Effects of Cu^{2+} and Detergents on the Autoxidation of Hydroxylamine Involving Superoxide Anion Radicals

Teruhiro TAKABE, Masako MIYAKAWA and Sueki NIKAI

*Department of Chemistry, Faculty of Science and Technology,
Meijo University, Tenpaku, Nagoya 468*

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The reduction rate of nitro-blue tetrazolium by superoxide anion radicals during the autoxidation of hydroxylamine ($20\text{ }\mu\text{M}$) was inhibited by 67% in the presence of $5\text{ }\mu\text{M}$ Cu^{2+} . However, upon the addition of a cationic micelle of hexadecyltrimethylammonium bromide or a nonionic micelle of polyoxyethylene dodecyl ether, the reduction rate markedly increased with an increase of the concentration of Cu^{2+} . The coexistence of $10\text{ }\mu\text{M}$ Cu^{2+} and 5 mM cationic or nonionic micelle accelerated this reaction about 50- or 75-fold, respectively, although the absence of Cu^{2+} accelerated the reaction rate only about 8.0- or 6.0-fold, respectively. On the other hand, the generation rate of nitrite during the autoxidation of hydroxylamine was increased by the addition of Cu^{2+} and decreased by the addition of the micelles. The co-existence effect of Cu^{2+} and micelles was not observed in the case of nitrite formation reaction. The microenvironmental effects on these reactions were discussed.

Although the superoxide anion radical (O_2^-) is known to be produced by a number of biologically significant oxidations,¹⁾ the micro-environmental effects on the reactivity of O_2^- have not yet extensively studied. In previous papers,^{2,3)} we reported that both the generation rate of O_2^- and the electron transfer rate from O_2^- to nitro-blue tetrazolium (NBT) during the autoxidation of thiol (dithiothreitol (DTT) and glutathione) and polyphenol (pyrogallol and catechol) compounds were markedly accelerated in the hydrophobic regions such as micelles, and were drastically effected by the transition metals such as Cu^{2+} .

Hydroxylamine is an intermediate in the oxidoreduction reactions between ammonia and nitrate.^{4,5)} It has been reported that the oxidation of hydroxylamine to nitrite by molecular oxygen in aqueous alkaline solution is catalyzed by Cu^{2+} , and a possible role of peroxonitrite as a species of intermediates involved in hydroxylamine oxidation has been proposed.^{6,7)} It has also shown that O_2^- is generated by the autoxidation of hydroxylamine at alkaline pH higher than 8.0.⁸⁾

In this paper, we are going to report drastic effects of Cu^{2+} and micelles on the electron

transfer rates from O_2^- to NBT using the auto-oxidation reaction of hydroxylamine as an O_2^- -generating system.

MATERIALS AND METHODS

NBT and superoxide dismutase (SOD) were purchased from Sigma Chem. Co. Hydroxylamine hydrochloride and polyoxyethylene dodecyl ether (Brij 35) were obtained from Katayama Chem. Co. These materials were used without further purification. Hexadecyltrimethylammonium bromide (CTAB) was recrystallized from methanol. Other chemicals were reagent grade. Water was deionized and distilled.

Spectroscopic measurements were made with a Shimadzu UV-200S recording spectrometer under aerobic conditions at 25°C . The reduction of NBT was measured from the increase of absorbance at 560 nm due to the accumulation of blue formazan.⁹⁾ The reaction was started by the addition of hydroxylamine to the buffered reaction mixture. Nitrite formation from hydroxylamine was determined under the following conditions.¹⁰⁾ Reaction mixture contained $25\text{ }\mu\text{M}$ hydroxylamine in 2 ml of 0.1 M sodium carbonate buffer at $\text{pH } 10.2$. 0.5 ml of the above reaction mixture was taken at appropriate time and added to 0.5 ml sulfanilic acid solution. Then 0.5 ml α -naphthylamine solution was added and shaken. After standing at room temperature for 20 min , the optical density of the mixture was determined at 530 nm in the spectrometer.

RESULTS

Recently, O_2^- has been shown to be generated during the autoxidation of hydroxylamine and the following reaction mechanism has been proposed.⁸⁾

As the autoxidation of hydroxylamine involves O_2^- (eq. 2), hydroxylamine is able to transfer electron to NBT *via* O_2^- under the aerobic conditions. Based on this mechanism, the autoxidation of hydroxylamine has often been assayed by the reduction of NBT. Figure 1 shows the effects of Cu^{2+} , EDTA, and micelles on the reduction of NBT by O_2^- coupled with the autoxidation of hydroxylamine at pH 10.5. The rate of accumulation of formazan in solution containing NBT and hydroxylamine was 0.0008 A_{560} units per min. The addition of 5 mM CTAB and Brij 35 to the above reaction

mixture accelerated the reduction rate of NBT by factors of 8.0 and 6.0, respectively. However, by the addition of Cu^{2+} , the reduction rate of NBT was decreased and 5 μM Cu^{2+} inhibited the rate by about 67%. Since it has been reported that Cu^{2+} catalyzes the dismutation of O_2^- with the rate constant of $k_2 = 2.7 \times 10^9$,¹¹⁾ this result suggests that free Cu^{2+} competes with NBT for O_2^- in a similar manner to SOD. Figure 1 also shows that 20 μM EDTA accelerated the reduction rate of NBT by factor of 2.5 compared with the case of the absence of EDTA, but at the higher concentration of Cu^{2+} larger than that of EDTA, the rate was markedly inhibited by free Cu^{2+} in the reaction medium. These results may also indicate that EDTA accelerates the reduction rate of NBT by chelating trace metal(s) such as iron, as well as inhibits the catalytic function of Cu^{2+} for the dismutation of O_2^- . Indeed the chelate compound of Fe^{2+} or Fe^{3+} with EDTA accelerated the reduction rate of NBT during the autoxidation of hydroxylamine as shown in Table I. Although the

FIG. 1. The Reduction Rate of NBT *via* O_2^- by the Autoxidation of Hydroxylamine as a Function of Cu^{2+} Concentration.

The reaction mixture contained 20 μM hydroxylamine, 100 μM NBT, 0.1 M sodium carbonate buffer at pH 10.5, and the indicated concentration of Cu^{2+} . ○, no addition; ●, plus 20 μM EDTA; △, plus 5 mM CTAB; □, plus 5 mM Brij 35.

TABLE I. EFFECTS OF Fe^{2+} , Fe^{3+} , EDTA, AND DETERGENTS ON THE REDUCTION OF NBT DURING THE AUTOXIDATION OF HYDROXYLAMINE

The reaction mixture contained 20 μM hydroxylamine, 100 μM NBT, and 0.1 M sodium carbonate buffer at pH 10.5. Control rate was 0.0008 A_{560} units per min.

Control	100
Fe^{2+} 10 μM	350
Fe^{3+} 10 μM	213
EDTA 20 μM	263
Fe^{2+} 10 μM + EDTA 20 μM	588
Fe^{3+} 10 μM + EDTA 20 μM	575
CTAB 5 mM	825
Brij 35 5 mM	625
Fe^{2+} 10 μM + CTAB 5 mM	938
Fe^{3+} 10 μM + CTAB 5 mM	825
Fe^{2+} 10 μM + Brij 35 5 mM	625
Fe^{3+} 10 μM + Brij 35 5 mM	625

reduction rate of NBT was strongly inhibited by Cu^{2+} , the drastic acceleration was observed in the presence of both Cu^{2+} and CTAB or Brij 35. Its rate increased with an increase of the concentration of Cu^{2+} and the coexistence

FIG. 2. The Rate of Nitrite Formation Accompanying the Autoxidation of Hydroxylamine as a Function of Cu^{2+} Concentration.

The incubation mixture contained 25 μM hydroxylamine and 0.1 M sodium carbonate buffer at pH 10.2 in a total volume of 2 ml. ○, no addition; ●, plus 20 μM EDTA; △, plus 5 mM CTAB; □, plus 5 mM Brij 35. After reactions, nitrite formed by the autoxidation of hydroxylamine was determined colorimetrically as described in MATERIALS AND METHODS.

of 10 μM Cu^{2+} and 5 mM CTAB or Brij 35 accelerated this reaction about 50- or 75-fold, respectively compared with the case of the absence of both Cu^{2+} and the micelles.

In order to examine which reaction step(s) in the oxidoreduction reactions from hydroxylamine to NBT were effected, we investigated the effects of Cu^{2+} and micelles on the generation rate of nitrite during the autoxidation of hydroxylamine as shown in Fig. 2. In sharp contrast to the case of NBT reduction, the generation rate of nitrite increased with an increase of the concentration of Cu^{2+} . Since 5 mM of CTAB and Brij 35 inhibited the nitrite formation by about 53%, the acceleration of NBT reduction by CTAB and Brij 35 was the process of the electron transfer from O_2^- to NBT, and not that of the generation of O_2^- . CTAB and Brij 35 (5 mM each) accelerate the rates of electron transfer from O_2^- to NBT by 17- and 13-folds, respectively. The coexistence effect of Cu^{2+} and micelles, which was observed in the case of NBT reduction, did not occur in the case of nitrite formation. Namely, the coexistence of 10 μM Cu^{2+} and 5 mM CTAB or Brij 35 accelerated the nitrite formation only

by 1.5- or 1.6-fold, respectively. Therefore, these systems accelerate the process of electron transfer from O_2^- to NBT by 33- and 47-folds, respectively.

Next, to determine whether or not O_2^- , which is generated during the autoxidation of hydroxylamine, practically reduces NBT in the presence of Cu^{2+} and micelles, the effect of SOD on the reduction of NBT was examined. In the presence of Cu^{2+} , Cu^{2+} -CTAB, and Cu^{2+} -Brij 35, SOD inhibited the reduction of NBT about 80, 75, and 45%, respectively. Although these results strongly suggest that NBT was reduced *via* O_2^- by the autoxidation of hydroxylamine even in the presence of Cu^{2+} , CTAB, and Brij 35, a possibility can not be excluded that some unknown intermediates produced by the autoxidation of hydroxylamine reduce NBT in the Cu^{2+} -Brij 35 system.

It has been reported that hydroxyl radicals can be generated by the following reaction involving O_2^- and/or metals.¹²⁻¹⁵⁾

Therefore, it is interesting to investigate a possibility that hydroxyl radicals participate in the autoxidation of hydroxylamine. The addition of benzoate and mannitol, scavengers

FIG. 3. Inhibition of the Reduction of NBT during the Autoxidation of Hydroxylamine by SOD.

The reaction mixture contained 20 μM hydroxylamine, 100 μM NBT, and 5 μM Cu^{2+} , and 0.1 M sodium carbonate buffer at pH 10.5. ○, no addition; △, plus 5 mM CTAB; □, plus 5 mM Brij 35.

of $\text{OH}\cdot$,¹⁶⁾ did not affect the autoxidation of hydroxylamine even in the presence of Cu^{2+} and micelles (data not shown). These results may indicate that the hydroxyl radicals formed by Harber-Weiss reaction^{12~14)} or Fenton type reaction¹⁵⁾ do not participate in the autoxidation of hydroxylamine.

DISCUSSION

In the previous paper,³⁾ we reported that Cu^{2+} increased the generation rate of O_2^- during the autoxidation of DTT and the autoxidation ceased when all DTT molecules were chelated by Cu^{2+} . And we proposed that chelate compounds of Cu^{2+} such as Cu^{2+} -DTT complex and Cu^{2+} -EDTA complex accelerate the autoxidation of DTT. In the case of hydroxylamine, the autoxidation was accelerated monotonously with an increase of the concentration of Cu^{2+} (Fig. 2). The catalytic function of Cu^{2+} was reduced by the addition of cationic or nonionic micelle and almost eliminated by the addition of EDTA. On the other hand, the electron transfer from O_2^- to NBT was remarkably accelerated by the coexistence of Cu^{2+} and CTAB or Brij 35. And its acceleration by the coexistence of Cu^{2+} and micelles was much larger than that by the existence of only micelles. Therefore, it must be noted that the dismutation activity of Cu^{2+} for O_2^- appeared to be inhibited by these micelles. All these results strongly suggest that there exists an interaction between Cu^{2+} and these micelles. In contrast to the case of the absence of Cu^{2+} , in the presence of Cu^{2+} , the acceleration by Brij 35 was larger than that by CTAB. This may be due to the repulsive interaction between Cu^{2+} and the cationic micelle, CTAB. Since it is known that the life time of O_2^- becomes larger in hydrophobic region compared with aqueous region,¹⁷⁾ all above data may suggest *in vivo* Cu^{2+} catalyzes the dismutation of O_2^- in a protic environment and catalyzes the electron transfer from O_2^- to some electron acceptor molecule in a hydrophobic environment such as in mem-

brane system. Therefore, it may be interesting to investigate the reactivity of O_2^- which is generated in various tissues, in connection with these results.

Other transition metals such as Fe^{2+} and Fe^{3+} also accelerated the autoxidation of hydroxylamine, but did not inhibit the reduction of NBT as shown in Table I. These are compatible with the result that Fe^{2+} and Fe^{3+} do not catalyze the dismutation of O_2^- .¹⁸⁾

Finally, the hydroxyl radical of which generation mechanism has been proposed by Harber-Weiss reaction^{12~14)} or Fenton type reaction¹⁵⁾ does not participate in the autoxidation of hydroxylamine regardless absence or presence of Cu^{2+} , CTAB, and Brij 35.

REFERENCES

- 1) A. M. Michelson, J. M. McCord and I. Fridovich, ed. "Superoxide and Superoxide Dismutase," Academic Press, New York, 1977.
- 2) T. Takabe, M. Miyakawa and S. Nikai, *Bull. Chem. Soc. Jpn.*, **51**, 321 (1978).
- 3) T. Takabe, H. Ishikawa, M. Miyakawa and S. Nikai, *Agric. Biol. Chem.*, **42**, 593 (1978).
- 4) A. Nason, *Bacteriol. Rev.*, **26**, 16 (1962).
- 5) A. B. Falcone, A. L. Shug and D. J. D. Nicholas, *Biochim. Biophys. Acta*, **77**, 199 (1963).
- 6) M. N. Hughes and H. G. Nicklin, *J. Chem. Soc. A*, **1970**, 925.
- 7) M. N. Hughes and H. G. Nicklin, *Biochim. Biophys. Acta*, **222**, 660 (1970).
- 8) Y. Kono, *Arch. Biochem. Biophys.*, **186**, 189 (1978).
- 9) C. Beauchamp and I. Fridovich, *Anal. Biochem.*, **44**, 276 (1971).
- 10) E. F. Elstner and A. Heupel, *ibid.*, **70**, 616 (1976).
- 11) R. Brigelius, R. Spottl, W. Bors, E. Lengfelder, M. Saran and U. Weser, *FEBS Lett.*, **47**, 72 (1974).
- 12) F. Haber and J. Weiss, *Proc. R. Soc. London, Ser. A*, **147**, 332 (1934).
- 13) C. Beauchamp and I. Fridovich, *J. Biol. Chem.*, **245**, 4641 (1970).
- 14) J. J. Van Hemmen and W. J. A. Meuling, *Arch. Biochem. Biophys.*, **182**, 743 (1977).
- 15) H. J. Fenton, *J. Chem. Soc.*, **65**, 899 (1894).
- 16) L. M. Dorfman and G. E. Adams, "Reactivity of Hydroxyl Radical in Aqueous Solutions," National Bureau of Standard, 1973.
- 17) J. S. Valentine and A. B. Curtis, *J. Am. Chem. Soc.*, **97**, 224 (1975).
- 18) B. Halliwell, *FEBS Lett.*, **56**, 34 (1975).