Magnetic Resonance Studies of Fredericamycin A: Evidence for O_2-Dependent Free-Radical Formation†

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ABSTRACT: Fredericamycin A, a newly described potent antitumor antibiotic, exhibits unusual spectroscopic and physical properties. The drug shows a striking color change from red to blue on exposure to O_2, with the appearance of an optical absorption band at 675 nm; on addition of acid these changes are readily reversed. ^1H and ^{13}C NMR spectra of fredericamycin A show that the resonances from the quininoid half of the molecule disappear after exposure to O_2 but reappear on acidification in parallel with the observed optical spectral shift. These unusual NMR data are explained by electron spin resonance studies which demonstrate that fredericamycin A spontaneously forms an oxidized free radical with electron transfer to O_2. The observed hyperfine structure of this radical is consistent with one-electron oxidation of the quininoid group. After fredericamycin A is exposed to O_2, an EPR signal is observed with axial symmetry with temperature and power saturation behavior suggestive of $^{\bullet}O_2^-$. Spin-trapping EPR studies demonstrate that the drug reduces O_2 to $^{\bullet}O_2^-$ and H_2O_2 to $^{\bullet}OH$. This spontaneous mechanism of O_2 reduction with the generation of oxidized drug free radicals and reduced oxygen free radicals is unprecedented among anticancer drugs, suggesting that fredericamycin A could be the forerunner of a new class of anticancer drug.

Fredericamycin A (NSC 305263) is an antitumor antibiotic produced by *Streptomyces griseus* (FCRF-48) (Pandey et al., 1981). In addition to possessing in vitro activity against Gram-positive bacteria and fungi, fredericamycin A was shown to be cytotoxic in vitro and active in vivo against several transplantable tumors in mice (Warnick-pickle et al., 1981). The structure of fredericamycin A was established by single-crystal X-ray analysis (Misra et al., 1982). Its spiro ring structure (Figure 1A) is unique for antibiotics. The biosynthesis of fredericamycin A has been studied by tracer methods using ^{14}C- and ^{13}C-labeled precursors (Byrne et al., 1985). The difficulty of rationalizing the abnormal appearance of the NMR and UV–visible spectra in particular led us to investigate whether fredericamycin A might form a free radical.

In this paper we report conclusive evidence demonstrating that fredericamycin A forms a stable oxidized free radical on exposure to oxygen. This accounts for the unusual properties of the drug. Furthermore, we discuss the implications of this discovery on possible mechanisms of action of the drug.

Experimental Procedures

(*1*) *UV.* UV–visible spectra were obtained using a Hewlett-Packard HP-8405A diode array spectrophotometer. Samples were contained in a 0.2-mm cell from Precision Cell, Inc. Spectra were the averages of 10 1-s acquisitions. Samples were aliquots from those samples used to obtain the proton NMR spectra below, without further treatment.

(*2*) *NMR.* ^1H NMR spectra were obtained at 300.074 MHz on a Nicolet NT-300 wide-bore spectrometer using a 5-mm fixed-tune probe, locked on deuterium of the solvent, hexadeuteriodimethyl sulfoxide (Merck) and referenced by Me_4Si[1] (0.00 ppm). Spectra were recorded at ambient temperature. Nuclear Overhauser enhancement (NOE) experiments were performed using flip angles of about 45°, a pulse width of ca. 1 s for selective presaturation of selected resonances, and an 8-s recycle time. All lines to be irradiated were assembled into a list of offset frequencies, and then data were acquired by interweaving frequencies. Difference spectra were then obtained to check for an NOE effect. Spectra (200 MHz) were obtained on a Varian XL-200 spectrometer equipped with the Advance data system and a 5-mm carbon–proton switchable probe.

(*3*) *EPR.* EPR spectra were recorded with a Varian E-9 spectrometer operating at X-band with a TE104 cavity. Microwave frequency measurements performed with a Hewlett-Packard 5342A microwave frequency counter and magnetic field measurements with a Bruker-IBM ER 035M proton probe Gauss meter. The spectrometer is interfaced to an IBM-PC via a DT2801 A to D converter. Signal acquisition and integration were performed by the EPRDAS software package from Adaptable Laboratory Software, Inc. Spectral simulations were performed on an IBM-PC using a program that assumes isotropic **g** and **A** tensors. Free-radical quantitation was performed by comparing the integrated signal intensity with that of a known concentration of freshly prepared DPPH dissolved in benzene in an identical precision EPR tube.

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[1] Abbreviations: Me_4Si, tetramethylsilane; EPR, electron paramagnetic resonance; Me_2SO, dimethyl sulfoxide; TFA, trifluoroacetic acid.

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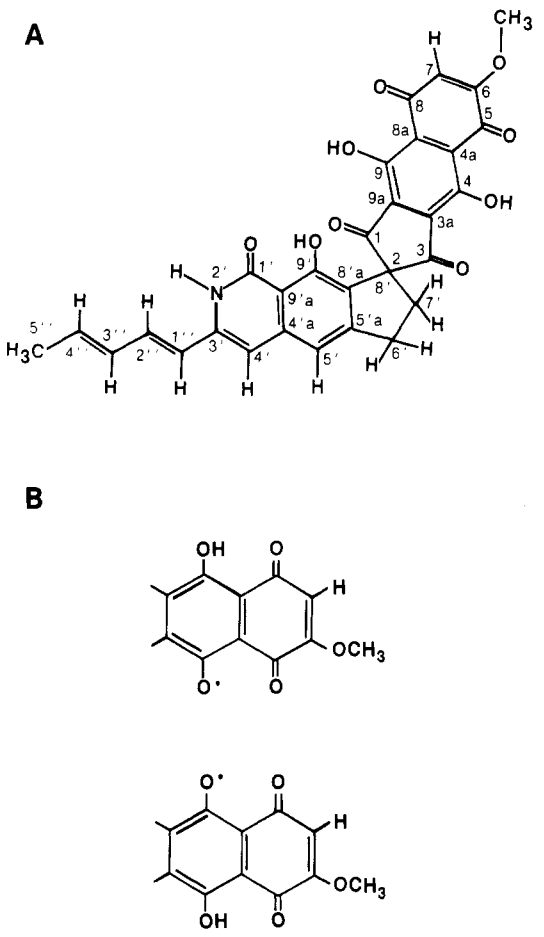

FIGURE 1: (A) Structure of fredericamycin as determined by X-ray crystallography. (B) Proposed partial structures for free-radical forms of fredericamycin.

Spin-trapping studies were performed with the spin trap 5,5-dimethyl-1-pyrroline oxide, DMPO, purchased from Aldrich Chemical Co. The DMPO was purified by filtration of aqueous DMPO through charcoal (Buettner & Oberly, 1978). The spin-trapping experiments were performed at a final concentration of 100 mM DMPO.

(*4*) *Fredericamycin.* All experiments were performed with the same preparation of fredericamycin A (FCRC-A48-NSC-305263), isolated from a strain of *S. grieseus* (FCRC-48). Fredericamycin A was purified on Prep LC-500 using silica cartridges and the solvent system chloroform–methanol–acetic acid in the proportion 87:3:3. The fredericamycin A was then repeatedly washed with acetonitrile to yield the final product. The course of isolation and purification was monitored by HPLC assay.

(*5*) *Atomic Absorption Studies.* Samples of 1.84 mg/mL of fredericamycin A in Me_2SO were tested for metal contamination on a Perkin-Elmer 5000 atomic absorption spectrophotometer.

(*6*) *General Note.* Experiments were generally carried out in reduced light or total darkness. Samples of fredericamycin A (red form) are stable on exposure to room temperature and light for up to 1 year.

RESULTS

Fredericamycin A is a violet powder, and when this powder is dissolved in anaerobic nitrogen-equilibrated Me_2SO, the resulting solution is initially red-violet. When this solution is allowed to stand in air for several minutes, the color gradually shifts to a very dark blue. This color change is greatly

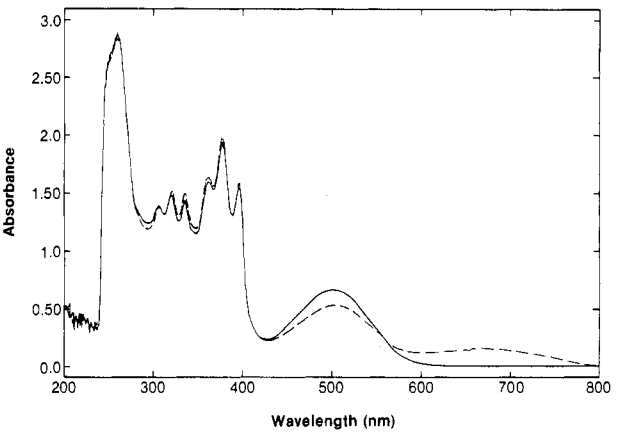

FIGURE 2: UV–visible spectra of a 1 mM solution of fredericamycin in Me_2SO: (---) freshly prepared aerobic solution; (—) solution after addition of a trace of TFA.

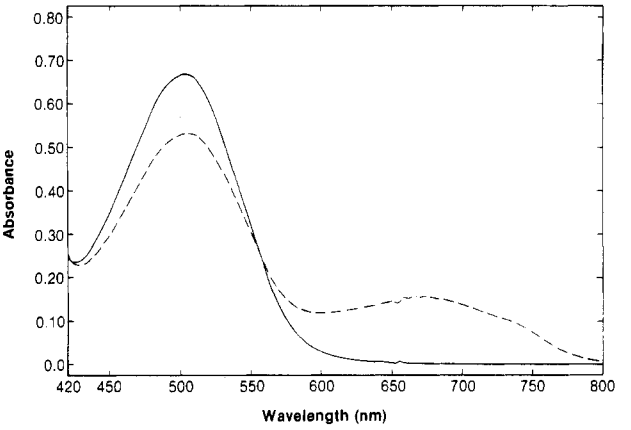

FIGURE 3: Expanded visible region of the spectra in Figure 2.

accelerated, occurring in seconds, on bubbling with air or oxygen gas. On addition of trace TFA the color of the fredericamycin A solution immediately shifts to a bright red, and on subsequent bubbling with oxygen the color shifts back to dark blue. The UV–vis optical absorption spectrum of fredericamycin A in Me_2SO solution is shown in Figure 2 after both equilibration with air (dashed line) and subsequent addition of trace TFA (solid line). On equilibration of the anaerobic solution with air, marked changes are observed in the visible region with a decrease in the 500-nm peak and the appearance of an absorption band centered at 675 nm. On the addition of trace TFA these spectral changes are reversed with the 675-mm absorption band disappearing and the 500-nm peak increasing (Figure 3). This change in color and optical obsorption spectrum is similarly observed in other solvents including pyridine, dimethylformamide, and chloroform. In water the red form of fredericamycin A is slightly soluble; however, on exposure to oxygen a blue precipitate is formed. Resolubilization of the drug is then possible by acidifying the solution with trace TFA.

The proton NMR spectrum of fredericamycin A 1 mM in Me_2SO before and after addition of TFA is shown in Figure 4, parts A and B, respectively. These spectra are similar to spectra obtained in other solvents. The NMR data are summarized in Table I. Assignments of the proton resonances were made by standard techniques including selective homonuclear decoupling, selective nuclear Overhauser experiments, and carbon-13 proton heteronuclear decoupling experiments using the carbon assignments of Byrne et al. (1985). Dramatic changes in the proton spectrum are evident as the color change occurs; the changes are restricted to the quininoid portion of

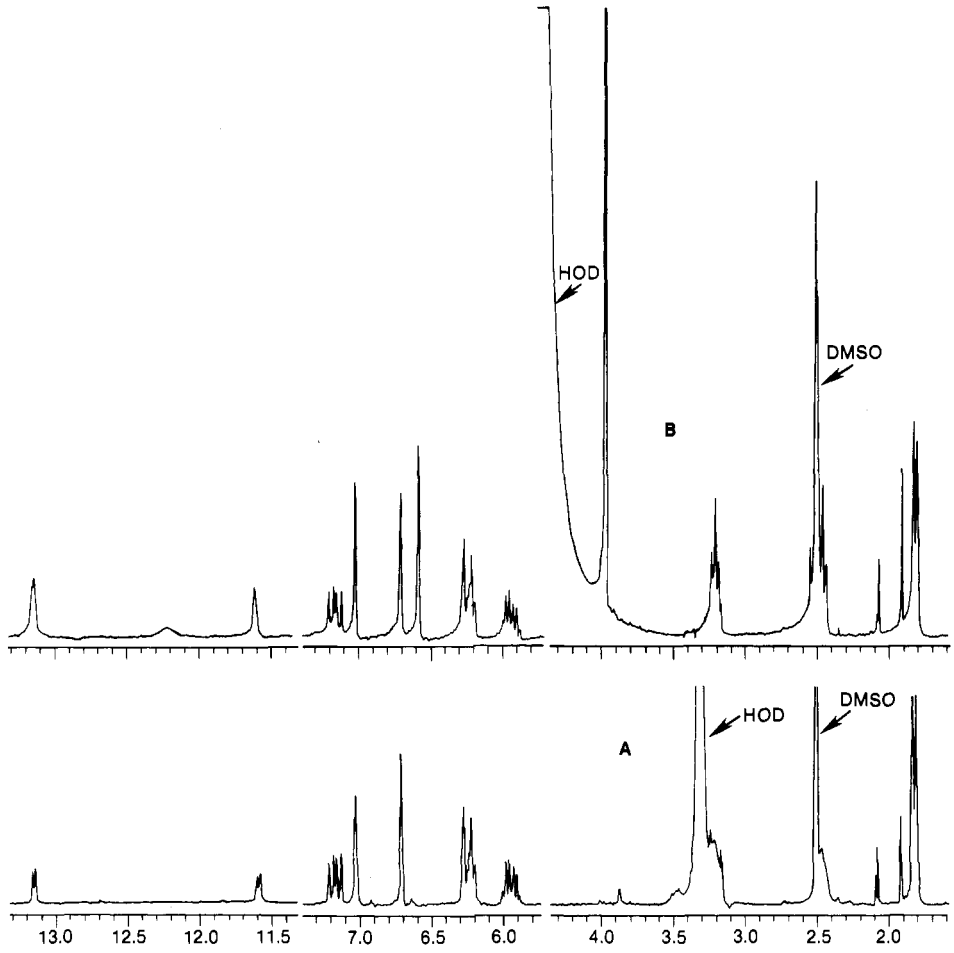

FIGURE 4: (A) Proton NMR spectrum (300 MHz) of freshly prepared 1 mM Me_2SO solution of fredericamycin. (B) Same sample after addition of a trace of TFA.

Table I: Proton NMR of Fredericamycin

proton	chemical shift[a] fresh	chemical shift[a] +TFA	multiplicity[b]	NOE[c]	proton–proton[d] coupling constants
9′-OH	13.15	13.15			
4,9-OH		12.2			
2′-NH	11.6	11.6	d		4′, 0.9
2″	7.15	7.17	dd		3″, 10.3; 1″, 15.9
5′	7.03	7.03		−4′	4′, <1
4′	6.72	6.71	s	−5′	
7		6.58		$-6OCH_3$	
1″	6.26	6.26	d		2″, 15.9
3″	6.21	6.21	ddq		2″, 10.3; 4″, 15.0; 5″, <1
4″	5.93	5.93	dq		3″, 15.0; 5″, 7.1
$6-OCH_3$		3.97	s	−7	
6′	3.20	3.20	t, br		ABXY system with 7′
7′	2.46	2.46	t, br		
5″	1.83	1.83	dd		4″, 7.1; 3″, <1

[a] In parts per million from Me_4Si (internal standard). [b] Key: d, doublet; dd, doublet of doublets; s, singlet; t, triplet, q, quartet; br, broad. [c] Resonances connected as determined by selective NOE experiments are listed. [d] The resonance coupled is followed by the coupling constant in hertz.

the molecule and the regions immediately adjacent (Table I). For example, proton 7 and the 6-methyl protons at 6.58 and 3.97 ppm, respectively, are not observed in the freshly prepared sample but appear as sharp singlets after acidification. The phenolic protons 4 and 9-OH appear in Figure 4B at 12.2 ppm but are not found in Figure 4A. Protons at carbons 6′ and 7′, 3.20 and 2.46 ppm, respectively, appear as broad humps before acidification and as sharp triplets after acidification.

These results are entirely consistent with the carbon-13 results (Figure 5). In general the quininoid carbons are not observable, or appear very broad, in preparations that have been exposed to air; however, all are observed after acidification and removal of oxygen. Carbon spectra of carbon-13-enriched fredericamycin A that have not been acidified show identifiable, broad resonances that correspond to the appropriate carbons of the quininoid portion of the molecule (Byrne et al., 1985). These include the 7′-methylene (34.4 ppm), 6-*O*-methyl (57.5 ppm), 7-methine (110.9 ppm), and the 8′a, 1, 2, 3, 3a, 4, 4a, 5, 6, 8, 8a, 9, and 9a quaternary (124.6, 199.0, 135.1, 153.4, 118.0, 183.3, 161.1, 188.6, 118.0, 152.2, and 136.9 ppm, respectively) carbons.

The sharp spectra of fredericamycin A can be obtained if solutions are deoxygenated by pumping on a vacuum line. Quininoid protons are observable, but broad, suggesting an intermediate state. The samples so obtained, or those obtained by acidification with TFA, are stable as monitored by NMR proton spectra for at least 1 year. Samples of fredericamycin A exposed to air appeared to be considerably less stable, although in many instances fredericamycin A could be recovered from apparently decomposed samples by acidification. Nevertheless, over short periods of time (several days or less) the conversion from blue to red form appeared entirely reversible; samples were cycled several times between the two forms without significant changes in the respective proton NMR spectra.

Electron paramagnetic resonance spectra of fredericamycin A were obtained to determine whether the observed oxygen-dependent changes in the NMR spectrum were due to the formation of a drug free radical. Fredericamycin A freshly

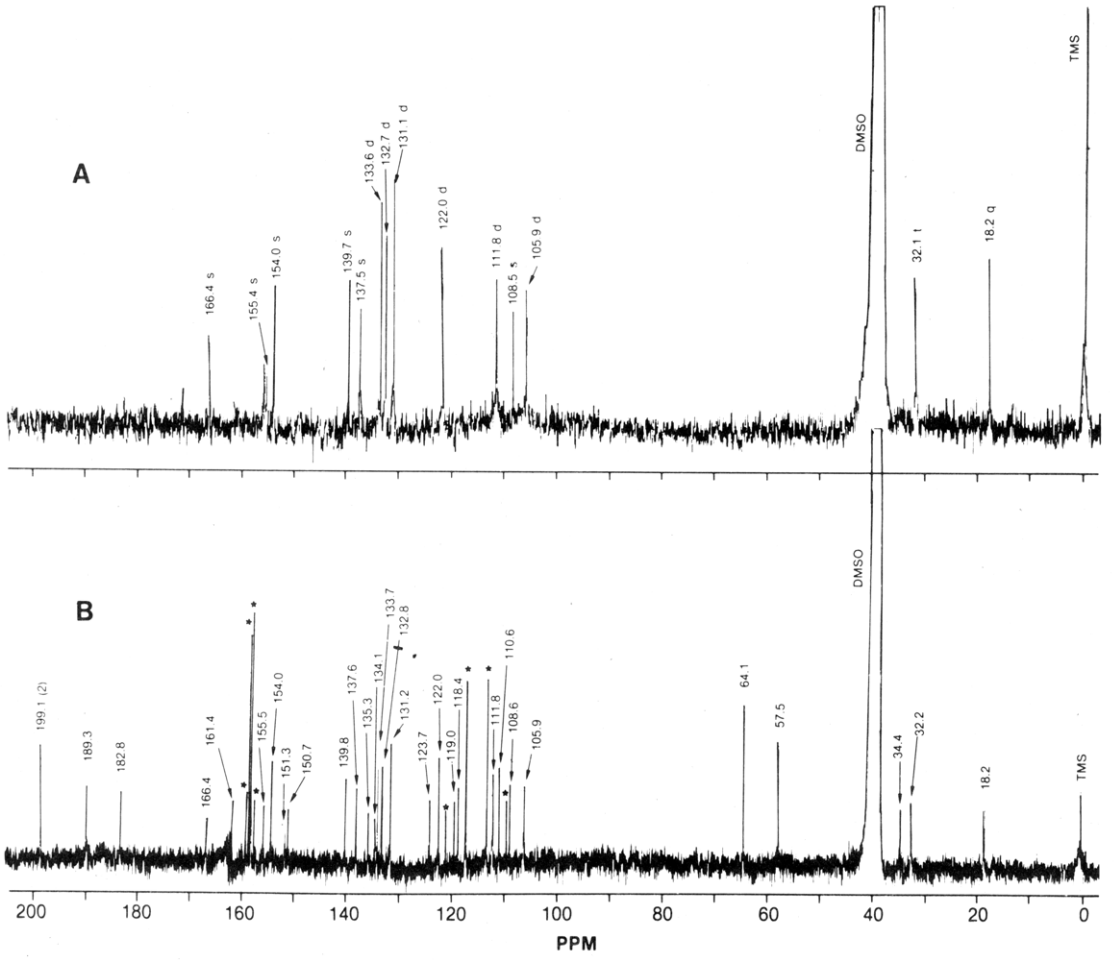

FIGURE 5: Proton noise-decoupled ^{13}C NMR spectra (75.4 MHz) of fredericamycin in Me_2SO-d_6 (A) containing 0.7% TFA-*d* (B). Signals due to TFA-*d* are designated with asterisks.

dissolved in Me_2SO (2.0 mM) gave rise to a prominent signal at $g = 2.004$ with a line width of 9 G at 77 K. Vigorous bubbling with air (prior to freezing the sample) increased the signal intensity fivefold (Figure 6A). On addition of TFA (trifluoroacetic acid) vapor by pipet (approximately 0.5 mL of vapor or 2×10^{-5} mol) the signal disappeared (Figure 6B). Liquid-phase spectra obtained at 24 °C exhibited a symmetric EPR signal at $g = 2.004$ with Gaussian lineshape and line width of 1.6 G. On bubbling with air, the signal intensity increased fivefold while on addition of TFA the signal disappeared. The spectrum of the solid powder of fredericamycin A exhibits a signal of $g = 2.004$ similar to that of the frozen solution. Quantitation of the amount of free-radical signal was performed at 77 K and at room temperature, respectively. The free-radical concentration in the 2 mM solution of fredericamycin A was 0.04 mM; after bubbling with air, the free-radical concentration rose to 0.2 mM. Sufficient H_2O_2 was then added to bring the concentration of H_2O_2 to 0.01%, and a further slow increase in the radical signal was observed. Addition of H_2O_2 led to the formation of a gradually increasing amount of blue precipitate; this rendered quantitation difficult. After 12 h, the signal had increased eightfold to a free-radical concentration of 1.6 mM. In addition to the increase in intensity, prominent hyperfine structure was observed (Figure 7A). Computer simulation of this spectrum was performed, and a best fit was obtained for a system of three identical protons with a coupling constant of $A_H = 0.8$ G, one proton with a coupling constant $A_H = 0.9$ G, and one proton with a coupling constant of $A_H = 0.45$ G (Figure 7B). The NMR spectrum of this sample after addition of TFA showed that

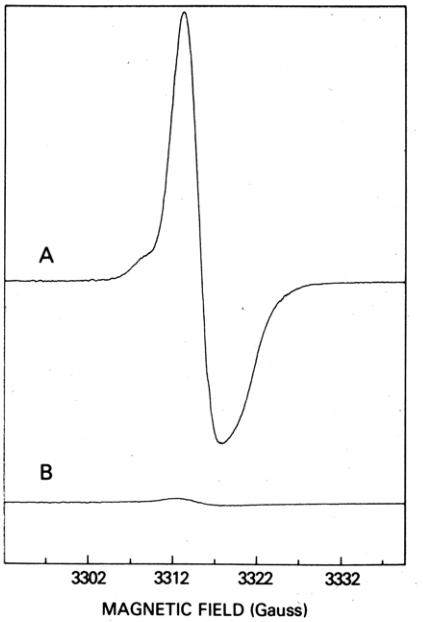

FIGURE 6: EPR spectra recorded at 77 K of 2.0 mM fredericamycin freshly dissolved in Me_2SO: (A) initial spectrum; (B) spectrum after addition of TFA. Conditions: microwave frequency, 9.295 GHz; microwave power, 1.0 mW; modulation amplitude, 2.5 G in both species.

the fredericamycin A was intact.

When preparations of fredericamycin A dissolved in Me_2SO were allowed to equilibrate with air for several days at room

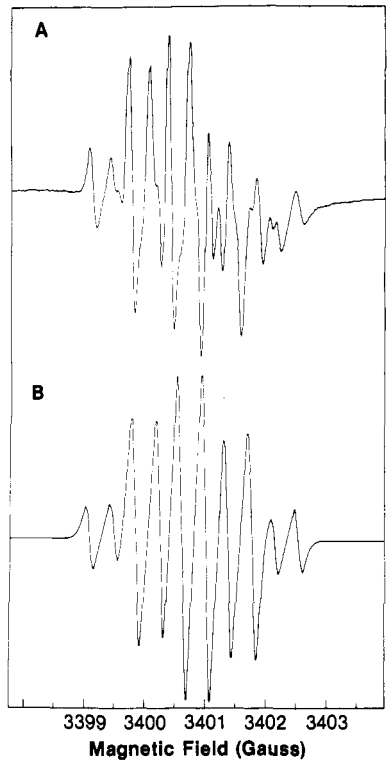

FIGURE 7: (A) EPR spectra of 2.0 mM fredericamycin after addition of H_2O_2 (final concentration, 0.01%; microwave frequency, 9.539 GHz; microwave power, 10 mW; modulation amplitude, 0.1 G). (B) Best fit computer simulation to the spectrum in A that was generated assuming three identical protons with A_H = 0.8 G, one proton with A_H = 0.9 G, and one proton with A_H = 0.45 G.

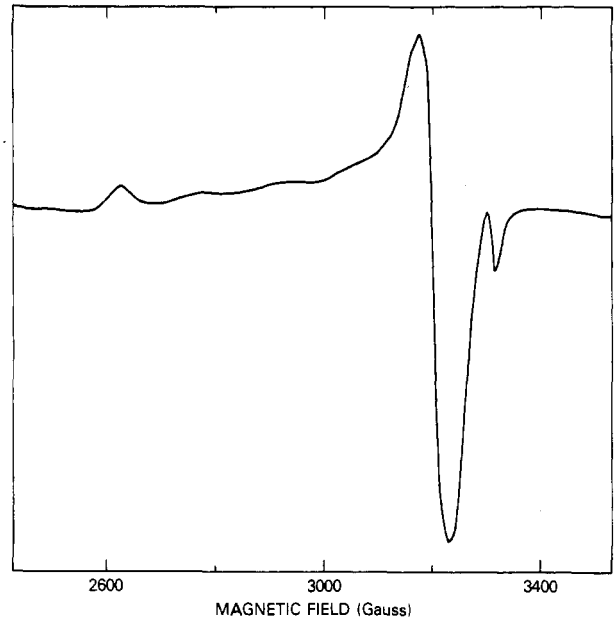

FIGURE 8: EPR spectrum at 77 K of a 2.0 mM preparation of fredericamycin dissolved in Me_2SO and exposed to air for 1 week (microwave frequency, 9.284 GHz; microwave power, 40 mW; modulation amplitude 5.0 G). A new spectrum is observed with axial symmetry $g_{\parallel}$ = 2.524 and $g_{\perp}$ = 2.073, located downfield from the residual saturated dry free-radical signal at g = 2.004.

temperature, a new broad anisotropic EPR signal was observed with axial symmetry $g_{\parallel}$ = 2.523 and $g_{\perp}$ = 2.073 (Figure 8). The $g_{\perp}$ region of this spectrum is located just downfield from the signal of the fredericamycin A free radical. In identical anaerobic preparations of fredericamycin A this signal was not observed. This axial EPR spectrum exhibits g values within the range expected for the superoxide anion free radical

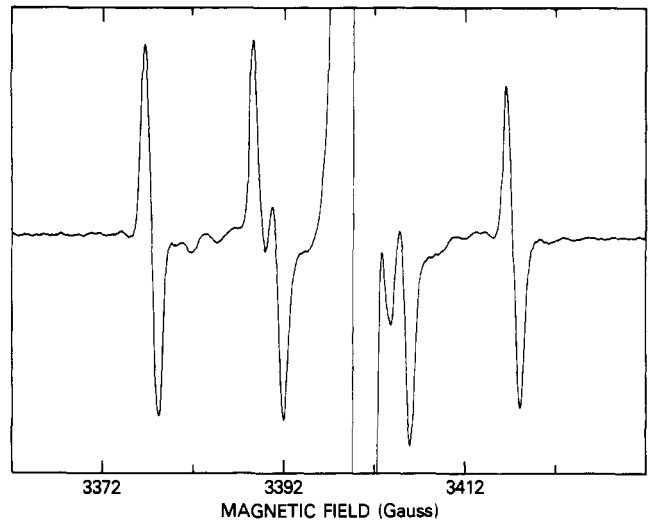

FIGURE 9: Spin-trapping experiment of a preparation of 1.0 mM fredericamycin in Me_2SO exposed to air (microwave frequency, 9.528 GHz; modulation amplitude, 1.0 G; microwave power, 20 mW). A quartet signal appears superimposed on the large signal of the fredericamycin free radical centered at 3397 G.

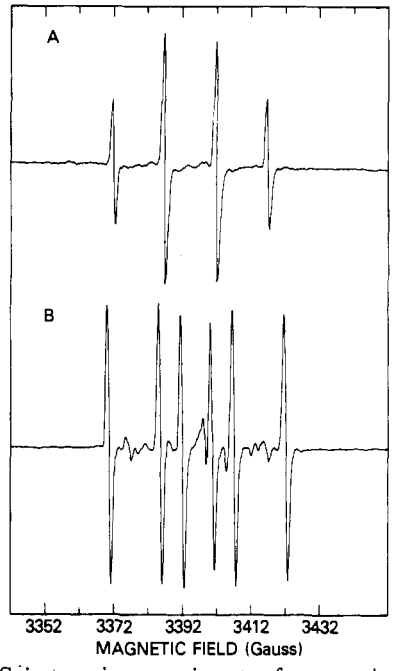

FIGURE 10: Spin-trapping experiments of preparation of 1.0 mM fredericamycin: (A) mixed with water in the presence of 0.1% H_2O_2; (B) dissolved in Me_2SO in the presence of 0.1% H_2O_2. Conditions: microwave frequency, 9.528 GHz; modulation amplitude, 1.0 G; microwave power, 20 mW.

(Knowles et al., 1979). Power saturation studies of this signal were performed at 77 K, and it was observed that saturation occurred only at microwave power greater than 50 mW.

Spin-trapping studies were performed using the spin trap DMPO in order to further determine if fredericamycin A generates oxygen free radicals. When fredericamycin A is dissolved in Me_2SO and exposed to air, a quartet signal is observed superimposed on the signal of the fredericamycin A free radical (Figure 9). The signal has hyperfine coupling constants a_N = 12.7G and a_H = 10.3G with a line width of 1.5 G. These coupling constants are the same as those of DMPO–O_2^- in Me_2SO (Janzen, 1980). The usual 1.3G a_H^{γ} splitting is not discernible due to the relatively broad linewidth of 1.5 G. On addition of sufficient 1% H_2O_2 to a solution of 1 mM fredericamycin A in Me_2SO to result in a final H_2O_2

concentration of 0.1% a sextet signal is observed (Figure 10B) with a_N = 16.0 and a_H = 22.7 characteristic of a trapped methoxy radical (Finkelstein et al., 1980). On addition of the solid fredericamycin A to an aqueous solution 0.1% in H_2O_2 a 1:2:2:1 quartet signal is observed (Figure 10A) with a_N = a_H = 14.9 G. This signal is characteristic of DMPO–OH (Finklestein et al., 1980). In control experiments in the absence of fredericamycin A only trace signals are observed with intensities less than one-tenth of those observed in the presence of fredericamycin A.

The possible presence of adventitious metal atoms was investigated by atomic absorption spectroscopy. Zn, Cu, Pb, and Mn all could not be detected at the limit of sensitivity (ca. 1 ppb). Iron was detected at a low level, less than 0.1% by weight vs. the drug.

Discussion

The studies reported clearly demonstrate that fredericamycin A exists in reversible equilibrium between reduced and oxidized forms. The reduced form is red with optical absorption band at 500 nm and sharp ^1H and ^{13}C NMR spectra containing resonances defining each hydrogen and carbon in the molecule. The oxidized form is blue with an additional absorption band at 675 nm, and the ^1H and ^{13}C resonances of the quininoid portion of the molecule are broadened beyond detection. EPR studies demonstrate that the oxidized form is a free radical, and this observation explains the unusual observed NMR behavior. The hyperfine structure of this free radical was best simulated by three identical protons with coupling constant A_H = 0.8 G, one proton with A_H = 0.9 G, and one proton with A_H = 0.45 G. The three identical protons with A_H = 0.8 G correspond to the OCH_3 group, the proton with A_H = 0.9 G corresponds to the C_7 proton, and the proton with A_H = 0.45 G is the C_9 or C_4 phenolic proton. Thus, the observed free-radical hyperfine structure suggests that the blue form of fredericamycin A is the one-electron-oxidized phenolate free radical (Figure 1B). The slight but noticeable asymmetry present in the hyperfine structure (Figure 4A) could be the result of the presence of two forms of the radical shown in Figure 1B or the trace presence of a free-radical degradation product of the drug. An important observation is that low levels of radical are sufficient to severely broaden all the quininoid resonances. At no time did we observe any evidence of the presence of two forms of fredericamycin A, one oxidized radical and one neutral, yielding broad and sharp signals, respectively. Thus, some mechanism for intermolecular exchange of the radical must exist, or less likely, the radical itself must be created at each molecule in solution continuously. Either of these processes must be fast on the NMR time scale; i.e., the radical must have a lifetime in a particular environment less than about 1 ms.

The characteristics of the proton and carbon NMR spectra of fredericamycin A are readily understood in terms of the presence of a free radical. The spiro carbon (2 in Figure 1) is known to act as a barrier to the delocalization of an unpaired electron (Cowell et al., 1963); thus, the protons of the benzidene portion of the molecule are readily observable even in the presence of considerable amount of radical, although the 6′- and 7′-methylene protons are broadened preceptibly in Me_2SO. Protons on the quininoid half of the molecule have their longitudinal relaxation times drastically reduced by the free radical, and their resonances are broadened to the point that they cannot be observed. Addition of trace amounts of TFA results in the sharpening of all of the protons of the quininoid half of the molecule, as well as the methylene protons 6′ and 7′. This is precisely as expected if the radical is reduced. In all cases the radical could be regenerated by bubbling the sample with air or oxygen, as evidenced in the NMR, EPR, and UV spectra. In several cases intermediate stages where only a very small amount of radical is present were observed. These conditions could not be readily reproduced in a quantitative manner so that application of the Solomon–Bloembergen equations was not practical (Solomon & Bloembergen, 1956). For example, samples of fredericamycin A freeze–pump–thawed on a vacuum line for several cycles and carefully handled to avoid the introduction of oxygen to the sample showed severely broadened 6-methoxyl and 7-protons (25 Hz). Such samples could readily be taken in either direction; i.e., addition of TFA sharpened their signals while addition of O_2 broadened them sufficiently that their signals could not be observed. A puzzling aspect of the proton spectrum in Me_2SO is that both the NH and the 9′-OH protons show a doublet character, not due to spin–spin splitting (confirmed by obtaining proton spectra at 200 MHz). We speculate that this results from two environments for these nuclei, resulting from intermolecular interactions between fredericamycin A molecules. In other solvents these lines appear as sharp singlets (DMF, $CDCl_3$, pyridine). Such an intermolecular interaction would have an uncertain effect on the ESR spectra obtained under similar conditions; it is possibly related to the slight asymmetry revealed in Figure 7.

The red, reduced, form of the drug is rapidly converted to the blue electron-oxidized form on exposure to oxygen. This reaction must result in electron transfer to molecular oxygen with the probable formation of the superoxide anion free radical:

$$\text{FMA–OH} + O_2 \rightleftarrows \text{FMA–O}^{\bullet} + H^+ + {}^{\bullet}O_2^-$$

In aerobic preparations an EPR signal with axial symmetry, $g_{\parallel}$ = 2.523 and $g_{\perp}$ = 2.073, was observed while an anaerobic preparation did not exhibit this signal. This signal was observed in the solid state at 77 K, and it disappeared in room-temperature measurements. Saturation of this signal was observed only at high power, greater than 50 mW. The observed g values, axial symmetry, temperature, and saturation behavior are all consistent with that expected for the superoxide anion free radical (Knowles et al., 1969). In spin-trapping experiments using the nitrone spin trap DMPO a DMPO–O_2^- signal was observed on exposure of fredericamycin A to O_2, which confirms the generation of superoxide anion. Spin-trapping experiments, after addition of H_2O_2 to the drug, demonstrated the generation of hydroxy radical in water and methoxy radical via hydroxy radical in Me_2SO. These observations suggest that the drug could generate the destructive hydroxyl radical in cells via cellular H_2O_2.

A number of the most effective anticancer drugs in clinical use are thought to exert their antitumor effects via the formation of reactive drug radicals or reduced oxygen radicals (Gianni et al., 1983; Carmichael et al., 1985). These include the anthracycline drugs adriamycin and daunomycin (Zweier, 1985). Free-radical generation is thought to be due to enzymatic reduction of the drug or to metal ion mediated oxidation. Adriamycin is activated by Fe^{3+} binding with the subsequent formation of reduced oxygen and drug free radicals (Zweier, 1984; Gianni et al., 1985). Fredericamycin A is a new structural class of antitumor drug. In preliminary studies it has shown potent tumoricidal activity with high therapeutic toxic index (Warnick-Pickle et al., 1981). In this paper we demonstrate that fredericamycin A is unique in that it spontaneously donates an electron to O_2 or H_2O_2, forming a one-electron-oxidized phenolate radical and superoxide or hydroxyl

radicals, respectively. This mechanism of drug and reduced oxygen free radical generation could explain the tumoricidal mechanism of the drug. The absence of an obligatory requirement for metal or enzyme activation could result in a unique spectrum of antitumor activity.

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Registry No. OH^-, 3352-57-6; fredericamycin A, 80455-68-1; fredericamycin A (radical at O-4), 103732-05-4; fredericamycin A (radical at O-9), 103732-06-5.

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Role of a Second Catabolite Activator Protein Molecule in Controlling Initiation of Transcription at the Galactose Operon of *Escherichia coli*†

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ABSTRACT: The molecular mechanisms whereby RNA polymerase, catabolite activator protein (CAP), and cyclic AMP (cAMP) participate in transcriptional regulation at the galactose operon have been probed by a variety of in vitro techniques. Interactions between purified proteins and promoter-containing DNA fragments were assayed by gel electrophoresis, by resistance to restriction endonuclease digestion, and by monitoring runoff transcripts. The data bear on the multiple functions that CAP performs in *gal* control. A CAP–cAMP complex can exclude RNA polymerase from one of the two overlapping promoter regions (P2), thereby targeting the enzyme to the other (P1); this process is markedly influenced by the cAMP level. In addition, a second CAP molecule is involved in a cooperative process, which, at low cAMP, is required for efficient formation of transcriptionally competent complexes at P1. This second CAP may serve to stabilize the 1:1:1 CAP–polymerase–*gal* DNA intermediate under physiological conditions, thus enhancing initiation from P1 relative to P2. Kinetic analysis reveals that the modest effect of CAP on the rate of P1 open complex formation can be resolved into about a 4-fold increase in the binding of RNA polymerase to the P1 region, plus a 1.5-fold elevation in the rate of isomerization of enzyme–promoter complexes to the open state.

Regulation of transcription at the *Escherichia coli* galactose operon is a complex process involving RNA polymerase, the catabolite activator protein (CAP) and its effector cAMP,[1] and the *gal* repressor (Adhya & Miller, 1979). In addition, transcription can occur from either of two overlapping promoter sites (Musso et al., 1977). In the absence of CAP–cAMP, RNA polymerase binds to the P2 promoter and initiates at nucleotide −5. When the intracellular concentration of cAMP rises, CAP activates P1 and enhanced levels of transcription occur from +1 start site. The binding of CAP at its primary site (−35 to −50) (Taniguchi et al., 1979; Busby et al., 1982) can, in principle, play two roles in stimulating

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[1] Abbreviations: CAP, catabolite activator protein; cAMP, adenosine cyclic 3′,5′-phosphate; bp, base pair; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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