Detection of Hydrogen Atom Adduct of Spin-Trap DEPMPO. The Relevance for Studies of Biological Systems

Miloš Mojović,[†] Ivan Spasojević,[‡,†] and Goran Bačić*,[†]

Faculty of Physical Chemistry, University of Belgrade, Studentski trg 12-16, 11000 Belgrade, and Center for Multidisciplinary Studies, University of Belgrade, Kneza Višeslava 1a, 11000 Belgrade

Received April 30, 2005

We proposed EPR spectroscopy using spin-trap DEPMPO as a novel method for the detection of a hydrogen atom (•H) produced by chemical and biological systems. In complex EPR spectra of DEPMPO adducts in biological systems, spectral lines of unknown origin have been observed. We have assumed (Bačić, G.; Mojović, M. *Ann. N. Y. Acad. Sci.* **2005**, *1048*, 230−243) that those lines represent the spectrum of a hydrogen atom (•H) adduct i.e., DEPMPO/H. An electrochemical system known to produce only •H radicals was used here in order to obtain a separate spectrum of the DEPMPO/H adduct. An acquired spectrum as well as a computer spectral simulation of the DEPMPO/H adduct showed considerable resemblance with additional lines in the EPR spectra of DEPMPO adducts in biological systems—plant plasma membranes and cell walls. This shows that such a radical is produced by plants as well as that DEPMPO is suitable for detection in both electrochemical and biological systems.

INTRODUCTION

EPR spin-trap spectroscopy is a frequently used technique for detection and quantification of different free radical species in both chemical and biological systems. The suitability of a certain spin-trap is defined through its ability to selectively trap a free radical of interest or that different trapped radicals can be easily distinguished by EPR spectra of their adducts. Numerous spin-traps are currently available for the detection of different types of free radicals. Among them, a DEPMPO spin-trap (a phosphorylated analogue of a previously widely exploited spin-trap DMPO)[1] attracted a lot of attention because of its ability to differentiate between various trapped radicals (Figure 1) due to additional ^{31}P hyperfine coupling. The EPR spectra of DEPMPO adducts are consequently rather complicated with the possible existence of diastereomers,[2] and spectral simulations of such adducts are difficult to perform especially in biological systems where many different radical types could be generated.[3]

Recently it has been shown that DEPMPO is particularly suitable for simultaneous spin-trapping of different oxygen radicals, i.e., •OH and $•O_2^-$, which are produced in the same biological system such as plant plasma membranes of maize roots and cell walls of pea.[3−5]

However, the EPR spectra of DEPMPO/OH and DEPMPO/OOH adducts obtained in those systems revealed an additional adduct characterized by extra spectral lines among which the most characteristic features were "wings" on both high and low field ends of the EPR spectra.[3] Such additional lines also have been recorded in some previous studies but were overlooked or dismissed as artifacts. We have assumed that they originate from an adduct of a hydrogen atom (•H)[3] i.e., DEPMPO/H, whose existence has been speculated earlier.[6] This study is aimed to clarify the production of such a radical by plant systems.

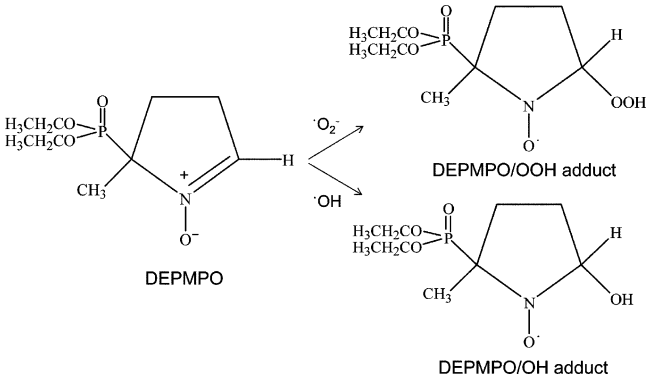

Figure 1. Structural formula of a spin-trap DEPMPO and some of its adducts.

A hydrogen atom (•H) is known to be generated biologically; however, few attempts have been made to develop practical methods for its detection. Although using EPR and DEPMPO seems to be a suitable method for the qualitative detection of a number of radicals produced by biological systems (•OH, $•O_2^-$, $•CO_2^-$, $•SO_3^-$ etc.),[1,7,8] the analysis of EPR spectra of DEPMPO adducts and their unambiguous assignment is rather complicated. It requires either elaborate biochemical manipulation of a biological system or complicated spectral simulations. An additional approach is an investigation of less complex systems, and therefore we primarily examined the production of radicals in a "pure •OH radical producing Fentone reaction". Besides an expected signal of the DEPMPO/OH adduct an additional signal which might correspond to the DEPMPO/H adduct has been observed.[4] However, the mechanism of production of free radicals in this system is still not fully understood, and some additional investigations have to be performed (work in progress). Hence, in the present study we used an electrochemical system which is known to produce only •H radicals,[9] so that a separate spectrum of the DEPMPO/H adduct could be obtained. That spectrum and computer spectral simulation of the DEPMPO/H adduct were compared

* Corresponding author phone: +38111630796; fax: +38111187133; e-mail: ggbacic@ffh.bg.ac.yu.
† Faculty of Physical Chemistry.
‡ Center for Multidisciplinary Studies.

10.1021/ci050173d CCC: $30.25
Published on Web 10/08/2005

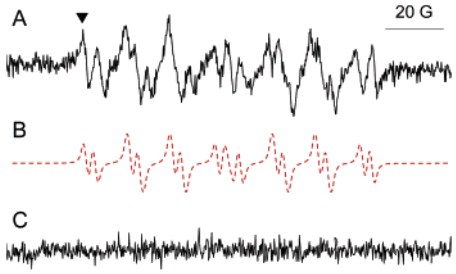

Figure 2. (A) EPR spectrum of DEPMPO/H adduct obtained in electrochemical system after 30 min (97% of CH_3CN and 3% of H_2O). (B) Computer spectral simulation of DEPMPO/H adduct: $a^P = 47.5$; $a^N = 14.0$; $a^H_\gamma(3H) = 0.27$; $a^H_\beta(1H) = 15.0$; $a^H_\beta(1H) = 3.0$; (▼) marks one of the characteristic EPR peaks of the DEPMPO/H adduct, later designated as the one of the "wings" in the EPR spectra of radicals in biological systems. (C) EPR spectrum obtained in the control electrochemical system in the absence of H_2O (100% of CH_3CN).

to additional signals in the EPR spectra of DEPMPO adducts obtained in biological systems.

MATERIALS AND METHODS

Chemicals. Spin-trap DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide) was purchased from Alexis Biochemical (Lausen, Switzerland). Spectroscopy grade CH_3CN was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO) and purified by double distillation over P_2O_5. Bidistilled 18MΩ deionized water was used.

Sample Preparation. Preparation of samples (except biological ones) and electrochemical experiments were performed in the nitrogen atmosphere, to eliminate the presence of atmospheric water. Samples for electrochemical experiments were composed of 97% CH_3CN and 3% H_2O in order to slow reverse reaction. Control experiments were performed on a system composed only of CH_3CN and DEPMPO. Undiluted DEPMPO was added to all samples before electrolysis at a final concentration of 100 mM. Plasma membranes were isolated from the maize root (inbred line VA35 of maize *Zea mays L.*), and the cell wall isolates were obtained from roots of pea (*Pisum sativum L.*) as described earlier.[4,5] DEPMPO (100 mM) was added to biological samples immediately prior to EPR measurements.

Electrochemical System. The electrolysis was performed in an electrochemical cell in which working and counter electrodes were made from thin golden plates, placed parallel to each other. Conditions for electrolysis were as follows: applied potential, −1.4 V; time of electrolysis, 30 s.

EPR Measurements. The EPR spectra were recorded using a Varian E104-A EPR spectrometer operating at X-band (~9.5 GHz) adjusted to the following settings: modulation amplitude − 2 G; modulation frequency − 100 kHz; microwave power − 10 mW; scan range − 200 G. Spectral simulations were performed using the computer program WINEPR SimFonia (Bruker Analytische Messtechnik GmbH).

RESULTS AND DISCUSSION

The EPR spectrum of DEPMPO adducts obtained in the $^{\bullet}H$ producing electrochemical system is presented in Figure 2A. The spectrum shows remarkable resemblance to the spectral simulation of the DEPMPO/H adduct (Figure 2B).

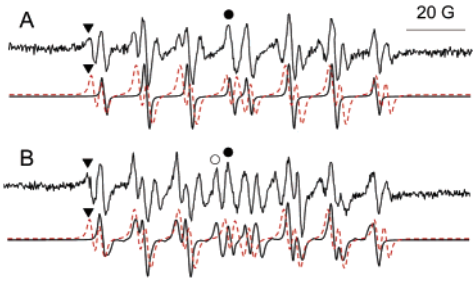

Figure 3. (A) EPR experimental spectrum of DEPMPO adducts obtained in cell walls of a pea roots sample (upper spectrum) and its computer simulation (lower spectrum, black solid line). Simulation was performed using the contribution of the DEPMPO/OH adduct ($a^P = 46.7$; $a^N = 13.9$; $a^H(1H) = 13.5$)[10] whose one of the characteristic signals in experimental spectrum is marked with "●". (B) EPR experimental spectrum of the DEPMPO adducts of a maize roots plasma membranes sample (upper spectrum) and its computer simulation (lower spectrum, black solid line). Spectral simulation is a combination of the contribution of the DEPMPO/OOH adduct (I isomer 55%: $a^P = 50.15$; $a^N = 13.0$; $a^H_\beta(1H) = 11.3$; $a^H_\gamma(1H) = 0.85$; $a^H_\gamma(1H) = 0.35$; $a^H_\gamma(3H) = 0.53$; II isomer 37%: $a^P = 48.68$; $a^N = 13.08$; $a^H_\beta(1H) = 0.88$; $a^H(1H) = 10.2$; $a^H_\gamma(1H) = 0.41$; $a^H_\gamma(1H) = 0.34$; III isomer 8.5%: $a^P = 40.8$; $a^N = 13.3$; $a^H(1H) = 10$; $a^H_\beta(1H) = 1.5$)[11] whose one of the characteristic signals in experimental spectrum is marked with "○", and the DEPMPO/OH adduct ($a^P = 46.7$; $a^N = 13.9$; $a^H(1H) = 13.5$) whose one of the characteristic signals in the experimental spectrum is marked with "●"; using ratio 1:4. Computer simulation of the DEPMPO/H adduct is the same as in Figure 2B (red, dotted line in both spectra) and shows supplemental spectral lines which exist in both experimental spectra of biological samples.

Attained hyperfine coupling constants a^P, a^N, and a^H_γ have values typical for DEPMPO adducts,[1,10,11] and an additional two a^H_β constants have different values than previously assumed for the DEPMPO adduct of the same type.[6] This is not surprising since those values have been obtained by complex manipulation of the EPR spectra of DEPMPO adducts obtained in a biological system.

The spectrum in Figure 2C shows that in the water-free control system composed only of CH_3CN and DEPMPO no free radicals could be trapped with DEPMPO. This is in agreement with the previous findings[9] that no free radicals can be generated in this system. Hence, the spectrum presented in Figure 2A can only originate from the DEPMPO/H adduct and not from DEPMPO adducts of some potential products of electrolysis of DEPMPO or CH_3CN.

Comparison of parts A and B of Figure 2 shows some additional lines, but their contribution to the overall spectral intensity is minor (roughly 10%). Unfortunately, due to the low *S*/*N* ratio of the experimental spectrum, it is virtually impossible to obtain a meaningful difference plot between experimental and simulation spectra to identify this additional adduct. Additional lines could be assigned to the presence of the DEPMPO/OH adduct or most likely to the presence of possible diastereomers, but that should not significantly influence the identification of the DEPMPO/H adduct generated in biological systems.

Figure 3 shows the EPR spectra of DEPMPO adducts obtained in biological systems: solution of cell walls isolated from pea roots (Figure 3A − upper spectrum) and purified plasma membranes isolated from maize roots (Figure 3B − upper spectrum). The cell walls isolated from pea roots are supposed to predominantly produce $^{\bullet}OH$ radicals,[5] and consequently the DEPMPO/OH adduct should be the domi-

nant spectral feature; however, the upper spectrum in Figure 3A shows some additional spectral features. Computer simulation shows that by overlaying the spectrum of the DEPMPO/H adduct over the spectrum of the DEPMPO/OH adduct (Figure 3A) one can easily explain the origin of additional lines (most notably "wings" in the cell wall spectrum).

In a more complex system containing the plant plasma membranes of maize roots which are known to produce $^{\bullet}OH$ and $^{\bullet}O_2^-$ radicals,[4] most of the prominent EPR lines in the spectrum of DEPMPO adducts could be reproduced by a computer spectral simulation which combines these two adducts (DEPMPO/OH and DEPMPO/OOH) as shown in Figure 3B. However, some additional spectral lines also occur on both sides of the spectra (one "wing" is marked with the triangle) as well as some inner lines which partially overlap with the principal lines of DEPMPO/OH and DEPMPO/OOH adducts. These could not be simulated even when all possible diastereomers of DEPMPO adducts were taken into consideration. However, when the spectrum of the DEPMPO/H adduct is taken into account, considerable overlapping occurs, especially when the wing area is concerned. It is also obvious that not all details in the spectrum in Figure 3B can be explained by simply including the spectrum of the DEPMPO/H adduct, but at least some notable spectral features clearly point out the production of $^{\bullet}H$ radicals. A detailed analysis of all spectral features would require the extensive use of chemical agents which can selectively inhibit the production of a certain radical (see refs 3 and 11 for details), but that is beyond the scope of this publication. Regardless, being able to distinguish the DEPMPO/H adduct from DEPMPO/OH and DEPMPO/OOH adducts is an important argument for the suitability of DEPMPO for the successful detection of $^{\bullet}H$ radicals even in such complicated biological free radical generating systems.

The importance of the fact that DEPMPO is able to distinguish $^{\bullet}H$ from other radicals can be briefly summarized as follows. It is beyond a doubt that the $^{\bullet}H$ radical is produced by biological systems, but the reliability of its detection is questionable. Although previous studies have demonstrated that some EPR spin-traps such as DMPO can unequivocally detect $^{\bullet}H$ radicals[12] in simple chemical radical generating systems under favorable conditions, such procedures cannot be used in complex biological systems and the hyperfine splitting and/or relative intensities of EPR lines in the spectra of various spin adducts are usually not sufficient to enable reliable identification of different radical species. The situation is even worse when plant systems are concerned. Previous EPR spin-trapping studies on plant systems[13−15] showed no evidence of the production of $^{\bullet}H$ radicals, but the simple explanation is that spin-traps used in those studies were not capable of detecting it. To the best of our knowledge, our study is the first to show the production of $^{\bullet}H$ radicals in plants, and this is due to the fact that we were able to compare the EPR spectra of adducts of the same spin-trap in simple electrochemical and complex biological systems.

CONCLUSIONS

In the present study we showed that using EPR spectroscopy and an appropriate spin-trap (DEPMPO) enables detection of the $^{\bullet}H$ radicals produced by plants. Although it seems that DEPMPO is currently the best spin-trap for detection of various free radical species, some other recently developed spin-traps (e.g. EMPO) can be efficient as well, but it would be advantageous to assess their performance by testing them in both, chemical and biological systems. Until then, EPR spectroscopy using spin-trap DEPMPO presents probably the most efficient method for detection of $^{\bullet}H$ radicals in biological systems.

ACKNOWLEDGMENT

We are thankful to Professor Željko Vučinić, Center for Multidisciplinary Studies, University of Belgrade and to Mirjana Vuletić, Maize Research Institute "Zemun Polje", Belgrade, for support and assistance. This work was supported by grant 1928 from the Ministry of Science and Environmental Protection of Republic of Serbia.

REFERENCES AND NOTES

(1) Frejaville, C.; Karoui, H. 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide: A new efficient phosphorylated nitrone for the in vivo spin trapping of oxygen-centered radicals. *J. Med. Chem*. **1995**, *38*, 258−265.

(2) Dikalov, S.; Landmesser, U.; Harrison, D. G. Geldanamycin leads to superoxide, formation by enzymatic and nonenzymatic redox cycling. *J. Biol. Chem*. **2002**, *277*, 25480−25485.

(3) Bačić, G.; Mojović, M. EPR spin trapping of oxygen radicals in plants: A methodological overview. *Ann. N. Y. Acad. Sci.* **2005**, *1048*, 230−243.

(4) Mojović, M.; Vuletić, M.; Bačić, G.; Vučinić, Ž. Oxygen radicals produced by plant plasma membranes: An EPR spin-trap study. *J. Exp. Bot.* **2004**, *55*, 2523−2531.

(5) Veljović-Jovanović, S.; Kukavica, B.; Cvetić, T.; Mojović, M.; Vučinić, Ž. Ascorbic acid and the oxidative processes in pea root cell wall isolates: Characterization by fluorescence and EPR spectroscopy. *Ann. N. Y. Acad. Sci.* **2005**, *1048*, 500−504.

(6) Chamulutrat, W. Activation of the superoxide-generating NADPH oxidase of intestinal lymphocytes produces highly reactive free radicals from sulfite. *Free. Rad. Biol. Med.* **1999**, *27*, 411−421.

(7) Karoui, H.; Hogg, N.; Frejaville, C.; Tordo, P.; Kalyanaraman, B. Characterization of sulfur-centered radical intermediates formed during the oxidation of thiols and sulfite by peroxynitrite. *J. Biol. Chem.* **1996**, *271*, 6000−6009.

(8) Liu, K. J.; Miyake, M.; Panz, T.; Swartz, H. Evaluation of DEPMPO as a spin trapping agent in biological systems. *Free Radical Biol. Med.* **1999**, *26*, 714−721.

(9) Endo, N.; Higashi, K.; Kanaori, K.; Tajima, K.; Makino, K. Electrochemical-ESR detection of hydrogen atom adducts of five-membered ring nitrone spin traps. *Bull. Chem. Soc. Jpn.* **2002**, *75*, 149−150.

(10) Vásquez-Vivar, J.; Kalyanaraman, B.; Martásek, P.; Hogg, N.; Masters, B. S. S.; Karoui, H.; Tordo, P.; Pritchard, K. A., Jr. Superoxide generation by endothelial nitric oxide synthase: The influence of cofactors. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9220−9225.

(11) Vásquez-Vivar, J.; Hogg, N.; Martásek, P.; Karoui, H.; Tordo, P.; Pritchard, K. A., Jr.; Kalyanaraman, B. Effect of redox-active drugs on superoxide generation from nitric oxide synthases: Biological and toxicological implications. *Free Radical Res.* **1999**, *31*, 607−617.

(12) Makino, K.; Mossoba, M. M.; Reisz, P. Chemical effects of ultrasound on aqueous solutions. Formation of hydroxyl radicals and hydrogen atoms. *J. Phys. Chem.* **1983**, *87*, 1369−1377.

(13) Kuchitsu, K.; Kosaka, H.; Shiga, T.; Shibuya, N. EPR evidence for generation of hydroxyl radical triggered by *N*-acetylchitooligosaccharide elicitor and a protein phosphatase inhibitor in suspension-cultured rice cells. *Protoplasma* **1995**, *188*, 138−142.

(14) Liszkay, A.; Van Der Zalm, E.; Schopfer, P. Production of reactive oxygen intermediates ($O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$) by maize roots and their role in wall loosening and elongation growth. *Plant Physiol.* **2004**, *136*, 3114−3123.

(15) Qiu, Q.-S.; Cheng, P.; Liang, H.-G. Characterization of the NAD(P)H oxidation by purified plasma membrane vesicles using a spin-trapping EPR method. *J. Plant Physiol.* **1995**, *146*, 445−449.

CI050173D