BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **230,** 54–57 (1997)
ARTICLE NO. RC965880

Quantification of Peroxynitrite, Superoxide, and Peroxyl Radicals by a New Spin Trap Hydroxylamine 1-Hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine

S. Dikalov,*[,1] M. Skatchkov, and E. Bassenge
**Institute of Chemical Kinetics & Combustion, Novosibirsk, Russia; and Institute of Applied Physiology, University of Freiburg, 7800 Freiburg, Germany*

Received November 15, 1996

The reactions of hydroxylamine 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H) with peroxynitrite, superoxide and peroxyl radicals were studied. It was shown that under these reactions TEMPONE-H is oxidized into a stable nitroxide 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidinoxyl (TEMPONE). The reactivity of TEMPONE-H towards reactive oxygen species was compared with the spin traps DMPO and TMIO as well as with DMSO and SOD. The rate constants of reactions of TEMPONE-H with peroxynitrite and superoxide radicals were 6×10^9 $M^{-1}s^{-1}$ and 1.2×10^4 $M^{-1}s^{-1}$, respectively. Using TEMPONE-H the sensitivity in the detection of peroxynitrite or superoxide radical was about 10-fold higher than using the spin traps DMPO or TMIO. Thus, TEMPONE-H may be used as a spin trap in chemical and biological systems to quantify peroxynitrite and superoxide radical formation.

It is well known that reactive oxygen species, particularly peroxynitrite, superoxide and peroxyl radicals, play an important role in the development of oxidative damage (1, 2). When studying the development of pathological conditions mediated by oxidative damage it is very important to quantify the formation of reactive oxygen species. Spin trapping techniques are one of the most direct methods for detection of oxygen radicals (3). However, in most cases only a relative comparison of ROS formation is possible. The determination of the absolute amount of ROS formation in biological systems by the spin trapping technique is still very difficult (4).

Previously it was supposed that hydroxylamines could be an effective spin trap for superoxide radical (4, 5). It was shown that hydroxylamines are oxidized by superoxide into corresponding stable nitroxide radicals (4). The amount of formed nitroxide radicals measured by ESR can be used to quantify superoxide radical production.

We supposed that hydroxylamines could be used to quantify the formation of peroxynitrite, as well as superoxide and peroxyl radicals. In this study we analized the reactions of hydroxylamine 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydro-chloride with peroxynitrite, superoxide and peroxyl radicals. The rate constants for the reactions of TEMPONE-H with peroxynitrite and superoxide radical were determined.

MATERIALS AND METHODS

The spin traps 3,5,5-trimethyl-imidazole-1-oxide (TMIO) and 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H) were kindly supplied by I. A. Kirilyuk (Institute of Organic Chemistry, Novosibirsk, Russia). TMIO and TEMPONE-H were synthesized as described in (6, 7). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO), dimethylsulfoxide (DMSO), DTPA, SOD, catalase and xanthine were obtained from Sigma (Germany). Peroxynitrite was from Alexis Corporation (USA).

Spin trapping experiments. All ESR samples were placed in 100 μl glass tubes and were prepared using 0.3M sodium phosphate buffer (PBS), pH=7.4. In order to inhibit reactions catalyzed by transition metals DTPA (2 mM) was added to all samples. The absence of paramagnetic impurities in the solutions of spin traps was checked by ESR spectroscopy. The ESR measurements were performed at room temperature using an EMX-A ESR spectrometer (Bruker). The ESR-settings were the following: field sweeped from 3444 G up to 3504 G, microwave frequency 9.72 GHz, microwave power 20 mW, modulation amplitude 2 G, conversion time 655 ms, detector time constant 5245 ms, magnetic field sweep time 671 s. Interpretations of ESR spectra were done according to hyperfine ESR splitting constants reported in (3, 10).

Superoxide radical generation. Xanthine oxidase superoxide generating system (8) contained xanthine oxidase (0.004 units activity), xanthine (100μM), DTPA (1mM) in 0.3M PBS, pH=7.4. The rate of superoxide radical generation was determined by cytochrome C assay (12).

[1] To whom correspondence should be addressed. Fax: 007-3832-352350; E-mail: Dikalov@kinetics.nsk.su.

0006-291X/97 $25.00

Spin trapping of peroxynitrite. As a source of peroxynitrite the stock solution of ONOONa (1mM stabilized in 0.3M NaOH) or 3-(4-morpholino)-sydnonimine (SIN-1) (10mM in 0.3M PBS, pH=7.4) (9) was used. Concentration of peroxynitrite established in 0.3M NaOH was determined spectrophotometrically using extinction $\epsilon(302nm)= 1670\ M^{-1}\,cm^{-1}$ (Alexis Corporation data sheet). Generation of peroxynitrite in SIN-1 solution was detected using 0.1 M DMPO (9) or 0.1 M TMIO (10) monitoring the formation of spin adducts DMPO-OH or TMIO-OH which was inhibited by addition of DMSO (5%) or SOD (3000 U/ml).

Peroxyl radical generation. As a source of peroxyl radical a 10mM solution of 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) in 0.3M PBS, pH=7.4, 37°C was used (11).

Determination of the rates of ROS generation. Using hydroxylamine TEMPONE-H as a spin trap the absolute rates of superoxide radical and peroxynitrite formation in xanthine oxidase or SIN-1 systems were determined. The calibration curve for scavenging of peroxynitrite by TEMPONE-H was obtained by quantification of TEMPONE formation after quick addition of TEMPONE-H solution (in 0.3M PBS) to small aliquots of peroxynitrite (in 0.3M NaOH). Final pH of mixtures was controlled.

Determination of rate constant by competitive kinetics. The rate constant for reaction of TEMPONE-H with superoxide radical was determined by competitive kinetics as described in using SOD as competitive reagent (4). The rate constant for the reaction of TEMPONE-H with peroxynitrite was determined using DMSO as competitive reagent for peroxynitrite. The rate constant was calculated from the dependence of TEMPONE-H oxidation by peroxynitrite on the concentration of DMSO using the competitive kinetics (4).

RESULTS AND DISCUSSION

Reaction of TEMPONE-H with Peroxynitrite

In the presence of peroxynitrite (10μM) or SIN-1 (1mM) using the spin traps DMPO or TMIO ESR signals corresponding to the spin adducts DMPO-OH or TMIO-OH were observed (Fig. 1a, b). After addition of peroxynitrite to TEMPONE-H an ESR signal of corresponding nitroxide TEMPONE was observed. The addition of SIN-1 to the solution of TEMPONE-H led to the continuous formation of the corresponding nitroxide TEMPONE (Fig. 1c). The signal/noise ratio of ESR signals obtained with TEMPONE-H was at least 10-fold better than in signals obtained with DMPO or TMIO (Fig. 1a, b, c).

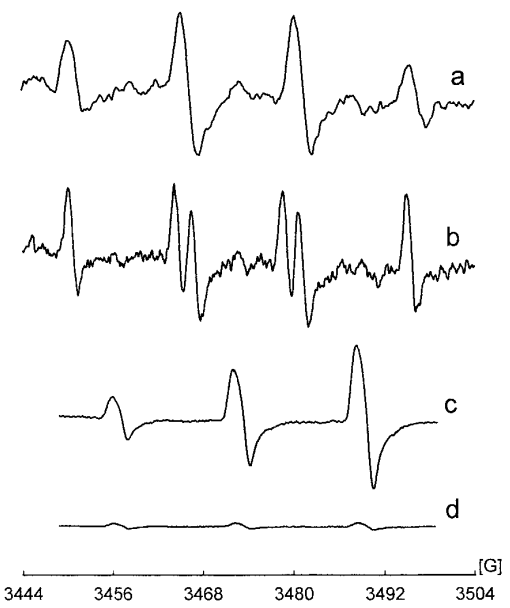

FIG. 1. ESR spectra of samples with 10mM SIN-1 containing 0.1 M DMPO (a); 0.1 M TMIO (b); 1 mM TEMPONE-H (c); 3000 units/ml SOD + 1 mM TEMPONE-H (d). ESR signal of TEMPONE was growing during scanning. ESR receiver gain was 5×10^5 (spectra (a) and (b)) or 5×10^4 (spectra (c) and (d)). Modulation amplitude was 1 G for spectrum (b), 2 G for spectra (a), (c), (d).

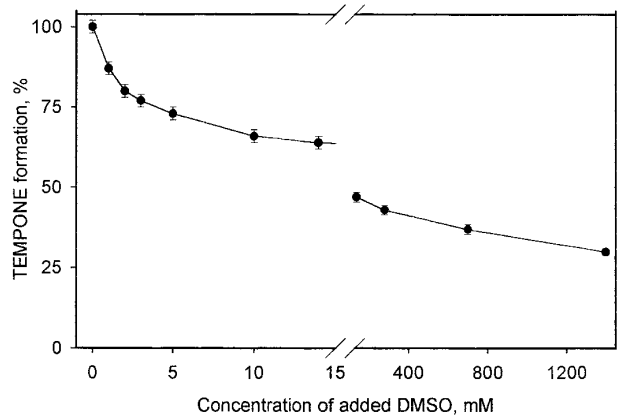

FIG. 2. Effect of DMSO on the TEMPONE formation during the reaction of TEMPONE-H and peroxynitrite. Two ranges of DMSO concentrations are associated with a characteristic inhibition of TEMPONE formation.

We used DMSO as a competitive inhibitor of TEMPONE-H oxidation by peroxynitrite in order to determine the rate constant for the reaction of TEMPONE-H with peroxynitrite. It was found that the inhibition of peroxynitrite induced oxidation of TEMPONE-H takes place within two different ranges of DMSO concentrations (fig. 2). An initial inhibition was observed in the range of 1÷15mM DMSO whereas a final inhibition occurred at a concentration of DMSO within the range of 300÷1500mM.

Peroxynitrite can oxidize organic compounds according to one or two electron reactions (2). The fact that DMSO inhibited oxidation of TEMPONE-H within two ranges of DMSO concentration points out that peroxynitrite oxidizes TEMPONE-H in two reactions. During the first reaction TEMPONE-H reduces peroxynitrite to NO_2 (reaction 1). During the second reaction TEMPONE-H reduces NO_2 to nitrite (reaction 2). Indeed, we found that during the reaction of TEMPONE-H with pure NO_2 nitroxide TEMPONE was formed (data not shown). Therefore one molecule of peroxynitrite finally forms two molecules of the stable nitroxide radical TEMPONE.

1. TEMPONE-H + ONOOH → TEMPONE + $^{\cdot}NO_2$ + H_2O
2. TEMPONE-H + $^{\cdot}NO_2$ → TEMPONE + HNO_2

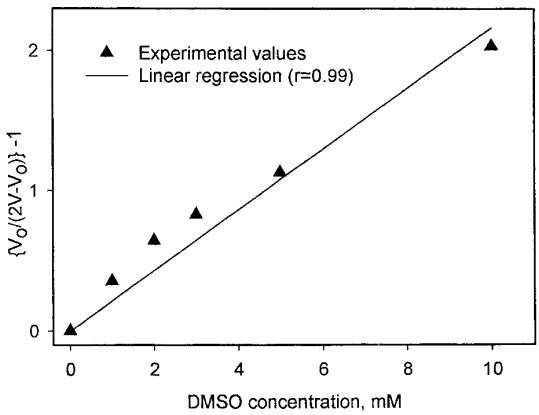

FIG. 3. Dependence of the rate of TEMPONE formation on DMSO concentration. V_o and V are the rates of TEMPONE formation during the reaction of TEMPONE-H with peroxynitrite in the absence and in the presence of DMSO, respectively.

Taking into account that the rate of reaction 1 is one half of the experimentally observed rate of TEMPONE formation (reaction 1 + reaction 2), we calculated the rate constant for the reaction of TEMPONE-H with peroxynitrite (when [DMSO]<15 mM) using the following equation: $\{V_o/(2V-V_o)\}-1= k_1^{\cdot}[\text{TEMPONE-H}]/k_{DMSO}^{\cdot}[\text{DMSO}]$, where V_o and V represent the rates of TEMPONE formation in the absence and presence of DMSO, respectively. The second order rate constants k_1 and k_{DMSO} are for the reaction of peroxynitrite with TEMPONE-H and DMSO, respectively.

It was found that the ratio of the rate constants k_{DMSO}/k_1 is 0,216 (Fig. 3), where $k_{DMSO} = 1{,}3\times10^9$ $M^{-1}s^{-1}$ (13). Therefore, we obtained $k_1=6\times10^9$ $M^{-1}s^{-1}$.

From the comparison of the amount of added peroxynitrite with the formation of nitroxide radical TEMPONE it was found that 6%, 8.7%, 14.5% of peroxynitrite was trapped by 0.1mM, 1mM, 10mM solutions of TEMPONE-H, respectively.

Reaction of TEMPONE-H with Superoxide Radicals

In the xanthine+xanthine oxidase superoxide radical generating system with the spin trap a DMPO characteristic ESR spectrum of DMPO-OOH spin adduct was observed (Fig. 4a). In the presence of the spin trap TMIO no spin adducts were detected (Fig. 4b) because TMIO does not react with the superoxide radicals (10). In the presence of TEMPONE-H the nitroxide radical TEMPONE was continuously formed (Fig. 4c). Formation of TEMPONE was inhibited by addition of SOD (Fig. 4d). The rate of TEMPONE formation was close to the rate of cytochrome C reduction. Therefore, the scavenging of superoxide radicals by TEMPONE-H was quantitative.

It was reported previously that superoxide radicals oxidize hydroxylamines to the corresponding nitroxide radicals (reaction 3) (4).

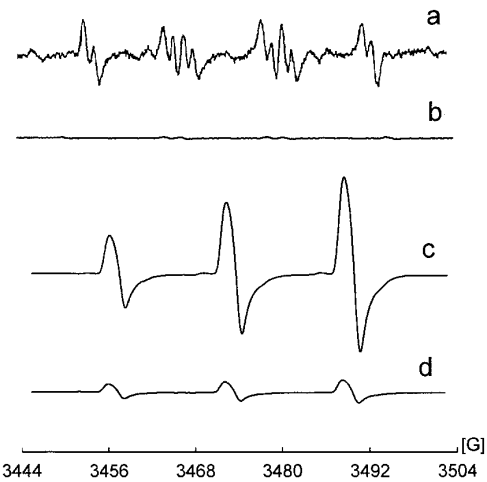

FIG. 4. ESR spectra of samples with a xanthine+xanthine oxidase superoxide generating system containing 0.2 M DMPO (a); 0.2 M TMIO (b); 2 mM TEMPONE-H (c); 100 units/ml SOD + 2 mM TEMPONE-H (d). Modulation amplitude was 0.5 G for spectrum (a), 1 G for spectrum (b), 2.0 G for spectra (c) and (d). ESR receiver gain was 5×10^5.

3. $\text{TEMPONE-H} + HO_2^{\cdot} \rightarrow \text{TEMPONE} + H_2O_2$

We used SOD as a competitive inhibitor of TEMPONE-H oxidation by superoxide radicals in order to determine the rate constant for the reaction of TEMPONE-H with superoxide radicals (Fig. 5). The concentration of TEMPONE-H was constant (2mM), while the concentration of SOD was varied. The observed rate constant for the reaction of TEMPONE-H with superoxide radicals at pH=7.4 was calculated using the following equation: $(V_o/V)-1= k_3^{\cdot}[\text{TEMPONE-H}]/k_{SOD}^{\cdot}[\text{SOD}]$, where V_o and V represent the rates of TEMPONE formation in the absence and presence of SOD, respectively. The second order rate constants k_3 and

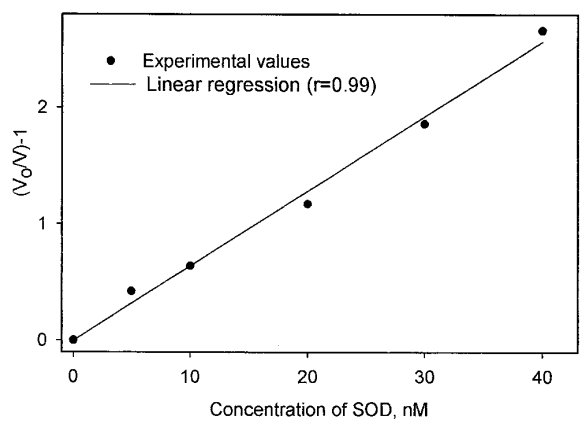

FIG. 5. Inhibition of superoxide-mediated oxidation of TEMPONE-H by SOD. V_o and V represents the rates of TEMPONE formation during the reaction of TEMPONE-H with superoxide radicals in the absence and presence of SOD, respectively.

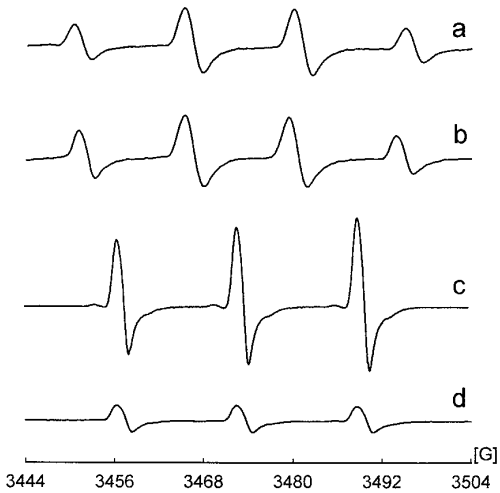

FIG. 6. ESR spectra of samples with 10 mM AAPH containing 0.2 M DMPO (a); 0.2 M TMIO (b); 2 mM TEMPONE-H (c); 2 mM TEMPONE-H at 4°C (d). ESR receiver gain was 5×10^5. Modulation amplitude was 2 G.

k_{SOD} are for the reaction of superoxide radical with TEMPONE-H and SOD, respectively.

The activity of SOD was standardized against cytochrome C (4). The ratio of the rate constants $k_3/k_{Cytochrome\ C}$ is 0.02, where $k_{Cytochrome\ C} = 6\times10^5\ M^{-1}s^{-1}$ (4). Therefore, we obtained $k_3=1.2\times10^4\ M^{-1}s^{-1}$.

Reaction of TEMPONE-H with Peroxyl Radical

In the AAPH peroxyl radical generating system with the spin traps DMPO or TMIO spin adducts with peroxyl radicals were formed (Fig. 6a, b). It was found that under the reaction of TEMPONE-H with AAPH derived peroxyl radicals the nitroxide radical TEMPONE was continuously formed (Fig. 6c). Formation of TEMPONE was inhibited by decreasing the temperature of a mixture of AAPH with TEMPONE-H at 4°C (Fig. 6c) due to inhibition of peroxyl radical formation (11). TEMPONE formation was proportional to the concentration of TEMPONE-H. Thus, we suggest that the scavenging of the peroxyl radicals by TEMPONE-H was not quantitative. Using the calculation of the rate of peroxyl radical generation by AAPH (11) the efficacy of peroxyl radical scavenging by 6mM TEMPONE-H was estimated to amount to 10±3%.

Earlier it was supposed that hydroxylamines could reduce peroxyl radicals (14). Our data support the idea that hydroxylamine TEMPONE-H reacts with peroxyl radicals with a formation of the nitroxide radical TEMPONE probably via reaction 4.

4. $\text{TEMPONE-H} + RO_2^{\cdot} \rightarrow \text{TEMPONE} + RO_2H$

CONCLUSION

It was shown that the hydroxylamine TEMPONE-H reacts with peroxynitrite, superoxide and peroxyl radicals with formation of stable nitroxide radical TEMPONE. The rate constants for reactions of TEMPONE-H with peroxynitrite and superoxide radicals are very high. Using TEMPONE-H the obtained sensitivity in the detection of peroxynitrite or superoxide radicals was about 10-fold higher than using the spin traps DMPO or TMIO. Therefore, the spin trap TEMPONE-H could be used to quantify peroxynitrite and superoxide radical formation in chemical and biological systems.

ACKNOWLEDGMENTS

This study was supported by ISIS PHARMA GmbH, Zwickau, Germany. We thank Dr. Bruno Fink for his help with the computer analysis of ESR spectra.

REFERENCES

1. Halliwell, B., and Gutterige, J. M. C. (1990) *in* Methods in Enzymology (Packer, L., and Glazer, A. N., Eds.), Vol. 186, pp. 1–85, Academic Press, London.
2. Pryor, W. A., Jin, X., and Squadrito, G. L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1173–1177.
3. Janzen, E. G., and Hare, D. L. (1990) *Advances in Radical Chemistry* **1,** 253–295.
4. Rosen, G. M., Finkelstein, E., and Rauckman, E. J. (1982) *Arch. Biochem. Biophys.* **215,** 367–378.
5. Kashkarov, K. P., Vasilyeva, E. V., and Ruuge, E. K. (1994) *Biokhimia (Russian)* **59,** 813–818.
6. Grigor'ev, I. A., and Volodarsky, L. B. (1974) *Zhurnal Organicheskoi Khimii (Russian)* **10,** 118–124.
7. Dikalov, S. I., Kirilyuk, I. A., Grigor'ev, I. A., and Volodarsky, L. B. (1992) *Bulletin of Russian Academy of Science* **41,** 834–837.
8. Fridovich, I. (1986) *in* CRC Handbook of Methods for Oxygen Radical Research (Greenwald, R. A., Ed.), pp. 51–53, CRC Press, New York.
9. Augusto, O., Gatti, R. M., and Radi, R. (1994) *Arch. Biochem. Biophys.* **310,** 118–125.
10. Dikalov, S. I., Kirilyuk, I. A., and Grigor'ev, I. A. (1996) *Biochem. Biophys. Res. Commun.* **218,** 616–622.
11. Niki, E. (1990) *in* Methods in Enzymology (Packer, L., and Glazer, A. N., Eds.), Vol. 186, pp. 100–108, Academic Press, London.
12. Fridovich, I. (1986) *in* CRC Handbook of Methods for Oxygen Radical Research (Greenwald, R. A., Ed.), pp. 121–122, CRC Press, New York.
13. Beckman, J. S., Beckman, T. W., Chen, J., Marchall, P. A., and Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1620–1624.
14. Mehlhorn, R. J., and Packer, L. (1984) *in* Methods in Enzymology (Packer, L., Ed.), Vol. 105, pp. 215–220, Academic Press, London.