fig. 3C).[2] These results, therefore, indicate that the light dependent reduction of TEMPOL is brought about by HP which existed in the cell organelles of yeast. The concentration of the reduced

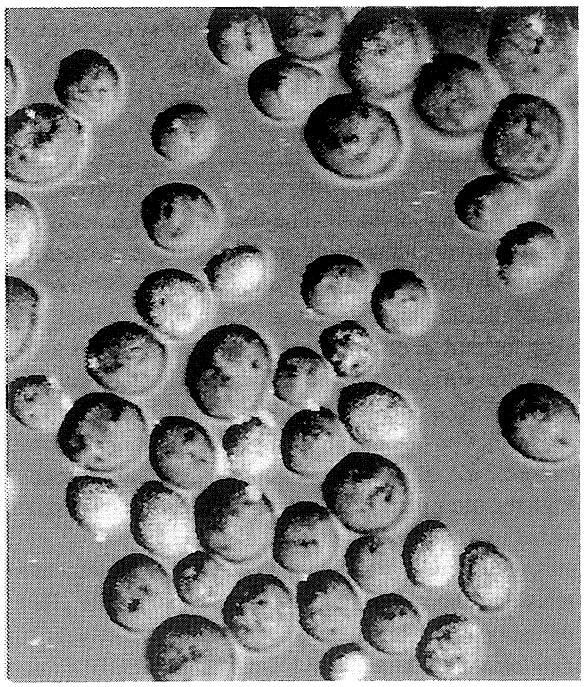

Fig. 2. A laser scanning microscopic image of the HP/Yeast system. Fluorescence image (bright spots) was drawn over the transmitted image of the HP/Yeast system.

Table 1. Pseudo First-Order Rate Constants of the TEMPOL Reduction Obtained from the HP/Yeast System in the Presence of Various Electron-Transfer Blockers under Illumination

		k / s^{-1}
1.	Control	$9.75 \pm 0.61 \times 10^{-3}$
2.	Mitochondrial electron-transfer blockers	
	50 mmol dm^{-3} NaN_3	$3.40 \pm 0.41 \times 10^{-4}$**
	1 mmol dm^{-3} PCMS	$2.49 \pm 0.21 \times 10^{-3}$**
	1 mmol dm^{-3} Antimycin A	$5.29 \pm 0.61 \times 10^{-3}$*
	10 mmol dm^{-3} Malonic acid	$5.10 \pm 0.73 \times 10^{-3}$*
3.	Ferredoxin blocker	
	10 mmol dm^{-3} Diphosphoric acid	$3.16 \pm 0.32 \times 10^{-3}$**
4.	Coenzymes	
	1 mmol dm^{-3} NADH	$1.46 \pm 0.10 \times 10^{-2}$**
	1 mmol dm^{-3} NAD^+	$9.87 \pm 0.14 \times 10^{-3}$
5.	SH-blockers	
	10 mmol dm^{-3} NEM	$1.52 \pm 0.14 \times 10^{-3}$**
	10 mmol dm^{-3} Iodoacetic acid	$7.48 \pm 0.44 \times 10^{-3}$**

Rate constants are presented as mean±S.D. (n = 3).
*: $p < 0.05$, **: $p < 0.01$. Content of HP: 0.02 mmol dm^{-3}. Concentration of TEMPOL: 0.1 mmol dm^{-3}.

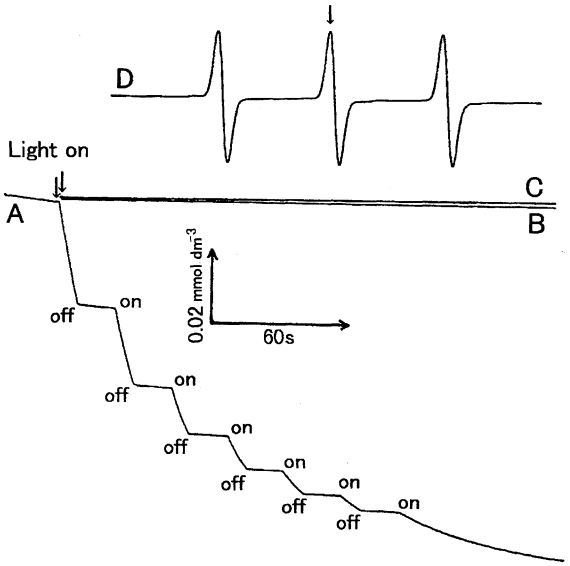

Fig. 3. Characteristics of the light dependent decay of TEMPOL found on the HP/Yeast system. A: HP/Yeast system, B: HP/Yeast system in the dark, C: HP on the separated cell wall of yeast, D: ESR spectrum of TEMPOL, concentration of HP: 0.02 mmol dm^{-3} concentration of TEMPOL: 0.1 mmol dm^{-3}, number of yeast: 1×10^9 cm^{-3}, illumination wavelength: 500—800 nm.

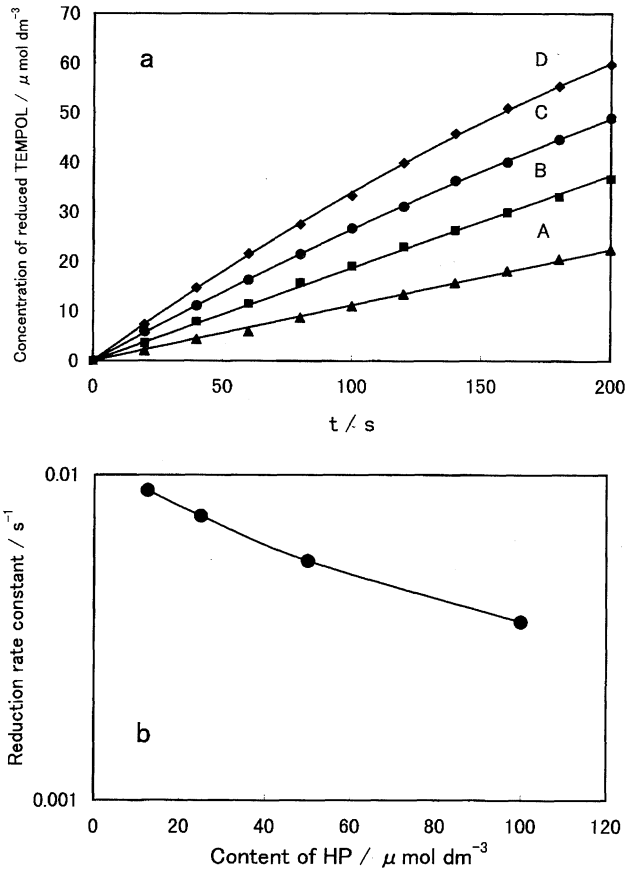

Fig. 4. Time dependence for the photoinduced reduction of TEMPOL caused by the HP/Yeast system with various contents of HP (a) and their estimated reduction rate constants (v_0/[HP], b). Content of HP (mmol dm^{-3}): 0.0125 (A), 0.025 (B), 0.05 (C), 0.1 (D), concentration of TEMPOL: 0.1 mmol dm^{-3}.

TEMPOL (ca. 0.1 mmol dm^{-3}) was enough larger than that of HP (ca. 0.02 mmol dm^{-3}) in the yeast cells. This means that several turnovers of a photoinduced charge-separation by HP have occurred.

Figure 4 shows the time dependence for the reduction of TEMPOL caused by the HP/Yeast system with various contents of HP under illumination (a) and their estimated reduction rate constants (b). An increase in the contents of HP in the system led to an increase in the reduction rates of TEMPOL (Fig. 4a). However, the rate constants for the reduction rate of TEMPOL decreased in the increase of the contents of HP in yeast cells (Fig. 4b). This result also

suggests that HP which exists in the specific regions of yeast cell plays a crucial role for the reduction of TEMPOL.

It has been established that mitochondria are the most damaged organelle in the photodynamic oxidation using HP and some of its derivatives.[8] In our study, HP adsorbed on the mitochondria in yeast cells may also play a crucial role on the reduction of TEMPOL. We then examined the effects of the mitochondrial electron-transfer blockers on the TEMPOL reduction brought about by the illuminated HP/Yeast system; sodium azide for a blocker of cytochrome *c* oxidase, PCMS for a blocker of NADH-coenzyme quinone reductase, malonic acid for a blocker of succinic acid-coenzyme quinone reductase, and antimycin A for a blocker of coenzyme quinol-cytochrome *c* reductase (see Fig. 5).

In general, ferredoxin and coenzymes such as NADH and NAD^+ play the role of electron transports on the reduction in biological systems. Therefore, these may also be related to the reduction of TEMPOL. We then examined the effects of a ferredoxin blocker of diphosphoric acid and the coenzymes on the TEMPOL reduction.

All of the reactions tested could be regarded as pseudo first-order reactions. The effects of the electron-transfer blockers on the TEMPOL reduction were estimated by using first-order rate constants. The rate constants estimated are listed in Table 1. All of the mitochondrial electron-transfer blockers decelerated the reduction rate of TEMPOL under illumination. The largest deceleration of the reduction rate of TEMPOL was observed in the case of 50 mmol dm^{-3} NaN_3, a blocker of cytochrome *c* oxidase existing in the hydrophobic medium of mitochondrial inner membrane. This value was nearly to the reduction rate estimated from the HP/Yeast system in the dark. The addition of a ferredoxin blocker of 10 mmol dm^{-3} diphosphoric acid about 70% inhibited the reduction of TEMPOL. The increase in the concentration of diphosphoric acid above 10 mmol dm^{-3} led to no increase of inhibition. This means the existence of other reduction paths. On the other hand, the addition of a mitochondrial inner membrane impermeable coenzyme of 1 mmol dm^{-3} NADH, which also acts as a blocker of ferredoxin-$NAD(P)^+$ reductase, accelerated the reduction rate of TEMPOL. From these results, one can conclude that HP adsorbed on the mitochondrial inner membrane surface may be related to the reduction of TEMPOL.

A mitochondrial electron transfer blocker of PCMS is also a mitochondrial inner membrane impermeable SH-blocker. Therefore, SH-compounds in the hydrophilic media of the mitochondrial intermembrane space may be blocked by the addition of PCMS. We then examined the effects of other SH-blockers on the reduction of TEMPOL. A large deceleration for the reduction rate of TEMPOL was observed by the addition of a mitochondrial inner membrane permeable SH-blocker of 10 mmol dm^{-3} NEM. On the other hand, a small deceleration for the reduction rate of TEMPOL was observed by the addition of a mitochondrial inner membrane impermeable SH-blocker of 10 mmol dm^{-3} iodoacetic acid. That is, SH-compounds in the mitochondrial inner membrane such as iron–sulfur complexes may also be related to the reduction of TEMPOL.

Discussion

In the following discussion, we disregarded the effects of oxygen or active oxygen species to the reduction of TEMPOL, because oxygen dissolved in the reaction mixture is consumed by the respiration of the HP/Yeast system (see results section).

An interaction between HP and cytochromes was con-

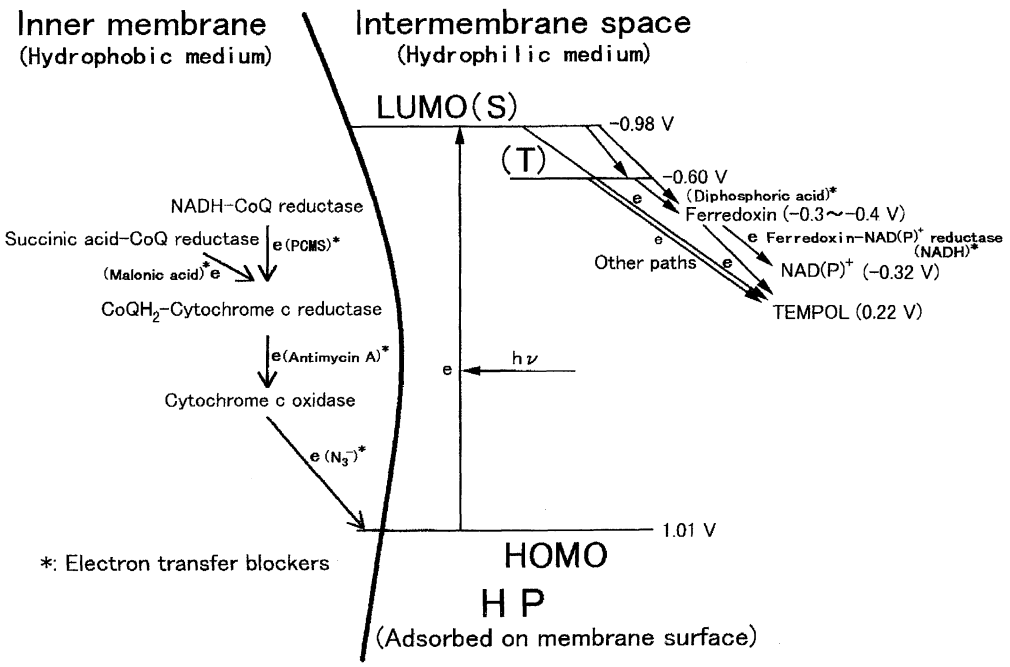

Fig. 5. An energetic illustration concerning the molecular orbitals of HP and the redox potentials of the components in yeast cell participating in the reduction of TEMPOL under illumination.

firmed from the analysis of the fluorescence and fluorescence excitation spectra (see Fig. 1). Moreover, our present results suggested that the light dependent reduction of TEMPOL by the HP/Yeast system might have occurred in the mitochondria (see Fig. 4 and Table 1). However, we could not clarify the adsorption structures of HP adsorbed on the mitochondrial inner membrane from the fluorescence spectra. In the following discussion, we hypothesized the adsorption structures of HP on the mitochondrial inner membrane as a monomeric HP.

Based on the analysis of the results observed in our study, a possible mechanism for controlling the TEMPOL reduction by the illumination of the HP/Yeast system can be proposed using an energetic illustration. Figure 5 shows the energy levels of HP (typical value for many porphyrins of natural origin)[9] and the redox potentials of the components in yeast cell and TEMPOL.[10—14] Generally, HP is first excited to its singlet state by illumination. The singlet state (S) of HP (−0.98 V) allows energy and/or electron transfer to acceptors or changing to a triplet state (−0.60 V) through intersystem crossing. The triplet state (T) of HP also allows energy and/or electron transfer to acceptors. The reduction of TEMPOL conducted mainly through ferredoxin may be caused by an electron-transfer from excited states of HP (S and/or T)[15—18] adsorbed on the mitochondrial inner membrane, because a ferredoxin blocker of diphosphoric acid about 70% decelerated the reduction rate of TEMPOL (see Table 1). This probability is supported by the fact that the reduction potential of TEMPOL (0.22 V) lies below both the LUMO (S and T) levels of HP (−0.98 and −0.60 V) and the redox potential of ferredoxin (−0.3——0.4 V). Further,minor electron transfer paths also relate to the light-dependent reduction of TEMPOL because it was not perfectly inhibited by the addition of diphosphoric acid. However, we could not clarify other electron transfer paths in the present study. On the other hand, it is presumed that HP cation (HP^+), which is produced after the electron-transfer, has been reduced by a mitochondrial electron-transfer system, because a cytochrome *c* oxidase blocker of sodium azide perfectly inhibited the light dependent reduction of TEMPOL (see Table 1). This probability is also supported by the fact that the HOMO level of HP (1.01 V) lies below the redox potentials of a mitochondrial electron transfer system (−0.38—0.39 V).[19]

Moreover, an electron transfer from the excited states of HP (S or T) to $NAD(P)^+$ (−0.32 V) conducted through ferredoxin-$NAD(P)^+$ reductase may also have occurred because the reduction rate of TEMPOL was accelerated by the addition of a ferredoxin-$NAD(P)^+$ reductase blocker of NADH (see Table 1). This phenomenon is quite similar to the photosystem I (PSI) in photosynthesis.

In conclusion, we have demonstrated that the reduction of TEMPOL conducted mainly through ferredoxin (ferredoxin-$NAD(P)^+$ reductase) can be controlled through the illumination of HP connected with a mitochondrial electron-transfer system of yeast cells. Our results will give useful information for the control of biological functions. Moreover, this may also give an useful artificial candidate for a photoinduced charge-separation and for photoredox systems.

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