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REACTION OF VANADYL WITH HYDROGEN PEROXIDE. AN ESR AND SPIN TRAPPING STUDY

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Vanadyl reacts with hydrogen peroxide forming hydroxyl radicals in a Fenton-like reaction. The hydroxyl radicals were spin trapped and identified using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The quantity of hydroxyl radicals spin trapped during the reaction between vanadyl and hydrogen peroxide are equal to half of the hydroxyl radicals spin trapped during the reaction between ferrous ions and hydrogen peroxide. Experiments in the presence of formate show that this hydroxyl radical scavenger effectively competes with DMPO preventing the formation of the DMPO-OH adduct. However, in experiments using ethanol as the hydroxyl radical scavenger it was not possible to completely prevent the formation of DMPO-OH. The formation of this additional DMPO-OH in the presence of ethanol does not depend on the concentration of dissolved oxygen, but does depend on the concentration of hydrogen peroxide added to the vanadyl solution. The results suggest that the additional DMPO-OH formed in the presence of ethanol originates from a vanadium (V) intermediate. This intermediate may oxidize DMPO leading to the formation of DMPO-O_2^- which rapidly decomposes forming DMPO-OH.

KEY WORDS: Vanadium, vanadyl, hydrogen peroxide, hydroxyl radicals, ESR, spin trapping.

INTRODUCTION

The oxycation of vanadium (IV), vanadyl (VO^{+2}), has been used for many years as a spin probe for the metal binding sites in proteins.[1,2] Since vanadyl has a single unpaired electron in its lowest nondegenerate d_{xy} orbital,[3] in a magnetic field this electron interacts with the ^{51}V nucleus (99.7 percent abundant) which has a nuclear spin, $I = 7/2$, producing a sharp isotropic eight line ESR spectrum at room temperature. An important property of vanadyl as a spin probe is the susceptibility of the VO^{+2} ESR spectrum to the motion of the cation in solution. For instance, the ESR spectrum of VO^{+2} bound to a large slowly tumbling protein is anisotropic. It resembles the ESR spectrum of immobilized VO^{+2} ions in a polycrystalline state or frozen solution. This difference between the bound and unbound VO^{+2} ESR spectrum has provided important information with regard to metal ion properties in metalloproteins.

In addition to these VO^{+2} spectroscopic properties, some aspects of the VO^{+2} chemistry may also prove to be important in biological studies. It has been suggested for some time that VO^{+2} participates, in the presence of H_2O_2, in a Fenton-like reaction generating hydroxyl radicals (·OH).[4] Brooks *et al.*[4] studied the kinetics of this reaction, however, their emphasis was on the VO^{+2} ESR. Recently, Keller *et al.*[5] using spin trapping have studied the effects of vanadium on lipid peroxidation in micelles of purified and partially peroxidized fatty acids. In this study it was shown than VO^{+2} was the active vanadium species that initiated conjugated diene formation and in the vanadium-catalyzed decomposition of fatty acid hydroperoxides. Further-

more, it was also implied that following the addition of VO^{+2} and H_2O_2 to a micellar suspension, ·OH was involved in the production of conjugated dienes. Keller *et al.* have also studied the vanadium-stimulated oxidation of NADH.[6]

The purpose of the present study is to address, using spin trapping, some important unanswered questions about the reaction between VO^{+2} and H_2O_2. These questions involve: (1) Is the product of the VO^{+2}/H_2O_2 reaction really ·OH or is it possible for other activated oxygen species to be produced; (2) Are other oxidizing species produced which may possible mediate free radical mechanisms; (3) If ·OH is produced, how effective is VO^{+2} in comparison with ferrous ions to produce these in a Fenton-like reaction?

MATERIALS AND METHODS

Vanadyl Sulfate and Ferrous Ammonium Sulfate were obtained from Fisher Scientific Co. (Fair Lawn, NJ). The concentration of vanadyl solutions was determined spectrophotometrically ($\lambda = 750\,nm$, $\varepsilon = 18\,M^{-1}cm^{-1}$).[7] Ferrous Ammonium Sulfate was titrated with a standard potassium permanganate solution to determine the ferrous ion concentration.[8] Hydrogen Peroxide was obtained from Sigma (St. Louis, MO) and its concentration was also determined by titration with potassium permanganate.[8] The spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Aldrich (Milwaukee, WI) and was purified following the method described by Buettner and Oberley.[9] This method consists of successively treating the DMPO with activated charcoal until all free radical impurities disappear as verified by ESR. The concentration of DMPO was measured spectrophotometrically ($\lambda = 227\,nm$, $\varepsilon = 8 \times 10^3\,M^{-1}cm^{-1}$).[10]

To eliminate any trace metals, all glassware was kept permanently soaking in a 1:1 mixture of concentrated sulfuric and nitric acids. Immediately prior to use, the glassware was rinsed with metal free water and dried under a stream of nitrogen. The metal-free water was prepared by further treating water obtained from a Sybron/Barnstead NANO pure system with a 0.001 percent dithizone (Sigma) solution in carbon tetrachloride. This treatment is carried out successively in a separating funnel until the green color of the dithizone persists. The water was then boiled to rid it of residual organic material.

Experiments requiring deaerated or oxygen-free conditions were carried out in an apparatus described by Russell *et al.*[11] and Evans.[12] This apparatus consists of a U-tube connected to an ESR quartz flat cell via a ground glass joint. Nitrogen bubbling through the samples was used to remove oxygen from the solutions. The required quantities of hydrogen peroxide mixed with metal-free water was placed in one stem of the U-tube and the DMPO, metal ions and scavengers ethanol or formate (when required) were placed in the other stem. Nitrogen bubbling was carried out for 20 minutes. However, in experiments requiring ethanol as a scavenger, the solutions and pure ethanol were saturated separately with nitrogen by vigorously bubbling prior to placing in the appropriate amounts in the U-tube. After sealing the U-tube, nitrogen was bubbled through the solutions for approximately 3–5 minutes to insure that the ethanol concentration remained fairly constant. Once nitrogen bubbling was completed, solutions were mixed in the U-tube and the ESR spectrum recorded.

Experiments carried out under air-saturated conditions were done by mixing DMPO, metal ions, ethanol or formate (when required) immediately prior to addition

of the appropriate amount of hydrogen peroxide. After addition of the hydrogen peroxide, the samples were rapidly mixed and transferred to an ESR quartz flat cell (60 × 10 × 0.25 mm) and their ESR spectrum recorded.

All ESR spectra were recorded on a Varian E-9 X-band spectrometer at 100 KHz magnetic field modulation. The magnetic field was set at 3350 G, microwave power, 10 mW; modulation amplitude, 0.5 G; and microwave frequency, 9.510 GHz. The hyperfine coupling constants were obtained by computer simulation generating a theoretical ESR spectrum that matches the experimental spectrum.

RESULTS AND DISCUSSION

When hydrogen peroxide is mixed with a vanadyl solution containing the spin trap DMPO, an ESR spectrum consisting of a 1:2:2:1 quartet is obtained. This quartet with hyperfine coupling constants, $a_N = a_H^\beta = 14.9$ G, corresponds to the DMPO–OH spin adduct.[13] In order to verify that the DMPO–OH spin adduct originates from the reaction of hydroxyl radicals with DMPO, two experiments were done using formate and ethanol as ·OH scavengers.[14] Figure 1 shows the results obtained after mixing H_2O_2 (1 mM) with the vanadyl solution (0.1 mM) each containing DMPO (100 mM) and different concentrations of formate. The ESR spectra from these solutions change from the DMPO–OH spectrum (Figure 1A), obtained when the concentration of DMPO is larger than the formate concentration by a factor of ten, to an ESR spectrum consisting of a triplet of doublets (Figure 1C) with hyperfine coupling constants, $a_N = 15.6$ G and $a_H^\beta = 18.7$ G. This ESR spectrum is observed when the concentration of formate is larger than the DMPO concentration by a factor of ten. The hyperfine coupling constants are consistent with those reported for the DMPO–CO_2^- spin adduct,[13] which is produced subsequent to the reaction of hydroxyl

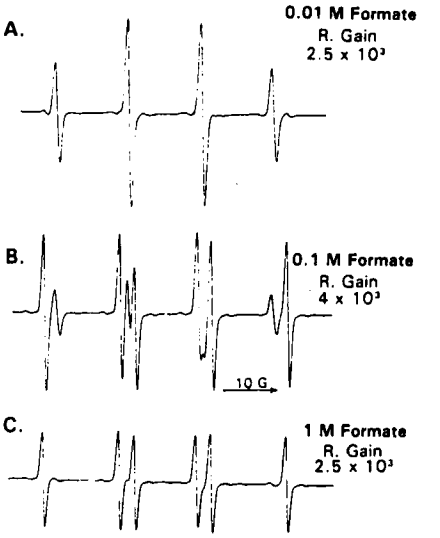

FIGURE 1 ESR spectra of DMPO–OH and DMPO–CO_2^- spin adducts obtained in the reaction of VO^{+2} with H_2O_2 in the presence of DMPO and varying concentrations of formate.

radicals with formate in the presence of DMPO. Figure 1B shows the ESR spectra of DMPO–OH and DMPO–CO_2^-. These spectra are obtained when the formate and DMPO concentration are approximately equal. The results shown in Figure 1 are consistent with DMPO and formate, which react with hydroxyl radicals at similar rates ($k > 10^9 m^{-1} s^{-1}$),[15] competing for hydroxyl radicals in the solution. Therefore, the results in Figure 1 verify that hydroxyl radicals are produced in the reaction between vanadyl and hydrogen peroxide.

Ethanol and DMPO also react with hydroxyl radicals at similar rates. However, the results obtained from experiments in which vanadyl and hydrogen peroxide are mixed in the presence of DMPO and varying concentrations of ethanol (Figure 2) are not consistent with the results obtained in the formate experiments. In the experiments using ethanol as a hydroxyl radical scavenger, the DMPO–OH ESR spectrum persists at high concentrations of ethanol. Figure 2A shows the DMPO–OH ESR spectrum obtained when vanadyl (0.1 mM) is mixed with hydrogen peroxide (1 mM) in the presence of DMPO (100 mM) and ethanol (10 mM). When the concentration of ethanol and DMPO are similar two spin adduct ESR spectra are obtained (Figure 2B). One corresponds to the DMPO–OH and the other consists of a triplet of doublets with hyperfine coupling constants, $a_N = 15.8$ G and $a_H^\beta = 22.8$ G. These parameters are consistent with the reported hyperfine coupling constants for the DMPO spin adduct obtained in the reaction between ethanol and hydroxyl radicals.[13] Figure 2C shows the ESR spectrum obtained following the reaction of vanadyl with hydrogen peroxide in the presence of 1.7 M ethanol. Although this concentration of ethanol is larger than the DMPO concentration by approximately a factor of 20, the DMPO–OH ESR signal persists. The formation of DMPO–OH at high concentrations of ethanol relative to DMPO occurs in air-saturated and nitrogen-saturated

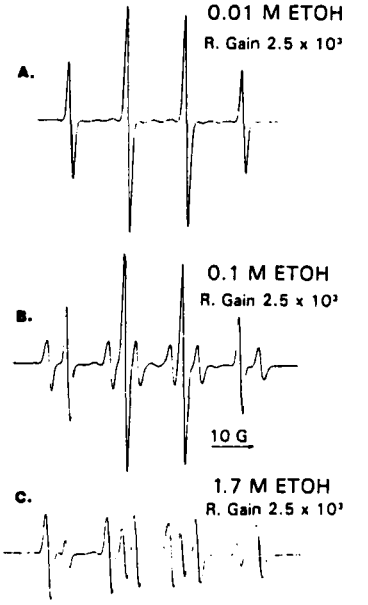

FIGURE 2 ESR spectra of DMPO–OH and DMPO–$CH(OH)CH_3$ spin adducts obtained in the reaction of VO^{+2} with H_2O_2 in the presence of DMPO and various concentrations of ethanol.

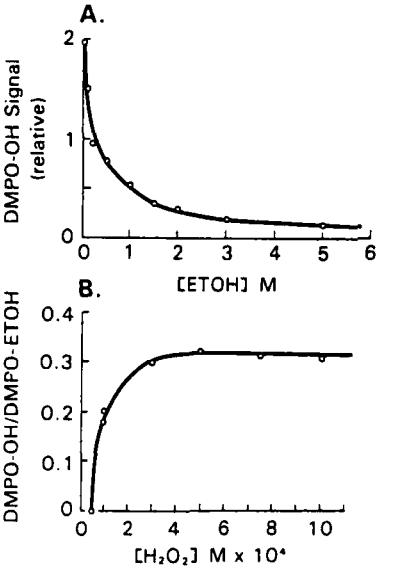

FIGURE 3 (A) DMPO–OH ESR signal intensity as a functions of increasing concentration of ethanol in the VO^{+2}/H_2O_2 reaction. (B) DMPO–OH ESR signal intensity as a function of hydrogen peroxide concentration obtained during the VO^{+2}/H_2O_2 reaction in the presence of a constant concentration of ethanol.

solutions, therefore, its production does not depend on the dissolved oxygen in the solution. Furthermore, although this property of the vanadyl/H_2O_2 reaction is not immediately obvious when formate is used as a hydroxyl radical scavenger, it does occur when other reagents less polar than water such as dimethylsulfoxide are added to the vanadyl solution. It is possible that this property of the vanadyl/H_2O_2 reaction is due to solvent effects, occurring in solvents less polar than water. The results in Figure 2 suggest that when the reaction of vanadyl with hydrogen peroxide is carried out in the presence of ethanol, another species capable of forming DMPO–OH is generated in addition to hydroxyl radicals.

Since the formation of this additional DMPO–OH is not dependent on the dissolved oxygen, it is important to determine whether or not its production, prior to proposing possible mechanisms, depends on the concentration of hydrogen peroxide. Figure 3 shows the results of these experiments. Figure 3A shows the DMPO–OH ESR signal intensity as a function of ethanol concentration following the mixing of hydrogen peroxide (1 mM) with a vanadyl (0.1 mM) solution containing DMPO (100 mM). It should be noted that at ethanol concentrations above 1 M, virtually no DMPO–OH should be observed if only hydroxyl radicals were responsible for the DMPO–OH signal in the absence of ethanol. Instead, at these concentrations of ethanol relative to DMPO only the triplet of doublets described in Figure 2 should be observed. Figure 3B shows the results obtained after varying the concentration of hydrogen peroxide mixed with several vanadyl solutions (1 mM) containing DMPO (100 mM) and a constant concentration of ethanol (1.7 M). Because the ESR signal intensity of the DMPO–OH and the CH_3–$\dot{C}$HOH adduct of DMPO simultaneously decrease as the hydrogen peroxide concentration is decreased, the ratio of both spin adducts is plotted against the hydrogen peroxide concentration. The results in Figure

3B indicate that the DMPO-OH originating from sources other than the addition of hydroxyl radicals to DMPO, formed directly in the vanadyl/H_2O_2 reaction, depends on the concentration of hydrogen peroxide in the solution. At a concentration of hydrogen peroxide larger than the vanadyl concentration by a factor of three, the formation of additional DMPO-OH remaind constant. At lower concentrations of hydrogen peroxide relative to vanadyl, the additional DMPO-OH decreases until disappearing. This occurs at a concentration of hydrogen peroxide five times lower than the vanadyl concentration.

Other than the reaction of directly formed hydroxyl radicals with DMPO, several pathways leading to the formation of DMPO-OH are possible. One alternative is that superoxide radicals are produced via a mechanism that does not involve dissolved oxygen. It is known that in an aqueous environment the superoxide spin adduct of DMPO, DMPO-O_2^-, rapidly decomposes forming DMPO-OH.[16] It is conceivable that superoxide could be produced via the following reaction:

$$\cdot OH + H_2O_2 \rightarrow H_2O + HO_2^{\cdot} \quad (1)$$

However, the rate of this reaction ($k = 2.6 \times 10^7 M^{-1}s^{-1}$) is relatively slow compared to the rate of the reaction between hydroxyl radicals and DMPO.[15] Therefore, for reaction (1) to occur in the experiments described, a concentration of hydrogen peroxide one hundredfold larger than the DMPO concentration would be required.

Another alternative that may cause the formation of DMPO-OH in the vanadyl/H_2O_2 reaction in the presence of ethanol could be a reactive vanadium intermediate. Several of these intermediates have been reported previously; however, one that seems possible is the vanadium (V) complex, $OVOO^{+2\cdot}$.[4] Although the ESR spectrum of this complex is not observed in the aqueous experiments described in this work, it has been observed when higher initial vanadyl concentrations are used.[4] However, in ethanol or DMSO and with the initial vanadyl concentration used in this work, an eight line ESR spectrum similar to the one reported for $OVOO^{+2}$ in water is observed.

There are two reaction schemes which could possibly explain the formation of DMPO-OH as a product of the reaction between DMPO and $OVOO^{+2}$. The first involves a direct addition of $OVOO^{+2}$ to DMPO followed by the decomposition of the spin adduct as shown in reactions (2) and (3):

$$OVOO^{+2\cdot} + DMPO \rightarrow DMPO\text{-}OOVO^{+2} \quad (2)$$

$$DMPO\text{-}OOVO^{+2} \xrightarrow{H^+} DMPO\text{-}OOH + VO^{2+} \quad (3)$$

The second scheme shown in reactions (4) through (6) involves the oxidation of DMPO by $OVOO^{+2}$. This reaction could form a $DMPO^+$ intermediate which rapidly adds a hydroxide anion to form DMPO-OH.

$$OVOO^{+2\cdot} + DMPO \rightarrow DMPO^+ + VO^{+2} + O_2^{\cdot -} \quad (4)$$

$$DMPO^+ + OH^- \rightarrow DMPO\text{-}OH \quad (5)$$

$$DMPO + O_2^{\cdot -} \rightarrow DMPO\text{-}O_2^- \quad (6)$$

The superoxide spin adduct of DMPO formed in reactions (3) and (6) would rapidly decompose generating DMPO-OH. Since the pH of the reaction mixtures ranged between pH 5 and pH 5.5, due to the instability of vanadyl at pH > 5.5, it would appear the reactions (2) and (3) are favored over reactions (4) through (6). However, at this time it is difficult to differentiate between both reaction schemes because only

the end products, which are the same for both schemes, are observed. A possible explanation for the hydrogen peroxide effect shown in Figure 3B is the following: at lower hydrogen peroxide concentrations relative to DMPO, the hydrogen peroxide is the limiting factor in the reaction and is consumed prior to sufficient formation of $OVOO^{+2}$ for its reaction with DMPO to be observed. However, at high concentrations of hydrogen peroxide relative to DMPO, vanadyl is being generated again (reaction 3 and 4) and continuously reacts with hydrogen peroxide until all the hydrogen peroxide is consumed. Therefore, it is possible that sufficient $OVOO^{+2}$ is produced allowing its effect to be observed. Since in water and at the initial vanadyl concentrations used in the experiments the $OVOO^{+2}$ intermediate is not observed, it is possible that this may be the reason why the results observed in the experiments containing ethanol (Figures 2 and 3) are not similar to those in which formate was used (Figure 1).

At this point, the results have shown that vanadyl in the presence of hydrogen peroxide effectively generates hydroxyl radicals. In addition, the results have suggested that at lower vanadyl concentrations and in environments less polar than water, a vanadium (V) intermediate may be produced which is capable of oxidizing DMPO leading to the formation of DMPO–OH. It is unclear at this time what implications, especially in biological systems, the formation of such an intermediate may have, It is possible that given the appropriate environment, such a vanadium (V) intermediate may act in a similar manner as the intermediate postulated for iron in its reaction with hydrogen peroxide.[17-19]

For biological purposes, it is of interest to determine how vanadyl compares with ferrous ions in its capacity to form hydroxyl radicals in the presence of hydrogen peroxide. Figure 4 shows the results obtained when separate solutions of vanadyl sulfate and ferrous ammonium sulfate of equal concentrations (0.1 mM) are mixed

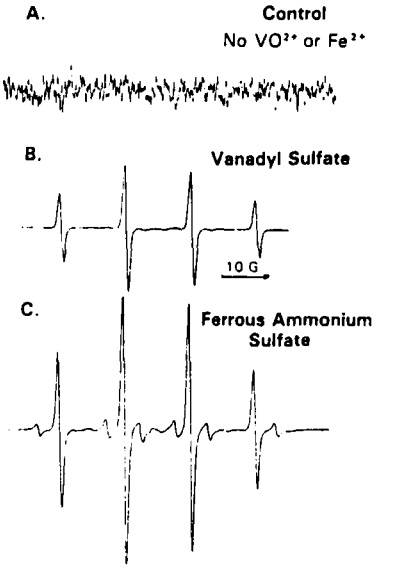

FIGURE 4 DMPO–OH ESR signal obtained in the reactions of vanadyl and ferrous ions with hydrogen peroxide at pH 5-5.5.

with hydrogen peroxide (1 mM) in the presence of DMPO (100 mM). Figure 4A is the control consisting of DMPO (100 mM) mixed with hydrogen peroxide (1 mM) and indicates that no spin adducts are formed. When the metal ions are included in the solutions the results show that the DMPO-OH originating from vanadyl (Figure 4B) is approximately half the DMPO-OH originating from the ferrous ions (Figure 4C). In addition, another DMPO spin adduct is also observed in Figure 4C. This spin adduct consists of a triplet of doublets with hyperfine coupling constants, $a_N = 15.5$ G and $a_H^\beta = 22.8$ G. These parameters are identical to the hyperfine coupling constants reported by Floyd *et al.*[20] for the 1-nitrosopyrroline spin adduct of DMPO. Therefore, it is possible that the additional spin adduct observed in Figure 4C corresponds to the interaction of a DMPO radical with DMPO. This triplet of doublets is also observed at higher vanadyl concentrations when reacted with hydrogen peroxide in the presence of DMPO.

Although the experimental results shown in Figure 4 indicate that in the presence of hydrogen peroxide ferrous ions are more efficient than vanadyl in producing hydroxyl radicals, it must be kept in mind that these reactions were carried out using solutions of plain inorganic salts. It remains to be determined if this property is true when these metal ions are chelated by complex biological systems.

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