MECHANISM OF INACTIVATION OF THE POLYENE ANTIBIOTIC AMPHOTERICIN B

EVIDENCE FOR RADICAL FORMATION IN THE PROCESS OF AUTOOXIDATION

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(Received for publication September 17, 1984)

Radical formation during autooxidation of the polyene antibiotic amphotericin B was monitored by following the kinetics of decay of the ESR signal of a stable nitroxide. The kinetics were seen to depend both on antibiotic and on nitroxide concentration. The radicals formed were studied by spin trapping. Three preparations — clinical Fungizone, amphotericin B suspended in buffer, and amphotericin B in buffer - 10% dimethyl sulfoxide — yielded spin adducts of different nature and/or concentrations. In the absence of dimethyl sulfoxide, amphotericin B yielded at least two carbon-centered radicals whose spectra indicated restricted mobility. This suggests that the radicals arise from the whole amphotericin B molecule located in molecular aggregates present in the preparations, and not from smaller and possibly more water-soluble fragments of the antibiotic. In the presence of dimethyl sulfoxide the spin adducts derived from secondary carbon radicals originated from this solvent. Their spectra were indicative of fast tumbling. Direct evidence for autooxidation was obtained by measuring oxygen consumption. All processes examined occurred at the same time scale observed for drug inactivation, in agreement with the idea that loss of activity is due to antibiotic autooxidation.

The autooxidation of polyene antibiotics has been reported[1,2] and is thought to be a possible mechanism of inactivation[3]. The addition of antioxidants has been shown to delay the inactivation of amphotericin B[4,5], but no chemical studies or evidence for the involvement of free radicals in the autooxidation process have been presented.

RICKARDS *et al.*[2] have studied the autooxidation of the polyene antibiotics filipin and lagosin. It was established that the major degradation products of those polyenes were the corresponding epoxides, which were proposed to originate from a process involving radical formation. However, a direct ESR examination of the reaction systems failed to yield spectra of radicals, possibly because of their low steady state concentration.

In the present work, radical formation during the autooxidation of amphotericin B (AB) was monitored using the two following approaches: 1) The reaction between paramagnetic species generated in the process and the stable nitroxide 2,2,6,6-tetramethyl-4-hydroxy-*N*-oxylpiperidine (TEMPOL), which led to the loss of TEMPOL ESR signal; and 2) the reaction between the former radicals and a spin trap, phenyl-*t*-butyl nitrone (PBN). Autooxidation was monitored by measurement of oxygen consumption. Radical formation and autooxidation occurred at the same time scale as antibiotic inactivation[4,5].

Materials and Methods

ESR spectra were obtained with a Varian E-4 X-band spectrometer at room temp. Flat quartz

Fig. 1. Effect of AB concentration on the kinetics of TEMPOL ESR signal decay at 27°C in 260 mOsm phosphate buffer - 10 % DMSO, pH 7.4.
TEMPOL=5 μM. AB (mM): ○, 1.0; ●, 2.5; □, 5.0; ■, 7.5; △, 10.

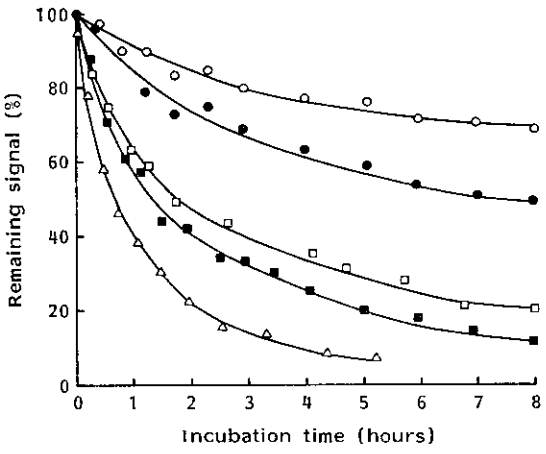
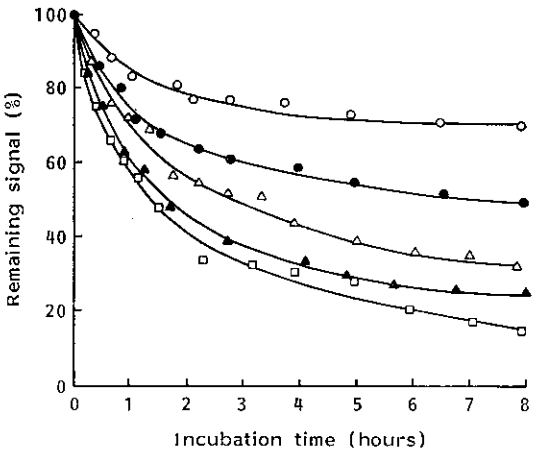


Fig. 2. Effect of initial TEMPOL concentration on the kinetics of TEMPOL ESR signal decay at 27°C in 260 mOsm phosphate buffer - 10% DMSO, pH 7.4.
AB=5 mM. TEMPOL (μM): ○, 25; ●, 10; △, 7.5; ▲, 5.0; □, 2.5.



cells for aqueous solutions came from "James Scanlon", Costa Mesa, California. Oxygen consumption was measured in an oxygen biological monitor Model 53, from Yellow Springs Instruments.

AB, type II, was a gift of Squibb Indústria Química S.A., São Paulo, Brazil. It was stored at −10°C in the dark. Dimethyl sulfoxide (DMSO, spectroscopic grade) was purchased from Merck, Quimitra Comércio e Indústria Química S.A., São Paulo, Brazil. TEMPOL was a gift from Dr. ROLF MEHLHORN from the Department of Physiology and Anatomy, University of California, Berkeley. PBN was purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconcin, U.S.A., all other reagents were analytical grade.

Unless otherwise stated, AB solutions were freshly prepared in DMSO and diluted to a final 10% (v/v) DMSO concentration with 260 mOsm NaCl-phosphate buffer, pH 7.4. AB was also used as the clinically employed preparation, Fungizone, which consists of AB: deoxycholate, 1: 2, on a molar basis. In one experiment (Fig. 3b), AB was directly dispersed in buffer.

Results

Kinetics of the Decay of TEMPOL ESR Signal

In the presence of AB, the amplitude of the TEMPOL ESR signal decreases with time. This was ascribed to a reaction between the nitroxide and free radicals formed as intermediates during the autooxidation of the antibiotic. That the process requires oxygen was verified by the lack of signal loss when the reaction was carried out under nitrogen.

The effect of time on the amplitude of the TEMPOL ESR signal was monitored both as a function of AB concentration (Fig. 1) and of TEMPOL concentration (Fig. 2).

The kinetics are complex, as expected for chain radical reactions, and do not seem to follow any simple rate equation. The rate of signal decay increases with AB concentration, but has an opposite behavior with respect to TEMPOL concentration. The reason for this will be analyzed in the discussion.

PBN Spin Adducts of Different AB Preparations

In the presence of AB, PBN gives rise to spin adducts whose nature and/or quantity depend on the method of sample preparation.

Fig. 3. ESR spectra of PBN spin adducts.
a: Fungizone (10 mM AB), the arrows indicate the broader sub-spectrum.
b: 10 mM AB directly dispersed in 260 mOsm phosphate buffer, pH 7.4.
c: 10 mM AB in 260 mOsm phosphate buffer - 10 % DMSO (v/v), pH 7.4, sample prepared by adding buffer to AB previously dissolved in DMSO. Spectra were obtained after *ca.* 20 hours. PBN=0.1 M.

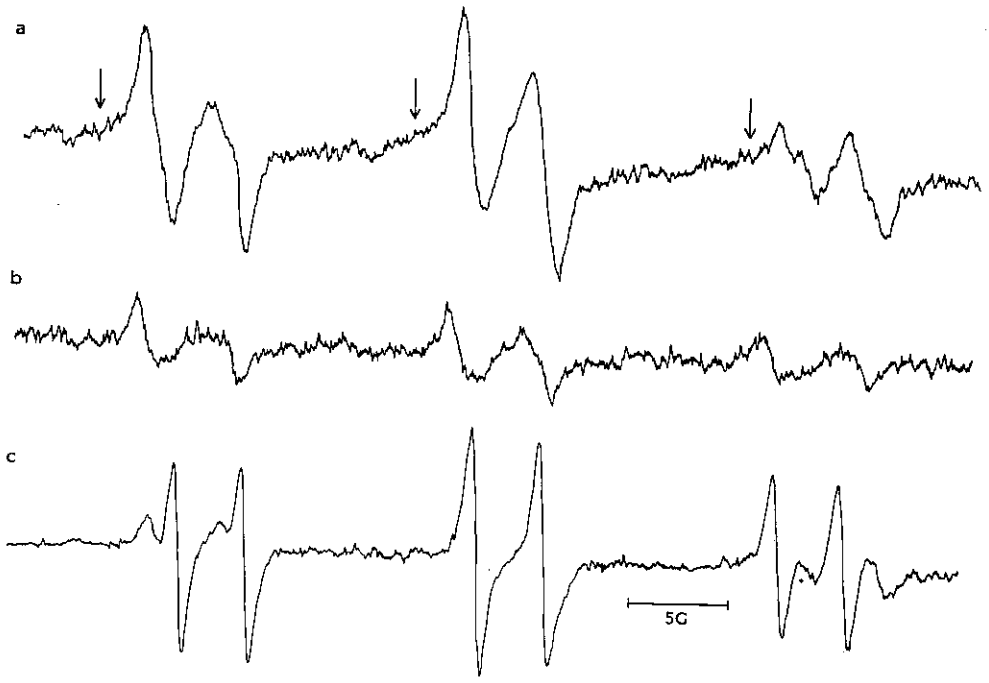


Table 1. Hyperfine splitting constants of PBN spin adducts.

System	Spectrum	a^N (Gauss)	a^H (Gauss)
Fungizone (Fig. 3a)		16.10±0.10	3.50±0.10
AB in phosphate buffer - 10 % DMSO (Fig. 3c)	A	15.05±0.05	3.35±0.05
		15.10*	3.42*
	B	16.40±0.05	3.70±0.05
		16.46*	3.60*

* Taken from ref 9.

The spectra yielded by Fungizone and AB dispersed in buffer are shown in Figs. 3a and 3b, respectively. Although present in smaller amount, the spectrum in Fig. 3b resembles very closely that in Fig. 3a. The low concentration of radicals formed by AB directly suspended in buffer is probably due to its very low solubility in H_2O. Fungizone provides a much more soluble preparation of the antibiotic, thus enhancing radical formation. Control experiments with deoxycholate showed that, for the same periods of time (*ca.* 20 hours), no signal was detected in the presence of PBN.

At least two sub-spectra are seen in Fig. 3a. Both display differentially broadenced lines, indicative of a slowly tumbling paramagnetic species. Due to the large linewidths and low intensity of one of the sub-spectra (see arrows in Fig. 3a) it was impossible to measure its hyperfine parameters. The spectral parameters for the narrower sub-spectrum are given in Table 1.

Depending on the PBN batch, variable amounts of paramagnetic degradation products of PBN

could also be detected. Their spectra were seen to correspond to the benzoyl adduct of PBN[6,7] and to *t*-butyl nitroxide[8].

AB is highly insoluble in H_2O; its solubility can be improved by first dissolving it in DMSO and then adding buffer. In such system, containing 10% DMSO (which was also used for the studies with TEMPOL), the spectra (Fig. 3c) display hyperfine splittings (Table 1) very similar to those reported by SAPRIN and PIETTE for carbon radicals originating from DMSO (Table 1 and ref 9). The spectrum in Fig. 3c was obtained after 20 hours. A similar spectrum is obtained for PBN in buffer - 10% DMSO after much longer periods (*ca.* 40 hours). The DMSO-PBN adducts yield spectra with narrow lines, indicative of fast tumbling.

Kinetics of Oxygen Consumption

Autooxidation of amphotericin B was monitored directly through the measurement of oxygen consumption. This process occurred at the same time scale as that for the decay of the TEMPOL ESR signal. In the presence of 5 mM AB, in buffer - 10% DMSO, a 5 μM TEMPOL solution lost 30% signal in 41 minutes (calcd from Fig. 1), while 30% oxygen consumption took place in 114 minutes. The difference could be due to the involvement of the nitroxide in secondary reactions. This reasoning is supported by the fact that in the presence of DMSO the spin adducts are derived from this molecule.

Discussion

Fig. 1 shows that the rate of decay of the TEMPOL ESR signal increases with increasing antibiotic concentration, whereas Fig. 2 indicates that this rate seems to be faster for lower spin label concentrations. This result can be rationalized in the following manner; the rate determining step is the formation of radicals by AB. Since these are formed at a very low steady state concentration, it is easier to detect them when they react with low spin label concentrations. In this latter case, a larger fraction of total spin label will react, while a smaller fraction will react when the spin label concentration is high, leading to an apparently anomalous effect of TEMPOL concentration on the reaction rate.

The nature and/or quantity of PBN spin adducts depended on the method of sample preparation (Fig. 3). The amount of radical formed by AB directly dispersed in buffer (Fig. 3b) is much smaller than either that formed by Fungizone (Fig. 3a) or by samples where the antibiotic was first dissolved in DMSO (Fig. 3c). Aggregation properties of AB and the effect of aggregation on autooxidation will be discussed in a further publication (M. T. LAMY-FREUND, V. F. N. FERREIRA, A. FALJONI-ALÁRIO and S. SCHREIER, in preparation).

In the absence of DMSO (Figs. 3a and 3b), the hyperfine parameters measured for the narrower sub-spectrum (Table 1) indicate that a carbon-centered radical was trapped[10]. The broader spectrum (for which hyperfine parameters could not be measured) is probably also due to a carbon-centered radical since the hydrogen hyperfine splitting seems to be similar to that of the narrower spectrum (Figs. 3a and 3b). The two sub-spectra in Fig. 3a and the two sub-spectra in Fig. 3b show differentially broadened lines, indicative of slow tumbling. This is probably due to the location of the spin adducts in sites where mobility is restricted. These sites could be provided by the (different) aggregates present in the preparations. The results also suggest that the radicals correspond to spin adducts of the whole AB molecule, since fragments of the antibiotic, due to their smaller size, and possibly to their increased water partitioning, should display narrow line spectra. We do not have at present an explanation for the different linewidths of the two sub-spectra displayed by the spin adducts in Figs. 3a and 3b.

The findings that the spin adducts are carbon-centered radicals and the suggestion that the spin adducts were formed on the whole amphotericin B molecule are in agreement with the results of RICKARDS *et al.*[2] who have shown that the major products of autooxidation of the polyene antibiotics filipin and lagosin were the corresponding epoxides. The authors also showed that the epoxides were formed at the more substituted ends of the polyene systems, probably due to the high activation energy

that would be required to remove the planarity of the all *trans* pentaene system in the large lactone ring. However, the authors failed to detect intermediary free radicals by ESR, probably because of their low steady state concentration. The carbon radicals trapped by PBN could be intermediates in epoxide formation.

In the presence of 10% DMSO, the spin adducts display the spectra shown in Fig. 3c. The hyperfine parameters of these spectra (Table 1) are very similar to those obtained by SAPRIN and PIETTE (Table 1, ref 9) during studies of lipid peroxidation by microsomes in the presence of DMSO. The authors ascribed the spectra to two carbon radicals derived from the solvent. Although this interpretation was questioned by JANZEN[10], it seems to apply to our system. We employed DMSO at a much higher concentration (10%) than AB (5 mM) and the reaction of PBN with secondary radicals stemming from the former should be favored. Moreover, the narrow lines in Fig. 3c are indicative of fast tumbling, which would be expected for small radicals.

Measurements of oxygen consumption provided direct evidence for autooxidation and showed that this process occurs at the same time scale as radical formation.

Although autooxidation was studied in detail from the chemical point of view for the pentaenes filipin and lagosin, radical formation was not demonstrated[2]. Moreover, there have been no such studies for the heptaene amphotericin B. BEGGS and coworkers have shown, through the examination of microorganism viability that AB underwent inactivation with time[3], and that addition of antioxidants delayed this process[4,5].

Our results show that amphotericin B undergoes autooxidation and that free radicals are formed during this process. The time scale of these events, of the order of hours, is the same as that found for loss of drug activity, suggesting that autooxidation could be involved in the mechanism of antibiotic inactivation.

Acknowledgments

We are very grateful to Dr. O. AUGUSTO for long discussions and helpful criticism. M. T. LAMY-FREUND and S. SCHREIER are recipient of research fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). V. F. N. FERREIRA has an undergraduate schollarship from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). This project was supported by research grants from FAPESP and Financiadora de Projetos (FINEP).

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