5970 *Inorg. Chem.* **1994**, *33*, 5970−5973

Electron Paramagnetic Resonance Studies on the Formation and Decomposition of the Oxygenated Product of [Co^{II}(PMA)]$^+$, a Synthetic Analogue of Cobalt(II)−Bleomycin

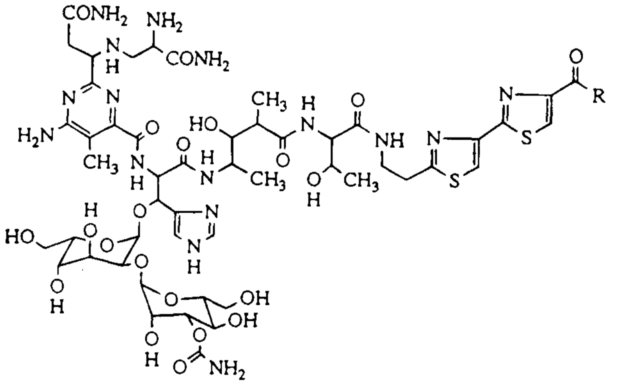
Edgardo Farinas, Narayan Baidya, and Pradip K. Mascharak*

Department of Chemistry and Biochemistry,
University of California, Santa Cruz, California 95064

Received June 22, 1994

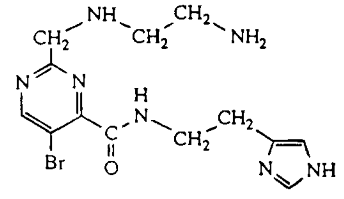
Introduction

Aerobic oxidation of mixtures of Co(II) salts and the antitumor antibiotic bleomycin (BLM, **1**) affords several Co(III) chelates of the drug which are often referred to as orange, brown, and green Co(III)−BLM.[1−4] Spectroscopic evidences indicate that BLM most possibly employs five N donor centers located in the primary and secondary amines, the pyrimidine and the imidazole ring, and the peptide moiety adjacent to the pyrimidine to bind the Co(III) center in these chelates.[5,6] The sixth coordination site is presumably occupied by a nitrogen donor, H_2O, and $-O_2H$ (hydroperoxy) group in the orange, brown, and green Co(III)−BLM respectively.[4,6] Though these chelates bind to DNA quite strongly,[7] the kinetically inert low-spin d^6 Co(III)−BLMs do not cause any oxidative DNA damage like the Fe−BLMs.[8] However, recently it has been shown that the Co(III) chelates induce DNA strand scission when illuminated with UV or visible light, and the process of photoinduced DNA damage is insensitive to dioxygen.[7,9−11]



BLM (1)

As part of our synthetic analogue approach to metallobleomycins, we have reported the structures and spectroscopic properties of three Co(III) complexes of a designed ligand PMAH (**2**, H is the dissociable amide H) which resembles the metal-chelating domain of BLM.[12] These Co(III) complexes of the composition [Co^{III}(PMA)(X)]$^{n+}$ (X = *N*-methylimidazole (*N*-MeIm), H_2O, or Cl^-) mimic the spectroscopic properties of the orange, brown, and green Co(III)−BLMs respectively and also inflict DNA damage under UV light. Results of the spin trapping and gel electrophoretic studies under aerobic and anaerobic conditions have revealed that (i) these positively charged complexes bind to DNA, (ii) UV irradiation gives rise to C/N-based radical(s) on the ligand framework of these complexes, and, (iii) in aqueous solution, the C/N based radical(s) rapidly produces •OH radicals in the vicinity of the DNA helix which in turn induce strand scission reactions. Control experiments with authentic samples of Co(III)−BLMs also suggest that the same mechanism could explain the DNA cleavage activity of the Co(III)−BLMs under UV illumination as well as the observed insensitivity of the photocleavage reaction to dissolved oxygen (the •OH radical is produced from H_2O). Recently, we have also reported the synthesis, structure, and spectroscopic properties of [Co(PMA)(Int-A)]Cl_2 (Int-A = acridine-9-carboxamido-*N*′-(3-propyl)imidazole) which inflicts photodamage to DNA at micromolar level much like the Co(III)−BLMs.[13]



PMAH(2)

Contrary to the thoroughly investigated [Co^{III}(PMA)X]$^{n+}$ complexes, the Co(II) complex(es) of PMAH remains unexplored so far. Since the chemistry of Co(II)−BLMs is quite extensive,[5,14] a comparative study of the Co(II) chelates of PMAH and the Co(II)−BLMs is necessary to further substantiate the merits of the model compounds. We report in this account the synthesis and spectral characterization of [Co^{II}(PMA)]$^+$. Also reported are (i) the reaction of [Co^{II}(PMA)]$^+$ with dioxygen and characterization of the oxygenated product [Co^{III}(PMA)(O_2^-)]$^+$ and (ii) identification of the decomposition products of [Co^{III}(PMA)(O_2^-)]$^+$ namely, the Co(III) chelate [Co^{III}(PMA)(H_2O)]$^{2+}$ and superoxide.[15]

---

(1) DeRiemer, L. H.; Meares, C. F.; Goodwin, D. A.; Diamanti, C. I. *J. Med. Chem.* **1979**, *22*, 1019.

(2) (a) Vos, C. M.; Westera, G. *J. Inorg. Biochem.* **1981**, *15*, 253. (b) Vos, C. M.; Westera, G.; Schipper, D. *J. Inorg. Biochem.* **1980**, *13*, 165. (c) Vos, C. M.; Westera, G.; van Zantem, B. *J. Inorg. Biochem.* **1980**, *12*, 45.

(3) (a) Albertini, J. P.; Garnier-Suillerot, A. *Biochemistry*, **1982**, *21*, 6777. (b) Garnier-Suillerot, A.; Albertini, J. P.; Tosi, L. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 499.

(4) Chang, C.-H.; Meares, C. F. *Biochem. Biophys. Res. Commun.* **1983**, *110*, 959.

(5) Sugiura, Y. *J. Am. Chem. Soc.* **1980**, *102*, 5216.

(6) Dabrowiak, J. C.; Tsukayama, M. *J. Am. Chem. Soc.* **1981**, *103*, 7543.

(7) Chang, C.-H.; Meares, C. F. *Biochemistry* **1984**, *23*, 2268.

(8) (a) Petering, D. H.; Byrnes, R. W.; Antholine, E. W. *Chem.-Biol. Interact.* **1990**, *73*, 133. (b) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107.

(9) (a) Saito, I.; Morii, T.; Sugiyama, H.; Matsuura, T.; Meares, C. F.; Hecht, S. M. *J. Am. Chem. Soc.* **1989**, *111*, 2307. (b) Chang, C.-H.; Meares, C. F. *Biochemistry* **1982**, *21*, 6332.

(10) (a) Suzuki, T.; Kuwahara, J.; Goto, M.; Sugiura, Y. *Biochem. Biophys. Acta* **1985**, *824*, 330. (b) Suzuki, T.; Kuwahara, J.; Sugiura, Y. *Nucl. Acids Res.* **1984**, *15*, 161.

(11) Subramanian, R.; Meares, C. F. *J. Am. Chem. Soc.* **1986**, *108*, 6427.

(12) (a) Tan, J. D.; Hudson, S. E.; Browm, S. J.; Olmstead, M. M.; Mascharak, P. K. *J. Am. Chem. Soc.* **1992**, *114*, 3841. (b) Brown, S. J.; Hudson, S. E.; Olmstead, M. M.; Mascharak, P. K. *J. Am. Chem. Soc.* **1989**, *111*, 6446.

(13) (a) Farinas, E. T.; Tan, J. D.; Baidya, N.; Mascharak, P. K. *J. Am. Chem. Soc.* **1993**, *115*, 2996−2997. (b) Tan, J. D.; Farinas, E. T.; David, S. S.; Mascharak, P. K. *Inorg. Chem.* **1994**, *33*, 4295.

(14) (a) Sugiura, Y.; Takita, T.; Umezawa, H. *Met. Ions. Biol. Syst.* **1985**, *19*, 81. (b) Dabrowiak, J. C. *Adv. Inorg. Biochem.* **1983**, *4*, 69.

(15) Though Co(III) complexes (including Co(III)−BLMs) are generally synthesized via oxygenation of the Co(II) species,[16−18] initial attempts to synthesize the Co(III) complexes of PMAH by this route failed.[12]

(16) Cotton, F. A.; Wilkinson, G. In *Advanced Inorganic Chemistry*, 5th ed.; Wiley: New York, 1988; pp 735−738.

(17) Shibata, M. In *Modern Syntheses of Cobalt (III) Complexes*; Springer-Verlag: New York, 1983; p 5.

(18) House, D. A. *Coord. Chem. Rev.* **1977**, *23*, 254.

0020-1669/94/1333-5970$04.50/0

Experimental Section

Preparation of Compounds. PMAH was synthesized by following the published procedure.[19] 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Fluka and was purified according to the literature procedure.[20] $(CH_3)_4NOH{\cdot}5H_2O$ and Superhydride (lithium triethylborohydride, 1.0 M solution in tetrahydrofuran) were purchased from Aldrich Chemical Co. $[Co(H_2O)_6](ClO_4)_2$ was procured from Alfa. The strongly acidic cation exchanger SP-C50-120 and Superoxide dismutase (SOD) were both purchased from Sigma Chemical Co. DMF (Burdick and Jackson) was purified[21] before use. Though most of the reactions discussed below occur in solvents like methanol, DMSO, and DMF, all physical measurements were performed with DMF solutions, unless specified, in order to keep the reaction conditions uniform throughout this study.

$[Co^{II}(PMA)](ClO_4)$. This complex was synthesized under a dry N_2 atmosphere (O_2 content < 10 ppm) and with the use of thoroughly degassed solvents. A solution of 0.1 g (0.28 mmol) of $[Co(H_2O)_6](ClO_4)_2$ in 5 mL of DMF was mixed with a solution of 0.11 g (0.30 mmol) of PMAH in 5 mL of DMF and to this mixture was added 10 μL of 0.9 M solution of $(Me)_4NOH{\cdot}5H_2O$ in ethanol. The clear cherry-red solution thus obtained was stirred for 30 min and then the solvent was removed in vacuo. Recrystallization of the residue from DMF/methanol mixture afforded 100 mg of brown microcrystals. Selected IR bands (KBr pellet, cm^{-1}): 3315 (s, br), 3000 (s), 2905 (s), 1600 (ν_{CO}, vs), 1484((s), 1337 (m), 1220 (m), 1105 (ClO_4, vs), 950 (s), 662 (m).

$[Co^{III}(PMA)(H_2O)](NO_3)_2$ from Oxygenation of $[Co^{II}(PMA)]^+$. Under a dry N_2 atmosphere, a solution of 0.16 g (0.63 mmol) of $[Co(CH_3COO)_2]{\cdot}4H_2O$ in 10 mL of methanol was added to a solution of 0.26 g (0.70 mmol) of PMAH in 5 mL of methanol when a cherry-red solution was obtained. After 30 min of stirring, 50 mg of activated charcoal was added to it and O_2 was bubbled through the mixture for 1.5 h. The dark mixture was stirred for another 12 h. Next, it was acidified with two drops of concentrated hydrochloric acid and filtered. The deep brown filtrate was evaporated to dryness. The residue was then dissolved in H_2O and the brown solution was loaded onto a Sephadex SP C-50-120 column (18 cm long) and eluted with 0.2 M KCl solution. The first fraction was collected and evaporated to dryness. The residue was desalted with ethanol. The salt (KCl) was filtered off and the filtrate was evaporated to dryness. The deep green residue thus obtained was washed with acetonitrile. IR and NMR measurements indicated that the green solid was indeed $[Co^{III}(PMA)Cl]Cl$.[12] The chloro complex was then converted to the aquo complex $[Co^{III}(PMA)(H_2O)]^{2+}$ by following the reported $AgNO_3$ procedure.[12] A batch of 60 mg of $[Co^{III}(PMA)(H_2O)](NO_3)_2$ was obtained as brown microcrystalline solid.

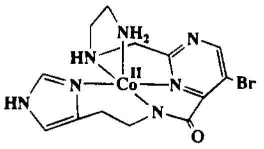
Physical Measurements. Infrared spectra were obtained with a Perkin-Elmer 1600 FTIR spectrophotometer. Absorption spectra were measured on a Perkin-Elmer Lambda 9 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a General Electric GN-300 (300-MHz) instrument. Samples were dissolved in $(CD_3)_2SO$ (99.9 atom % D). EPR spectra were monitored at X-band frequencies by using a Bruker ESP-300 spectrometer. Spin trapping experiments (293 K) were performed with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as the spin trap.

Sample Preparations for the EPR Measurements and the Spin-Trapping Experiment. Under a dry N_2 atmosphere, a solution of $[Co^{II}(PMA)]^+$ in DMF was prepared (from $[Co(H_2O)_6](ClO_4)_2$, PMAH and $(Me)_4NOH{\cdot}5H_2O$) and transferred into an EPR tube. The tube was properly stoppered and taken out of the glovebox, and the spectrum of the Co(II) complex was recorded at 110 K. The sample was then thawed and exposed to the atmosphere for a very brief period (5 s) and was frozen back immediately. The EPR spectrum of the superoxide adduct ($[Co^{III}(PMA)(O_2^-)]^+$) thus produced was recorded at 110 K. The superoxide adduct was also prepared via reduction of the Co(III) complex $[Co^{III}(PMA)(H_2O)]^{2+}$ with superhydride and reoxidation with O_2 in DMF, and its EPR spectrum was monitored at 110 K. Trapping of the superoxide ion was performed by thawing a sample of $[Co^{III}(PMA)(O_2^-)]^+$ in presence of an excess of DMPO. The sample was then transferred into a capillary tube and its EPR spectrum was recorded at 298 K.

The enzymatic reactions were performed as follows: 100 μL of a 5.0×10^{-3} M solution of $[Co^{II}(PMA)]^+$ in DMF was introduced into 100 μL of a 1.0 M solution of DMPO in 3.0 mM phosphate buffer, pH = 7 at room temperature. The sample was transferred into a capillary tube and the ESR spectrum was recorded at 298 K. The reaction described above was repeated, but the DMPO solution was treated with 20 μL of 15 units per μL of superoxide dismutase (SOD) in 3.0 mM phosphate buffer, pH = 7. The reaction mixtures were transferred into capillary tubes and the ESR spectra were recorded at 298 K.

Results and Discussion

Under strictly anaerobic conditions, reactions of Co(II) salts with PMAH in presence of 1 equiv of a base afford the Co(II) complex $[Co^{II}(PMA)]^+$ in which the deprotonated PMA^- ligand is coordinated to the Co(II) center in a pentadentate fashion. Ligation of the deprotonated peptido N center to Co(II) in $[Co^{II}(PMA)]^+$ is evident from the ν_{CO} frequency (1600 cm^{-1}) which is identical to that noted for $[Cu^{II}(PMA)]^+$ previously reported by us.[19,22] One equivalent of a base is required in the reaction mixture for successful ligation of the PMA^- framework to the Co(II) center. The cherry-red color (λ_{max} (ϵ, M^{-1}, cm^{-1}) in DMF: 530 nm (430)) of $[Co^{II}(PMA)]^+$ is developed only when the base is added. Also, the EPR signal of $[Co^{II}(PMA)]^+$ is not fully generated until at least 1 equiv of base is added. The structure of $[Co^{II}(PMA)]^+$ has been assigned on the basis its EPR spectrum (Figure 1a) $[Co^{II}(PMA)]^+$ displays nearly axial EPR spectrum with eight-line hyperfine splitting in the $g_{\parallel}$ region due to ^{59}Co ($I = {}^7/_2$) nucleus (Figure 1). This EPR feature is characteristic of low-spin d^7 ($S = 1/2$) Co(II) in 5-coordinated square-pyramidal geometry with the unpaired electron in the d_{z^2} orbital.[23] The presence of one axial N ($I = 1$) donor gives rise to the three-line superhyperfine splitting in the parallel region. The EPR parameters of $[Co^{II}(PMA)]^+$ ($g_{\perp} = 2.268$, $g_{\parallel} = 2.024$, $A_{\parallel}^{Co} = 87.09$ G, and $A_{\parallel}^{N} = 13.01$ G) are very similar to those reported for Co(II)-BLM ($g_{\perp} = 2.272$, $g_{\parallel} = 2.025$, $A_{\parallel}^{Co} = 92.5$ G, and $A_{\parallel}^{N} = 13$)[5] and other 5-coordinated square-pyramidal complexes.[23,24] Reduction of the structurally characterized $[Co^{III}(PMA)(H_2O)]^{2+}$ with lithium triethylborohydride also affords $[Co^{II}(PMA)]^+$ (confirmed by EPR spectroscopy, Scheme 1) which provides further evidence for the proposed structure of $[Co^{II}(PMA)]^+$.



The EPR spectrum of $[Co^{II}(PMA)]^+$ transforms dramatically upon exposure to dioxygen (Figure 1b). The axial ($g_{\perp} > g_{\parallel}$) EPR signal of $[Co^{II}(PMA)]^+$ changes to a more symmetrical ($g_{\parallel} > g_{\perp}$) signal at $g \sim 2$ and the $A_{\parallel}^{Co}$ value drops to 17.3 G. This latter fact suggests that the unpaired electron density resides mostly on the dioxygen moiety in the oxygenated species. The

---

[19] Brown, S. J.; Stephan, D. W.; Mascharak, P. K. *J. Am. Chem. Soc.* **1988**, *110*, 1996.

[20] Buettner, G. R. In *Handbook of Methods for Oxygen Radical Research*, Greenwald, R. A., Ed., CRC Press: Boca Raton, FL, 1985; pp 151–155.

[21] Perrin, D. D.; Armarego, W. L. F. In *Purification of Laboratory Chemicals*, 3rd ed.; Pergamon Press: Oxford, 1988; p 157.

[22] The ν_{CO} of PMAH (1670 cm^{-1}) is shifted to lower energy upon binding to M(II) centers.[19]

[23] Jones, R. D.; Summerville, D. A.; Basolo, F. *Chem. Rev.* **1979**, *79*, 139.

[24] Smith, T. D.; Pilbrow, J. R. *Coord. Chem. Rev.* **1981**, *39*, 295.

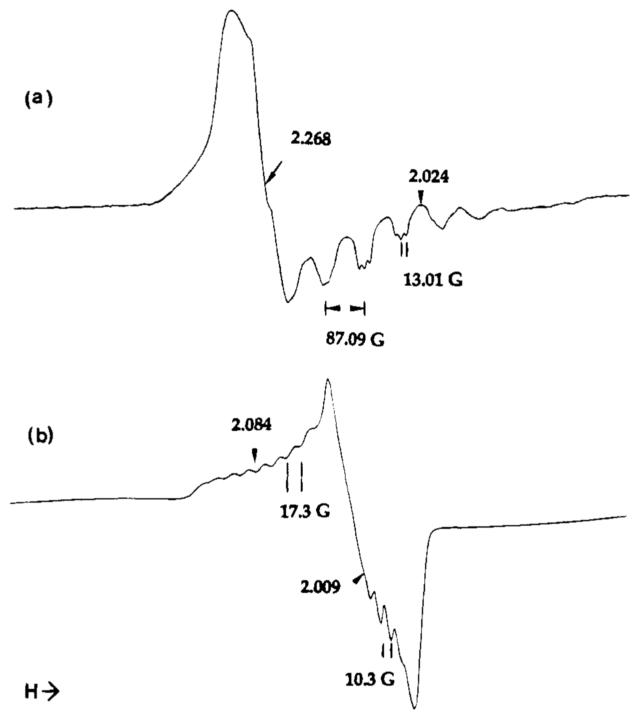
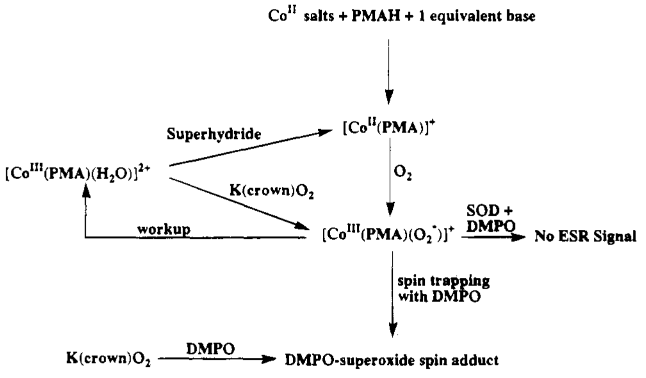


Figure 1. X-band EPR spectra of (a) $[Co^{II}(PMA)]^+$ and (b) $[Co^{III}(PMA)(O_2^-)]^+$ in DMF glass (110 K). Selected spectral parameters are shown. Spectrometer settings: microwave frequency, 9.42 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 2 G.

Scheme 1



EPR spectrum of the oxygenated species (Figure 1b) is typical of monomeric Co(III)-superoxide complexes[23,24] and identifies it as $[Co^{III}(PMA)(O_2^-)]^+$. This assignment is further confirmed by the fact that the same EPR spectrum is obtained when $[Co^{III}(PMA)(H_2O)]^{2+}$ is allowed to react with KO_2 (with added 18-crown-6) in DMF solution (Scheme 1). The EPR parameters of $[Co^{III}(PMA)(O_2^-)]^+$ ($g_\perp = 2.009$, $g_\parallel = 2.084$, $A_\parallel{}^{Co} = 17.3$, and $A_\perp{}^{Co} = 10.3$) are again very similar to those of Co(III)−BLM−(O_2^-) (2.007, 2.098, 20.2, and 12.4 respectively).[5]

Decomposition of $[Co^{III}(PMA)(O_2^-)]^+$ in DMF solution at 298 K in the presence of the spin trap DMPO affords the six-line ESR spectrum of the DMPO−superoxide spin adduct (Figure 2). The ESR parameters of the DMPO-superoxide in DMF at 298 K are as follows: $a_H = 17.6$, $a_N = 14.4$, and $g = 2.009$. The identity of the spin adduct was confirmed in the following way. First, the EPR spectrum of the superoxide radical was recorded at 110 K in DMF solution under N_2 atmosphere by using a 1:1 mixture of KO_2 and 18-crown-6 (at 110 K, $g_\parallel = 2.09$; $g_\perp = 2.008$).[25] Subsequently, DMPO was added and the mixture was allowed to thaw to 298 K. The DMPO−superoxide adduct thus obtained exhibited the same six-line EPR spectrum derived from the decomposition $[Co^{III}(PMA)(O_2^-)]^+$ in the DMF solution. Second, decomposition of $[Co^{III}(PMA)(O_2^-)]^+$ in DMF/aqueous buffer (3 mM phosphate buffer, pH = 7) in the presence of the spin trap DMPO also affords the six line ESR spectrum of DMPO-superoxide adduct. The ESR parameters of the DMPO-superoxide in DMF/aqueous buffer are as follows: $a_H = 18.6$, $a_N = 15.2$, and $g = 2.009$. The six-line spectrum is *not* obtained when the decomposition of $[Co^{III}(PMA)(O_2^-)^+$ is performed in DMF/aqueous buffer in the presence of DMPO and superoxide dismutase (a superoxide scavenger). It is thus evident that oxygenation of $[Co^{II}(PMA)]^+$ gives rise to $[Co^{III}(PMA)(O_2^-)]^+$ from which superoxide diffuses out from the metal coordination sphere and enters the bulk solution. The vacant coordination site on the Co(III) center is eventually filled by a solvent molecule. In aqueous solution, these steps lead to $[Co^{III}(PMA)(H_2O)]^{2+}$ as the final product (vide Experimental Section). We suspect that damage of PMAH by the superoxide (and radicals derived from it in aqueous solution) is responsible for the low yield of $[Co(^{III}(PMA)(H_2O)]^{2+}$ in the oxygenation procedure. $[Co^{III}(PMA)(H_2O)]^{2+}$ is better synthesized from the Co(III) starting material $Na_3[Co(CO_3)_3]$.[12]

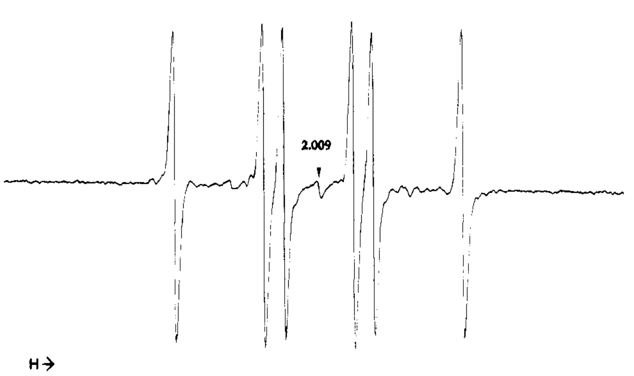


Figure 2. ESR spectrum of the DMPO−superoxide spin adduct in DMF (298 K). The spin adduct was generated by thawing a sample of $[Co^{III}(PMA)(O_2^-)]^+$ in presence of excess DMPO.

Summary and Conclusions

The following are the principal results and conclusions of this investigation.

(i) In $[Co^{II}(PMA)]^+$, the Co(II) complex of the tailored ligand PMAH, the deprotonated PMA^- framework employs five N donor centers located in the primary and secondary amines, pyrimidine and imidazole rings, and the deprotonated amido moiety to bind the Co(II) center. The complex exhibits a nearly axial EPR spectrum which is typical of low-spin Co(II) in 5-coordinated square-pyramidal geometry. The EPR parameters are very similar to those for Co(II)−BLM. In this respect, $[Co^{II}(PMA)]^+$ is a good model for Co(II)-BLM and provides further support to the proposed coordination structure of Co(II)−BLM.[5] Furthermore, $[Co^{II}(PMA)]^+$ can be generated by borohydride reduction from the structurally characterized $[Co^{III}(PMA)(H_2O)]^{2+}$ which affords $[Co^{II}(PMA)]^+$ in quantitative yield.

(ii) Exposure of $[Co^{II}(PMA)]^+$ to dioxygen results in the formation of the superoxide adduct, $[Co^{III}(PMA)(O_2^-)]^+$. This conclusion is supported by the fact that the structure characterized complex $[Co^{III}(PMA)(H_2O)]^{2+}$ reacts with KO_2 to produce the superoxide adduct that exhibits EPR features identical to those of $[Co^{III}(PMA)(O_2^-)]^+$ generated from the oxygenation reaction. Furthermore, $[Co^{III}(PMA)(O_2^-)]^+$ is generated when

---

(25) Sawyer, D. T.; Yamaguchi, K.; Calderwood, T. S. In *Handbook of Methods for Oxygen Radical Research*; Greenwald, R. A., Ed., CRC Press: Boca Raton, FL, 1985; p 65.

$[Co^{III}(PMA)(H_2O)]^{2+}$ is first reduced with borohydride and then reacted with O_2. The EPR parameters of $[Co^{III}(PMA)(O_2^-)]^+$ match well with those of the Co(II)-BLM-O_2 complex.

(iii) The coordinated superoxide ion eventually escapes from $[Co^{III}(PMA)(O_2^-)]^+$ into solution and $[Co^{III}(PMA)(H_2O)]^{2+}$ is produced. The superoxide has been trapped as the DMPO spin-adduct. The presence of O_2^- in $[Co^{III}(PMA)(O_2^-)]^+$ has further been confirmed by the bleaching of the ESR signal of the DMPO-superoxide spin adduct when $[Co^{III}(PMA)(O_2^-)]^+$ is allowed to decompose in a reaction mixture that also contain SOD, a superoxide scavenger.

Acknowledgment. We thank the American Cancer Society and the National Institutes of Health for support of this research under Grant No. CH-481 and No. CA53076. E.F. was partially supported by a NIH-MBRS grant (GM08132).