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Note

High-performance liquid chromatography–electrochemical detection of singlet oxygen by reaction with 2,2,6,6-tetramethyl-4-piperidone

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Singlet molecular oxygen ($^1O_2$) is a potentially toxic oxidant. During the past 10 years, the characteristic emission band at 1270 nm has been used to measure directly this reactant[1–3], but it must be monitored with an ultrasensitive spectrometer equipped with a liquid-nitrogen-cooled germanium detector because the near-infrared band is very weak. An indirect detection of $^1O_2$ using electron spin resonance (ESR) spectrometry has been proposed[4,5]. The stable nitroxide radicals generated by reaction of sterically hindered amines with $^1O_2$ were detectable down to 10 nM. ESR spectrometry is a well established method for detecting free radicals, however the spectrometer is an expensive instrument. An high-performance liquid chromatography (HPLC)–electrochemical detection (ED) system has been used to measure superoxide- and hydroxyl-generated DMPO adducts though they have short lifetimes[6,7]. The oxidation of sterically hindered heterocyclic amines by $^1O_2$ produces more stable nitroxide radicals which are known as spin labels.

We have tried to detect and quantify the $^1O_2$ adduct of 2,2,6,6-tetramethyl-4-piperidone (TEMP) using the corresponding spin label, namely 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TEMPO), as a standard by an HPLC–ED method. The HPLC system attached to a silicon polymer-coated silica gel column enabled us to measure the spin adduct as sensitively as by ESR spectrometry. The method was applied to the measurement of $^1O_2$ induced by a porphyrin-photosensitized oxidation.

EXPERIMENTAL

TEMPO and TEMP were obtained from Aldrich, catalase from Sigma. The concentration of TEMPO was calculated from the absorbance at 235 nm due to the $>N^{\cdot}-O$ group, using $\varepsilon_{235} = 2500\ M^{-1}\ cm^{-1}$ (ref. 8). α, β, γ, δ-Tetrakis(4-N-methylpyridyl)porphine (TMPyP) from Dojindo (Kumamoto, Japan) was used for the photosensitized production of $^1O_2$. The photoirradiation was carried out at 25°C in an oxygen electrode cell, YSI Model 5301 (Yellow Springs Instrument, Yellow Springs, OH, U.S.A.), placed at the centre of a 28 W toroidal lamp of the daylight fluorescent type. The oxygen consumption during photoirradiation was measured with a Clark oxygen electrode YSI 5331. The sample solution was prepared in calcium- and magnesium-free Dulbecco phosphate-buffered saline(PBS), pH 7.5 or pD 7.5, made using water or [$^2H_2$]water. The HPLC analysis was performed on a LC-6A HPLC in-

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strument (Shimadzu, Kyoto, Japan) equipped with a Capcell Pak C_{18} (5 μm) 150 × 4.6 mm I.D. column (Shiseido, Tokyo, Japan) packed with silicon polymer-coated silica gel, a Shimadzu L-ECD-6A electrochemical detector and a 10-μl sample loop. The detector potential was set at + 0.8 V *versus* an Ag/AgCl reference electrode so as to get the maximum response for TEMPO (Fig. 1). The retention time and peak integration were recorded by a Shimadzu C-R4A chromatograph integrator. The eluent consisted of 0.05 *M* citric acid, 10% acetonitrile and 4% methanol adjusted to pH 3.5 with 10 *M* sodium hydroxide, containing 10 mg Na_2 EDTA per litre. Elution was accomplished with a flow-rate of 1 ml/min through an ERC-3312 degasser (Erma, Tokyo, Japan).

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram of the spin label, TEMPO. The electrochemical response was linear over three orders of magnitude ranging from 50 n*M* to 50 μM. The linearity between the peak area and TEMPO concentration (μM) was represented by the following equation: $y = 108\,747x - 1373$, $r = 0.9999$. When a LiChrocart, Supersphere RP-18 (4 μm), 125 × 4 mm I.D. column (Merck) was used in place of the Capcell Pak column, the detection limit of TEMPO was increased 0.2 μM because of the broadening of the peak.

The oxygen consumption during the reaction between TEMP and $^1O_2$ was measured in water and [$^2H_2$]water (Fig. 3). The rate of oxygen consumption by the control system was 17.5 times faster in [$^2H_2$]water than in water. Sodium azide added in [$^2H_2$]water effectively inhibited the oxygen consumption as shown in Fig. 3 (right). These results show that TEMP reacts directly with $^1O_2$ since the lifetime of $^1O_2$ is about 17 times longer in [$^2H_2$]water than in water[9] and azide ion is a physical quencher of $^1O_2$ (ref. 10).

TEMP was treated with photochemically generated $^1O_2$ in the presence of sodi-

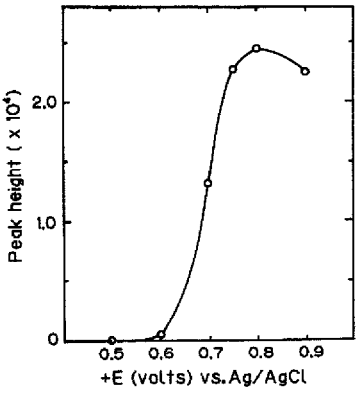

Fig. 1. Voltammogram of TEMPO (10 μM) measured by an electrochemical detector.

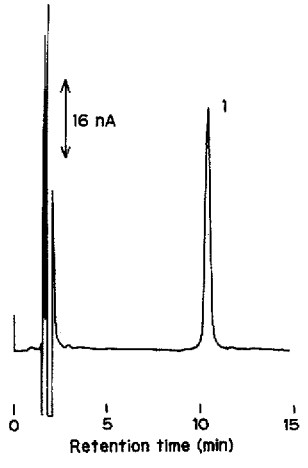

Fig. 2. Chromatogram of authentic TEMPO measured by an electrochemical detector. Peak 1 = 1 μM TEMPO.

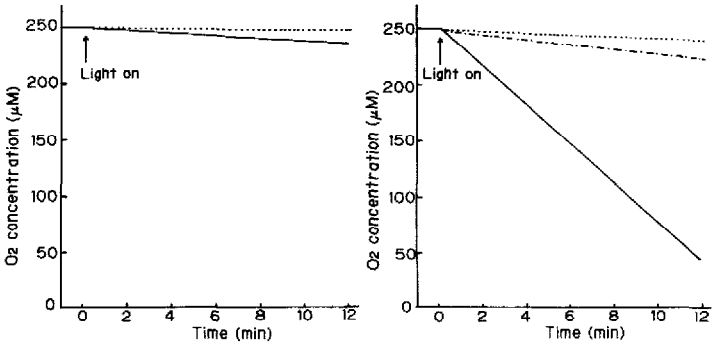

Fig. 3. Oxygen uptakes of TEMP during the TMPyP-photosensitized oxidation. The control solution contained 50 m*M* TEMP and 10 μM TMPyP in 3 ml PBS buffer, pH 7.5 or pD 7.5, made from water (left) or [$^2H_2$]water (right). The oxygen concentration of the air-saturated solution at 25°C was estimated as 250 μM. After thermal equilibration for 5 min, the sample solution was irradiated with a 28-W fluorescent lamp. (—), Control system; (· · ·), minus TMPyP; (– · –) plus 1 m*M* sodium azide.

um azide and mannitol in aerated water and [$^2H_2$]water. The chromatograms of the reaction mixture in [$^2H_2$]water are shown in Fig. 4. The peak near 10.5 min decreased dramatically in the presence of azide ion, a $^1O_2$ quencher, but was unchanged in the presence of mannitol, a specific hydroxyl radical scavenger. The peak was, therefore, due to TEMPO formed from TEMP and $^1O_2$.

Table I shows the amount of TEMPO calculated from the peak area using a calibration graph for authentic TEMPO. The TEMPO yields from both control systems were much lower than the 610 nmol in [$^2H_2$]water and 35 nmol in water expected on the basis of the molecular oxygen consumed, as shown in Fig. 3. The ratio of the TEMPO found in [$^2H_2$]water to that in water was 4.5. After the irradiation, TEMPO, which itself is very stable even upon photoirradiation for 90 min, decreased rapidly

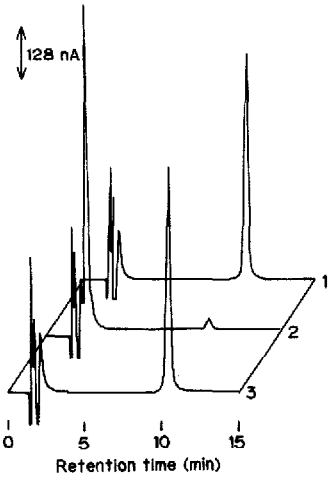

Fig. 4. Effect of sodium azide and mannitol on the TEMPO production during the TMPyP-photosensitized oxidation in PBS made from [$^2H_2$]water. 1 = Control prepared as described in Fig. 3; 2 = plus 1 m*M* sodium azide; 3 = plus 10 m*M* mannitol. Aerated sample solutions were irradiated at 25°C for 12 min with a 28-W fluorescent lamp.

with time. Externally added TEMPO also reduced through the TMPyP-photosensitized oxidation together with TEMP. However, it did not change in the absence of TEMP and was also uninfluenced by TEMP added immediately after cessation of the irradiation. The results suggest that photoinduced TMPyP radicals[11] and their TEMP adducts have little effect on the decrease of TEMPO and that TEMP causes the degradation of TEMPO formed during the photosensitization of TMPyP. Sodium azide surprisingly inhibited the formation of TEMPO in [$^2H_2$]water, but resulted in the lower inhibition in water. This is due to hydrogen peroxide produced by the dismutation reaction

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

of a small amount of superoxide anion ($O_2^{\cdot-}$) generated by electron transfer from excited states of porphyrin to moleculare oxygen, since hydrogen peroxide is used as the oxidizing agent for the synthesis of TEMPO from TEMP[12] and $O_2^{\cdot}$ is unreactive with TEMP[13]. Catalase reduced the formation of TEMPO to 87% in water, suggesting the participation of hydrogen peroxide, but dit not do so in [$^2H_2$]water. Mannitol scarcely inhibited the formation of TEMPO in both solvents. Although TEMP also reacts with hydroxyl radical generated via hydrogen peroxide to give TEMPO[13] and since mannitol is a specific quencher of hydroxyl radical, the results show that little hydroxyl radical is generated under the present experimental conditions. Therefore, the amounts of TEMPO resulting from the reaction of TEMP with $^1O_2$ were 6.07 nmol in water and 31.33 nmol in [$^2H_2$]water.

When using the TEMP spin-trapping to monitor $^1O_2$ and hydroxyl radical yields, one should study the solvent and quencher effects for nitroxide production to verify any active oxygen species involved. Our HPLC–ED system was easily capable of determining TEMPO with a 10-μl sample solution and within 15 min. Moreover,

TABLE I

EFFECTS OF SOLVENTS AND QUENCHERS ON THE TEMPO PRODUCTION DURING THE TMPyP-PHOTOSENSITIZED OXIDATION OF TEMP

Experiments were carried out as described in the legend to Fig. 4. Before irradiation, the amounts of nitroxide radicals present in the water and [$^2H_2$]water systems were 0.08 and 0.11 nmol, respectively. These amounts were subtracted from amounts of nitroxide radical obtained from the systems used. Catalase added was 39.6 units per 3 ml of reaction mixture.

System	*Amount of TEMPO*			
	Water		*[$^2H_2$]water*	
	nmol	*%*	*nmol*	*%*
Control	6.99 ± 0.14	100	31.54 ± 0.84	100
Control–10 μM TMPyP	0.06 ± 0.03	0.1	0.23 ± 0.12	0.7
Control + 1 mM sodium azide	2.27 ± 0.01	32.5	1.36 ± 0.03	4.3
Control + 1.25 nM catalase	6.07 ± 0.43	86.8	31.33 ± 0.10	99.3
Control + 1.25 nM heated catalase[a]	6.69 ± 0.01	100	31.47 ± 0.07	99.8
Control + 10 mM mannitol	6.69 ± 0.24	95.7	31.23 ± 0.13	99.0

[a] Catalase was heated to 95°C for 5 min.

the system can detect TEMPO at 10 n*M* range as sensitively as ESR spectrometry. The present method, which is reproducible and simple, may be very useful in the elucidation of $^1O_2$ and/or hydroxyl radical involvement in chemical and biological systems.

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