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Pure forms of the singlet oxygen sensors TEMP and TEMPD do not inhibit Photosystem II

Éva Hideg [a], Zsuzsanna Deák [a], Marja Hakala-Yatkin [b], Maarit Karonen [c], A. William Rutherford [d], Esa Tyystjärvi [b], Imre Vass [a], Anja Krieger-Liszkay [d,*]

[a] Institute of Plant Biology, Biological Research Center of the Hungarian Academy of Sciences, P.O. Box 521, 6701 Szeged, Hungary
[b] Molecular Plant Biology, Department of Biochemistry and Food Chemistry, University of Turku, FI-20014 Turku, Finland
[c] Department of Chemistry, University of Turku, FI-20014 Turku, Finland
[d] Commissariat à l'Energie Atomique (CEA) Saclay, iBiTec-S, CNRS URA 2096, Service de Bioénergétique, Biologie Structurale et Mécanisme, 91191 Gif-sur-Yvette Cedex, France

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ABSTRACT

In a recent article (Hakala-Yatkin and Tyystjärvi BBA 1807 (2011) 243–250) it was reported that the singlet oxygen spin traps 2,2,6,6-tetramethylpiperidine (TEMP) and 2,2,6,6-tetramethyl-4-piperidone (TEMPD) inhibit Photosystem II (PSII), the water oxidizing enzyme. O_2 evolution, chlorophyll fluorescence and thermoluminescence were measured and were shown to be greatly affected by these chemicals. This work casts doubts over an earlier body of work in which these chemicals were used as spin traps for monitoring $^1O_2$ production when PSII was inhibited by high light intensities. Here we show that these spin probes hardly affect PSII. We show that the commercial batches of TEMPD and TEMP used by Hakala-Yatkin and Tyystjärvi contained impurities and/or derivatives that inhibited PSII and caused the specific effects on fluorescence. Earlier work that used pure spin traps to measure $^1O_2$ during photoinhibition, thus remains valid. However, concern must be expressed towards using these spin traps without proper controls.

1. Introduction

The formation of $^1O_2$ by chlorophyll triplet in Photosystem II (PSII) has been investigated in a number of studies by using either the lipophilic spin probe 2,2,6,6-tetramethylpiperidine (TEMP) or the hydrophilic spin probe 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TEMPD-HCl). Both spin probes are specific for the detection of $^1O_2$ [1,2]. Using TEMP as spin probe it was shown that PSII is the main source of $^1O_2$ during illumination of thylakoid membranes [3]. In other studies the dependence of $^1O_2$ production on the midpoint potential of the primary quinone acceptor, Q_A, was demonstrated using TEMP [4,5] or TEMPD-HCl [6].

In a recent publication by Hakala-Yatkin and Tyystjärvi [7], the validity of these studies was questioned. TEMP and TEMPD were found to inhibit PSII and to affect charge recombination reactions of PSII. Here we show that the inhibition of PSII found in [7] was caused by impurities in the commercial products (from Sigma-Aldrich Co.) and not by the pure compounds.

2. Materials and methods

TEMP (purity 99%), TEMPD (95%) and TEMPD-HCl (98%) were bought from Sigma-Aldrich Co. TEMP was further purified by vacuum distillation [4]. The latter will be referred to as distilled TEMP. TEMPD and TEMPD-HCl were dissolved in distilled water, TEMP and TEMPO were first dissolved in ethanol, then diluted to the indicated concentration with distilled water.

Thylakoids were prepared according to Ref. [3]. Light-saturated electron rate of oxygen evolution was measured using shocked thylakoids from spinach with an oxygen electrode in the presence of 0.5 mM 2,6-dichloro-1,4-benzoquinone (DCBQ) and 10 mM NH_4Cl as uncoupler.

Room temperature chlorophyll fluorescence was measured using a pulse-amplitude modulation fluorometer (Dual-PAM, Walz, Effeltrich, Germany). The fluorescence levels F_0 (minimum fluorescence yield), Fv (variable fluorescence) and F_m (maximum fluorescence yield) were determined and the efficiency of PSII was assayed by calculating the ratio of Fv/Fm.

Flash induced Chl fluorescence was measured as described earlier using isolated spinach thylakoids [8].

Mass spectra were measured with micrOTOF$_Q$ ESI-mass spectrometer (Bruker Daltonics, Bremen, Germany). The mass spectrometer was controlled by Bruker Compass micrOTOF control software and

* Corresponding author at: CEA Saclay, iBiTec-S, Bât. 532, 91191 Gif-sur-Yvette Cedex, France. Tel.: +33 169081803; fax: +33 169088717.
E-mail address: anja.krieger-liszkay@cea.fr (A. Krieger-Liszkay).

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operated in the positive ion mode. Samples were dissolved in methanol:water (1:1, v/v) containing 0.1% acetic acid and infused using the syringe pump. The data were handled by Bruker Compass DataAnalysis (version 4.0).

For EPR spectroscopy 50 μL samples were measured in glass capillaries, using a MiniScope MS 200 (Magnettech GmbH, Berlin, Germany) ESR spectrometer. All spectra were recorded with the same parameters: 10 dB, 0.14 mT, 60 s sweep time, 335.0 mT centre field. Vertical bars in the figures represent equal signal strengths. Where indicated, commercially available TEMP was purified as described by Fufezan et al. [4] and used either promptly or kept at −80 °C under nitrogen gas atmosphere. TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl), a stable nitroxide radical was a kind gift from Prof. Tamás Kálai (University of Pécs, Hungary) and was used as reference spectrum.

2.1. Statistics

The data represent means or representative examples from measurements repeated 3–4 times. In Fig. 1 errors are given as standard deviation.

3. Results

Hakala-Yatkin and Tyystjärvi [7] reported that after 30 min incubation, 10 mM TEMP and 100 mM TEMPD inhibit oxygen evolution by 90% and 75% respectively. In contrast Fig. 1 shows that 100 mM TEMPD-HCl has no effect on O_2 evolution in thylakoids even after 30 min of incubation. Hideg et al. [3] reported that TEMP, synthesized and purified in the laboratory, did not affect PSII activity. After distillation, the TEMP batch used by Hakala-Yatkin and Tyystjärvi no longer inhibited oxygen evolution after 30 min incubation (data not shown). In [3] emphasis was put on avoiding long incubations with the spin trap.

Hakala-Yatkin and Tyystjärvi [7] also reported that TEMP and TEMPD decreased Fv/Fm and modified fluorescence decay kinetics and thermoluminescence curves in the presence and absence of DCMU. As shown in Fig. 2, distilled TEMP did not cause the same effect and the effect was greatly diminished when TEMPD-HCl was used. We did find that when thylakoids were incubated with TEMPD-HCl for 30 min, both F_0 and F_M were lowered by 40% and 45%, respectively, but the Fv/Fm value fell only to 86% of value measured in the absence of TEMPD-HCl. This effect of TEMPD-HCl on chlorophyll fluorescence is much smaller than the effect caused by the 95% TEMPD preparation used by Hakala-Yatkin and Tyystjärvi and is not associated with inhibition of oxygen evolution (Fig. 1) and thus seems irrelevant for the use of TEMPD-HCl to monitor $^1O_2$ formation.

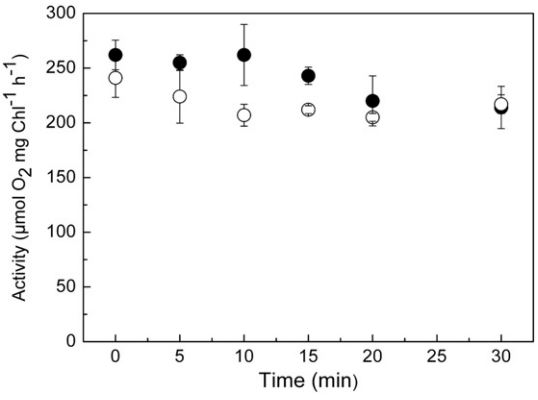

Fig. 1. Effect of 100 mM TEMPD on oxygen evolution during incubation of thylakoid membranes from spinach (closed symbols). Open symbol measurements were performed in the absence of TEMPD. Oxygen evolution was measured using 0.5 mM DCBQ as electron acceptor and 10 mM NH_4Cl as uncoupler. Each data point represents an average of three independent measurements, SD is given. The maximum rate of oxygen evolution was 250 ± 19 μmol O_2 mg Chl^{-1} h^{-1}.

The present data on TEMPD-HCl and TEMP contradict the observation of Hakala-Yatkin and Tyystjärvi [7] that TEMP and TEMPD severely inhibit PSII. A clear difference between the current work and that of Hakala-Yatkin and Tyystjärvi is that they, following recently published experimental procedures, used the commercial spin probes (99% pure TEMP and 95% pure TEMPD, both from Sigma-Aldrich Co.) without further purification. Some earlier work has specified the use of distilled TEMP (Fufezan et al. 2002); the details of the distillation procedure were given by Fufezan et al. [4]. In more recent studies [6,9–11], purification of commercially available TEMP from Sigma-Aldrich Co. was no longer specified although distilled TEMP was always used. The background EPR signal remaining after distillation was considered small, and therefore we believe that these batches of TEMP did not have serious side effects. With regard to TEMPD, Hakala-Yatkin and Tyystjärvi [7] used the TEMPD from Sigma-Aldrich Co. while Fischer et al. (2007) used TEMPD hydrochloride (98%). The use of the hydrochloride form was not specified in the publication by Fischer et al. (2007), because the non-chlorinated TEMPD is the chemical in solution.

We measured EPR spectra of the non-chlorinated form of TEMPD and the non-distilled TEMP from the specific batches that had been used in the experiments of Hakala-Yatkin and Tyystjärvi [7]. In both cases these showed a large background signal (Fig. 3) which was absent in purified TEMP and in TEMPD-HCl. In addition, this batch of TEMPD failed to react with $^1O_2$ (Fig. 4), possibly due to impurities.

In order to identify the impurity that causes specific effects on PSII in the commercial preparations of TEMP and TEMPD, we measured masses from the TEMP and TEMPD preparations. The mass spectrum of commercial 99% TEMP exhibited an intense $[M+H]^+$ ion at *m/z* 142.16 in addition to minor ions and a fragment ion at *m/z* 125.13. Distillation of TEMP produced an almost colourless TEMP fraction compared to the undistilled, yellowish compound. However, no clear difference was observed in the mass spectra of these two TEMP preparations. One explanation for the different biological effects of undistilled TEMP may be an ion at *m/z* 319 which was observed with higher intensity in the mass spectrum of TEMP in comparison to that of distilled TEMP. The mass spectrum of TEMPD (purity 95%) contained several ions. The main ions were at *m/z* 98.10 and 156.14 ($[M+H]^+$). The other ions observed were at *m/z* 170.16, 188.17, 254.10, 288.24 and 328.27. Less ions were observed in the mass spectrum of TEMPD-HCl (purity 98%): the molecular ion $[M+H]^+$ was detected at *m/z* 156.14 in additions to the ions at *m/z* 98.10, 170.16 and 188.17.

4. Discussion

We show here that the results of Hakala-Yatkin and Tyystjärvi [7] were compromised by impurities in the commercial TEMP and TEMPD. These impurities found in 99% TEMP and in non-chlorinated 95% TEMPD have an inhibitory effect on PSII activity and cause specific changes in fluorescence and thermoluminescence. Mass spectrometric analyses of TEMP, TEMP after distillation, TEMPD and TEMPD-HCl (all chemicals from Sigma Aldrich) showed a variation in constituents in all four preparations, which makes it difficult to identify the compound that affects PSII. We are working to identify the compounds and to test the hypothesis that the species that inhibits PSII is EPR active.

Experiments aimed at estimating $^1O_2$ concentrations should only be performed with purified TEMP or the hydrochloric salt of TEMPD. Furthermore, each batch of TEMP or TEMPD should be checked for effects on oxygen evolution activity and charge recombination