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IDENTIFICATION OF A KEY STRUCTURAL FEATURE OF COBALT(III)-BLEOMYCINS: AN EXOGENOUS LIGAND (e.g. HYDROPEROXIDE) BOUND TO COBALT

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SUMMARY. A series of cobalt(III) complexes of the anticancer antibiotic bleomycin has been prepared. Mass spectrometry and enzymatic analysis show that the green cobalt-bleomycin complex contains a hydroperoxide (-OOH) group bound to cobalt with unusual stability. Under appropriate conditions, cobalt-bleomycins containing other monodentate ligands to cobalt can be formed; fast-atom bombardment mass spectra of such complexes show peaks for cobalt-bleomycin at the expected mass, and also peaks for the intact complexes at the required higher mass.

INTRODUCTION: Bleomycin (BLM) is the name of a group of glycopeptide antibiotics used in the treatment of cancer (1); the drug is thought to act *in vivo* by chemically degrading intracellular DNA (2,3). Bleomycin forms complexes with transition-metal ions, and its biological activity appears to depend on metal complexation (4-7). Cobalt(III) complexes of bleomycin have the unusual property of accumulating selectively in the nuclei of certain types of cancer cells (8-12). Several different Co(III)-BLM complexes are formed upon air oxidation of aqueous solutions of Co(II) and BLM under various conditions (13-22). We have recently found that light can cause some of these Co(III)-BLMs to efficiently degrade DNA in vitro (18), and that different Co(III)-BLMs show significant differences in uptake by murine tumors in vivo (13). Understanding the molecular reasons for these differences should provide useful insight into bleomycin's biological activity.

Abbreviations: BLM, bleomycin; Co(III)-BLM, a cobalt(III)-bleomycin complex.

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MATERIALS AND METHODS

Blenoxane (bleomycin sulfate) was a gift from Bristol Laboratories. Horseradish peroxidase (type vi) and o-dianisidine were purchased from Sigma Chemical Co. Other chemicals were the purest commercially available reagents. Distilled, deionized water was used throughout.

Preparation of Co(III)-Bleomycins. A 5-ml aqueous solution containing Blenoxane (15 μmol) and a slight excess of $CoCl_2$ (or $CoSO_4$) was adjusted to pH 7.0 with dilute NaOH and allowed to stand at room temperature overnight. The resulting greenish-brown solution was then applied to a Sephadex C-25 column (1 x 50 cm, NH_4^+) and eluted with a 1-liter gradient of 0.05 to 0.5M ammonium formate, pH 5.7. Four colored bands, corresponding to the green A_2, green B_2, brown A_2, and brown B_2 fractions, were eluted at ionic strengths 0.19, 0.23, 0.33, and 0.43M. The previously reported (13) orange A_2 and B_2 complexes could be obtained by heating the original product mixture at 96°C for 3 h; these eluted from the column slightly before the respective green complexes. Other Co(III)-BLMs could be prepared from the original product mixture by incubating with an appropriate ligand. The thiocyanate adduct was prepared by adding NaSCN to 0.5M and incubating for 3 days at pH 3.6, room temperature; the nitrite and the azide adducts were prepared similarly. Progress of these reactions was monitored by HPLC; and products were purified on Sephadex C-25, followed by preparative HPLC.

Fast-atom bombardment mass spectra were taken by Dr. Ken Straub on a Kratos MS-50 mass spectrometer equipped with an FAB source using an Xe^+ ionizing beam (40 μA, 8 kV).

H_2O_2 Assays. The qualitative ferricyanide test was performed by adding a Co(III)-BLM (1 mM, 40 μl) to a solution containing 30 μl of 0.4% $FeCl_3$ (in 10 mM HCl) and 30 μl of 0.8% $K_3Fe(CN)_6$. Only the green Co(III)-BLM gave an immediate blue precipitate, as does H_2O_2. Horseradish peroxidase (23) was used to quantitate the H_2O_2 released upon gentle hydrolysis of green Co(III)-BLM. The assay solutions contained 0.9 ml of 10 mM phosphate, pH 6.0, to which were added 10 μl of o-dianisidine (0.5%, in CH_3OH), aliquots (0 - 90 μl) of 1 mM H_2O_2 or 1 mM Co(III)-BLM, and 20 μl of horseradish peroxidase (10 μg/ml). The absorbance at 460 nm was read 20 min after adding the enzyme.

RESULTS

Because the HPLC procedure separates Co(III)-BLMs of very similar structure (as shown in Figure 1), it is indispensable in checking their purity and useful in studying their reactions. In particular, the brown Co(III)-BLM fraction from the C-25 column was shown by HPLC to contain two different complexes.

These two brown Co(III)-BLMs interconvert slowly and reversibly as the formate concentration is changed (17), in the way expected for reversible replacement of cobalt-bound water by formate ion. The introduction of other anions such as acetate, thiocyanate, azide, or nitrite leads to the formation of new Co(III)-BLMs. This behavior is very similar to that observed for

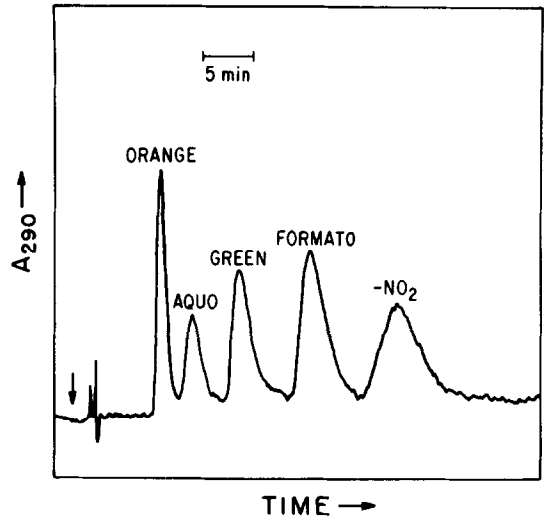

Figure 1. Separation of different cobalt(III)-bleomycin A_2 complexes by reverse-phase liquid chromatography on a Waters 0.4 x 30 cm C_{18} column, using an 86:14 (by volume) mixture of 0.1M aqueous ammonium acetate and acetonitrile (2 ml/min). Arrow indicates injection of sample.

acidopentammine cobalt (III) complexes, in which the acido ligand can be replaced by water or another anion under the appropriate conditions (24). The absorption spectra of the Co(III)-BLMs change in the manner expected for coordination of each anion directly to Co(III), as shown in Table I. These complexes also have characteristic NMR and CD spectra (25).

TABLE I. Visible Absorption Maxima of Cobalt(III)-Bleomycin Complexes

Species	Color	Lambda Max/nm[a]
HOO-Co(III)-BLM[b]	Green[c]	594
Aquo-Co(III)-BLM[b]	Golden Brown	544
Formate-Co(III)-BLM	Brown	560
NO_2-Co(III)-BLM	Orange	520[d]
SCN-Co(III)-BLM	Burgundy	552
N_3-Co(III)-BLM	Olive Brown	520,600[d]
Co(III)-BLM	Orange[c]	520[d]

[a]Each species (except the azide) also has an absorption maximum near 450 nm.

[b]As shown by the similarity of CD spectra and chromatographic behavior for corresponding species, the hydroperoxo- and aquo-Co(III)-BLMs are the type I and type II complexes of ref. 15. The hydroperoxo-Co(III)-BLM was formerly suggested to have a dinuclear μ-peroxo structure (20).

[c]Ref. 13.

[d]Shoulder.

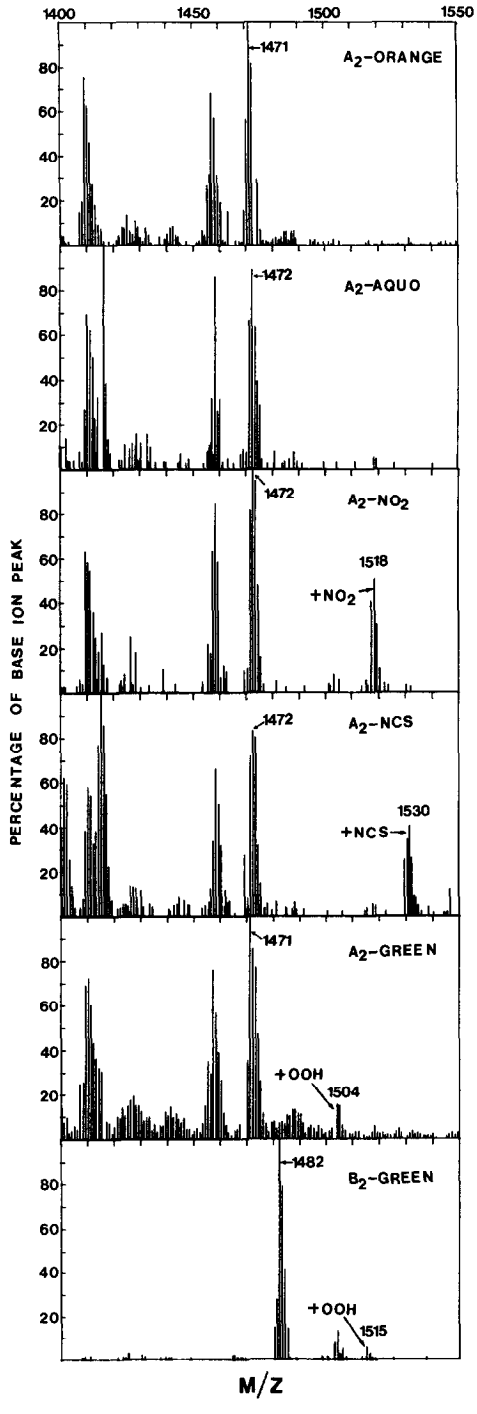

Figure 2. Fast-atom-bombardment mass spectra of cobalt(III) bleomycins. The pseudomolecular ion of cobalt-bleomycin A_2 yields a group of peaks at m/z 1470-1473, corresponding to its mass; that of cobalt-bleomycin B_2 occurs 11 mass units higher. Peaks due to cobalt-bleomycins bearing an exogeneous ligand occur at the expected higher mass, as shown clearly for the NO_2 and NCS complexes and suggested for the "green" hydroperoxide (OOH) complexes of cobalt-bleomycin A_2 and B_2.

The presence of a coordinated nitrite or thiocyanate group in the respective Co(III)-BLMs is strikingly confirmed by fast atom bombardment mass spectrometry, as shown in Figure 2. It should be noted that in a large molecule such as bleomycin (formula $C_{55}H_{84}N_{17}O_{21}S_3^+$ (26)), *groups* of peaks (rather than individual peaks) are seen in the mass spectrum because of the significant probability that a given molecule will contain one or more rare isotopes such as ^{13}C. The prominent peaks at m/z 1470-1473 are due to the pseudomolecular ion of cobalt-bleomycin A_2, containing no additional ligands; the bottom spectrum of a cobalt-bleomycin B_2 species has the pseudomolecular ion peak 11 mass units higher, which is the mass difference between bleomycins A_2 and B_2. The NO_2-Co(III)-BLM A_2 complex gives a peak at m/z 1518, as expected for the additional mass of the NO_2 group; likewise, the SCN-Co(III)-BLM A_2 complex gives a peak at m/z 1530. Orange Co(III)-BLM A_2 does not show significant peaks beyond the pseudomolecular ion mass, suggesting that it contains no additional ligand. However, aquo-Co(III)-BLM also shows no significant peaks at higher mass; this may be due to easy loss of water from the complex.

The mass spectra also led us to speculate that the green Co(III)-BLM contains coordinated hydroperoxide. In the bottom two spectra of Figure 2, small peaks 33 mass units heavier than the pseudomolecular ions may be seen. These peaks were quite reproducible in repeated experiments, but their low intensity does not provide the quality of evidence seen for the NO_2 and SCN adducts of Co(III)-BLM. Preparation of green Co(III)-BLM using $^{18}O_2$ in order to shift the adduct peak 4 mass units higher provided inconclusive results. Comparison of the infrared spectra of green Co(III)-BLM made with $^{16}O_2$ and $^{18}O_2$ also was inconclusive, due to the complexity of the spectra.

Chemical evidence that green Co(III)-BLM contains coordinated hydroperoxide was obtained by allowing this complex to stand at pH 2.7 in aqueous solution in the dark for several days -- which partly hydrolyzes it to aquo-Co(III)-BLM -- and then assaying the solution to determine whether H_2O_2 has been produced by the hydrolysis. Both the qualitative ferricyanide test

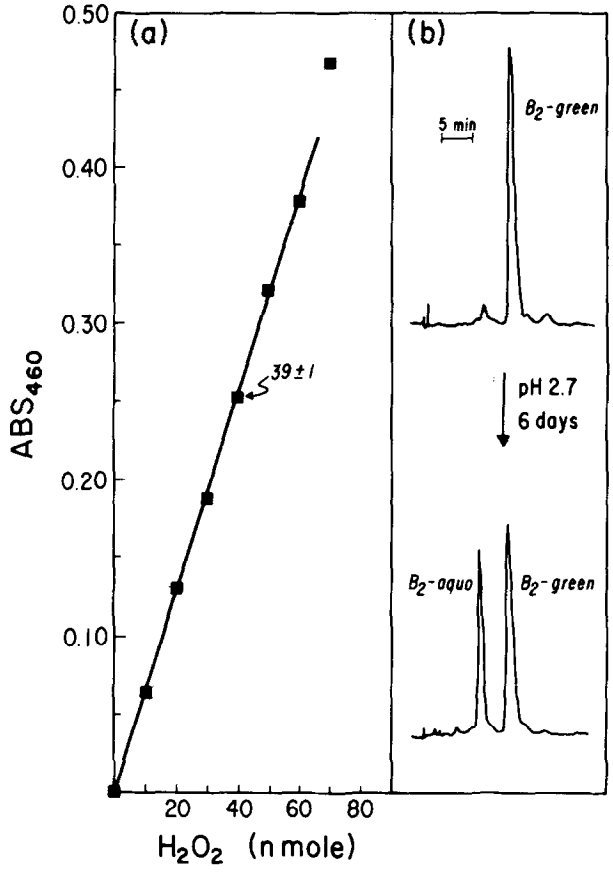

Figure 3. Enzymatic assay of the H_2O_2 produced by gentle hydrolysis of "green" cobalt-bleomycin B_2. (a) Calibration curve showing result of triplicate determination that 39 ± 1 nmol of H_2O_2 was produced. (b) Column trace showing "green" cobalt-bleomycin B_2 sample before and after partial hydrolysis to aquo-cobalt-bleomycin B_2; this produced 42 ± 1 nmol of aquo-cobalt-bleomycin B_2. Therefore, one mol of "green" cobalt-bleomycin B_2 contains 0.93 ± 0.05 mol of a ligand which yields H_2O_2 upon standing at pH 2.7. Other cobalt-bleomycins (aquo, nitro, formato, thiocyanato) did not yield H_2O_2.

and the quantitative assay using horseradish peroxidase (29,23) were positive. The latter (Figure 3) showed that this gentle acid treatment of green Co(III)-BLM released 0.93 ± 0.05 mols of H_2O_2 for each mol of green Co(III)-BLM hydrolyzed, and that none of the other Co(III)-BLMs (aquo, nitro, formato, or isothiocyanato) released a detectable amount of H_2O_2. This is the result expected for coordinated hydroperoxide in the green Co(III)-BLM, and the stoichiometry found rules out the possibility that the mol ratio of cobalt to O_2 is anything but one to one (20).

DISCUSSION

Since air oxidation of Co(II) complexes in the presence of nitrogen ligands usually proceeds via a dioxygen coordinated intermediate (30), it is possible for the green complex to have a ligand which originated from dissolved oxygen. The existence of a mononuclear dioxygen adduct of Co(II)-BLM is indicated by the epr studies of Sugiura (31); this could lead to the hydroperoxide. Similar reaction schemes have been suggested for iron-bleomycin (32-34).

The *in vitro* degradation of DNA by bleomycin involves an oxygen-requiring oxidation/reduction cycle of iron-BLM (5,35). A difference between iron- and cobalt-BLMs is that the Co(III) complexes will be kinetically inert; that is, they will not exchange ligands readily. While a ligand with reducing properties might be able to coordinate to a Fe(III)-BLM complex so that the redox cycle could repeat itself, such a process would happen slowly (or not at all) with Co(III)-BLM. Nevertheless, in the presence of light, green and brown Co(III)-BLMs are strikingly efficient in nicking supercoiled DNA (18). We are now investigating the mechanism of this reaction.

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REFERENCES

(1) Umezawa, H., Maeda, K., Takeuchi, T., and Okami, Y. (1966) J. Antibiotics, Ser. A, 19, 200-209.
(2) Suzuki, H., Nagai, K., Yamaki, H., Tanabe, N., and Umezawa, H. (1969) J. Antibiotics 22, 446-448.
(3) Terasima, T., Yasukawa, M., and Umezawa, H. (1970) GANN 61, 513-516.
(4) Sausville, E. A., Peisach, J., and Horwitz, S. B. (1978) Biochemistry 17, 2740-2746.
(5) Sausville, E. A., Stein, R. W., Peisach, J., and Horwitz, S. B. (1978) Biochemistry 17, 2746-2754.
(6) Freedman, J. H., Horwitz, S. B., and Peisach, J. (1982) Biochemistry 21, 2203-2210.
(7) Margusan, N., Ehrenfeld, G. M., and Hecht, S. M. (1982) J. Biol. Chem. 257, 8600-8603.

(8) Nouel, J. P., Renault, H., Robelt, J., Jeanne, C., Wicart, L. (1972) La Nouvelle Presse Medicale 1, 95-98.
(9) Kono, A., Kojima, M., Maeda, T., (1972) Radioisotopes 21, 118-120.
(10) Maeda, T., Kono, A., Kojima, M. (1972) Radioisotopes 21, 436-
(11) Kono, A., Matsushima, Y., Kojima, M., and Maeda, T. (1977) Chem. Pharm. Bull. 25, 1725-1731.
(12) Kono, A. (1977) Chem. Pharm. Bull. 25, 2882-2886.
(13) DeRiemer, L. H., Meares, C. F., Goodwin, D. A., and Diamanti, C. I. (1979) J. Med. Chem. 22, 1019-1023.
(14) Raban, P., Brousil, J., Svihovcova', P. (1979) Eur. J. Nucl. Med. 4, 191-197.
(15) Kikinuma, J., Kagiyama, R., Orii, H. (1980) Eur. J. Nucl. Med. 5, 159-163.
(16) Vos, C. M., Westera, G., van Zanten, B. O. (1980) J. Inorg. Biochem. 12, 45-55.
(17) Vos, V. M., Westera, G., Schipper, D. (1981) J. Inorg. Biochem. 15, 253-260.
(18) Chang, C-H., and Meares, C. F. (1982) Biochemistry 21, 6332-6334.
(19) Nunn, A. D. (1977) Int. J. Nucl. Med. Biol. 4, 204-205.
(20) Garnier-Suillerot, A., Albertini, J.-P., and Tosi, L. (1981) Biochem. Biophys. Res. Comm. 102, 499-506.
(21) Tsukiyama, M., Randall, C. R., Santillo, F. S., and Dabrowiak, J. C. (1981) J. Am. Chem. Soc. 103, 458-461.
(22) Dabrowiak, J. C., and Tsukuyama, M. (1981) J. Am. Chem. Soc. 103, 7543-7550.
(23) Worthington Enzyme Manual (1972) pp 43-45, Worthington Biochemical Corp., Freehold, N.J.
(24) Basolo, F., and Pearson, R.G. (1967) Chapter 3 in Mechanisms of Inorganic Reactions. A Study of Metal Complexes in Solution, 2nd Ed., John Wiley & Sons, New York.
(25) Chang, Dallas, and Meares, unpublished observations.
(26) Positive-ion FAB mass spectra have been reported for metal-free BLM A_2 and B_2, and for the ferrous complex of A_2 (27,28). The pseudo-molecular ion was observed for BLM A_2 at m/z 1414, corresponding to the sulfonium cation species ($C_{55}H_{84}N_{17}O_{21}S_3^+$).
(27) Barber, M., Bordoli, R. S., Sedgwick, R. D., Tyler, A. N., and Bycroft, B. S. (1981) Biochem. Biophys. Res. Comm. 101, 632-638.
(28) Dell, A., Morris, H. R., Hecht, S. M., and Levin, M. D. (1980) Biochem. Biophys. Res. Comm. 97, 987-994.
(29) Feigl, F. (1958) p 354 in Spot Tests in Inorganic Analysis, Elsevier, Amsterdam.
(30) Fallab, S. (1967) Angew. Chem. Internat. Edit. 6, 496-507.
(31) Sugiura, Y. (1980) J. Am. Chem. Soc. 102, 5216-5221.
(32) Kuramochi, M., Takahashi, K., Takita, T., and Umezawa, H. (1981) J. Antibiotics 34, 576-582.
(33) Burger, R. M., Peisach, J., Blumberg, W. E., and Horwitz, S. B. (1979) J. Biol. Chem. 254, 10906-10912.
(34) Burger, R. M., Peisach, J., and Horwitz, S. B. (1981) J. Biol. Chem. 256, 11636-11644.
(35) Lown, J. W., and Sim, S.-K. (1977) Biochem. Biophys. Res. Commun. 77, 1150-1157.