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ELECTRON SPIN RESONANCE DETECTION OF FREE RADICALS IN THE MERCAPTAN-ACTIVATION AND UV-INACTIVATION OF NEOCARZINOSTATIN

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Summary

Neocarzinostatin, an antitumor protein antibiotic containing an essential non-protein chromophore, causes single-strand breaks in DNA in vitro. Mercaptans are required for the DNA-cleavage activity. Irradiation of the protein by ultraviolet light destroys this activity. We demonstrate directly by EPR spectroscopy that mercaptan-activation of the drug and UV-inactivation involve the generation of free radicals. These radicals are chromophore-dependent and related to neocarzinostatin activity. They may be intermediates in the antitumor action of this drug. The ^1H NMR spectrum of neocarzinostatin shows unique features that can be related to activity.

Neocarzinostatin (NCS) is an antitumor protein antibiotic produced by a variant of Streptomyces carzinostaticus. The primary sequence of this protein (MW ~10,700) is known (1). NCS causes single strand breaks in DNA almost exclusively at adenylate and thymidylate residues by degrading the backbone sugars (2). The reaction is non-enzymatic; only a limited number of breaks are produced by a given concentration of NCS (2). The cleavage activity requires the presence of oxygen and mercaptans (3). NCS can be inactivated irreversibly by irradiation with ultraviolet light above 300 nm (4), exposure to heat (5), or preincubation with mercaptans prior to the introduction of DNA (3). It was recently discovered that NCS contains a non-protein chromophore (MW ~660) which shows UV absorbance above 300 nm and which can be extracted from the protein (6). The isolated chromophore exhibits the full DNA-cleavage activity of NCS, while the protein portion (apo-NCS) exhibits no activity (7-10). The activity of the isolated chromophore is UV- and heat-sensitive and also mercaptan-dependent (7).

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It appears that the release of chromophore from the protein, or at least a partial unfolding of the protein to expose the chromophore to solvent and allow intercalation of chromophore with DNA is necessary for activity (7,10,11). The protein probably serves to protect the chromophore from rapid inactivation in aqueous solution (10). The radical scavenger α-tocopherol acid succinate inhibits the cleavage reaction (3). However, superoxide dismutase, catalase and OH$^{\cdot}$ scavengers have no effect on the reaction; this rules out a mechanism involving diffusable O_2^-, H_2O_2, or OH$^{\cdot}$ radicals. It was inferred from this indirect evidence that the essential mercaptans may be necessary to induce a free radical on the chromophore (7). In this communication, we demonstrate by direct EPR detection that mercaptan-activation of NCS occurs via a free radical mechanism. UV-inactivation also proceeds via a free radical mechanism, involving a radical with different spectral characteristics. These radicals are chromophore dependent and may be related to the in vivo activity of NCS.

MATERIALS AND METHODS

Native NCS (Kayaku Antibiotics Research, Tokyo) was provided by Dr. W.T. Bradner (Bristol Laboratories, Syracuse, NY). It was stored at 4°C in 15 mM sodium acetate buffer (pH 5.0). All preparative steps were carried out in the dark so as to minimize exposure of NCS to light. Samples of stored NCS were dialyzed against distilled water, lyophilized, and redissolved in 50 mM Tris-Cl buffer (pH 8.0) (for EPR) or in distilled D_2O (for NMR) to a final concentration of $\sim 2 \times 10^{-3}$M (using $\varepsilon_{270} = 23{,}000\ M^{-1}cm^{-1}$). Apo-NCS was prepared by extracting the lyophilized powder many times with methanol, evaporating the methanol under reduced pressure and redissolving the methanol-insoluble material in buffer or D_2O, as needed. Under these extraction conditions virtually all the chromophoric material is removed.

EPR spectra were recorded at -160°C on a Varian E-4 X-band spectrometer. Time-averaging was used where necessary to improve the spectral signal-to-noise ratio. β-Mercaptoethanol (BME) was added to buffered samples of native NCS and apo-NCS to a final concentration of 20 mM (maximum NCS activity occurs at 10-20 mM BME (3)), the samples were incubated at room temperature for 3-5 minutes and then frozen in liquid nitrogen. Parallel samples containing up to 10 mM (per phosphate) sonicated calf thymus DNA were also run. Samples of native NCS and apo-NCS (in quartz EPR tubes) were irradiated by long-wave UV light (Mineralite UVSL-85; distance 2 cm) for 5 minutes, then quickly frozen in liquid nitrogen. Alternately, the samples were frozen and then irradiated while remaining frozen.

^1H NMR samples were run at 25°C on a Varian HR-220 FT NMR spectrometer at the University of Pennsylvania. Unbuffered NCS and apo-NCS samples in D_2O were adjusted to pH 8.0 (uncorrected for deuterium isotope effect) by the addition of DCl or KOD. BME in D_2O was added to native NCS to a final concentration of 20 mM. When needed native NCS samples were irradiated for 20 minutes.

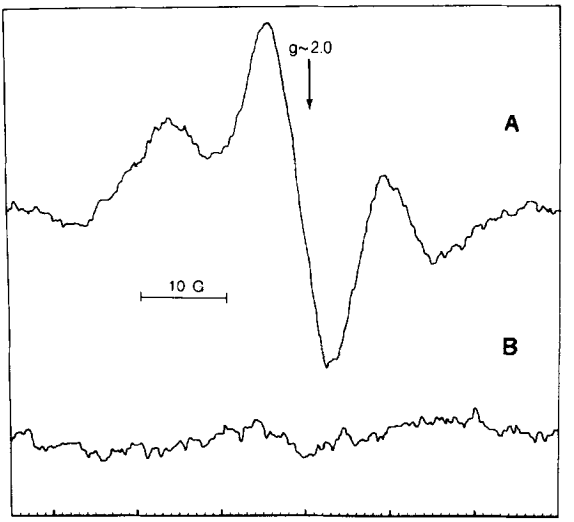

Figure 1A. The EPR spectrum of NCS incubated in the presence of 20 mM β-mercaptoethanol for 5 minutes at room temperature. Instrumental settings were: modulation amplitude, 10G; time constant, 0.3 sec; microwave power, ~1 mW; modulation frequency, 100 KHz; scan rate, 100 G/min. Sample contained ~ 2 x 10^{-3} M NCS, 50 mM Tris-Cl, pH 8.0. Ten traces were time-averaged to obtain this spectrum.
Figure 1B. Sample A refrozen after standing at 37° for 15 min.

RESULTS AND DISCUSSION

EPR-detectable free radicals were formed when native NCS was incubated with 20 mM BME, a mercaptan known to activate NCS (Figure 1A). The radicals were short-lived in thawed samples and decayed to undetectable levels upon incubation of the sample at 37°C for 15 minutes (Figure 1B). Only a fraction of total NCS contained a radical at any one time, judging from the small amplitude of the signal relative to the total NCS concentration. Further addition of BME to these pre-incubated samples did not generate any further radicals. The presence of 10 mM DNA had no appreciable effect on the intensity, shape, or decay rate of the EPR signal, although electrophoresis on 1.5% agarose gels showed that the calf thymus DNA in the samples had been cleaved. No BME-induced signal was detectable with apo-NCS or with NCS previously inactivated by UV-irradiation. Native NCS without BME also showed no radicals. The EPR signal showed a triplet hyperfine structure suggesting an interaction of the unpaired electron with two nearby protons.

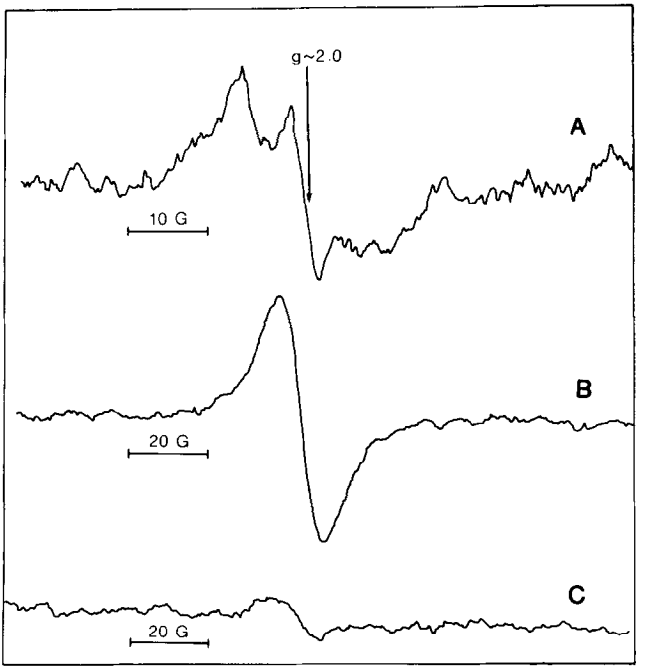

Figure 2A. The EPR spectra of NCS upon UV-irradiation. Irradiated ~5 min. at room temperature, then frozen to -160°C. Eight traces were time-averaged; modulation amplitude, 5G.
Figure 2B. Irradiated ~5 min. while frozen; no time-averaging.
Figure 2C. Sample B refrozen after standing at room temperature for 3 min; no time-averaging. Other EPR parameters were the same as in Figure 1A.

UV-irradiation of NCS at room temperature also generated chromophore-dependent EPR-detectable free radicals. The production of radicals in the UV-inactivation of NCS was not previously suspected. No signal was observed for irradiated apo-NCS or for NCS inactivated by preincubation with BME. The EPR signal showed a hyperfine structure with different relative peak intensities than that for BME-treatment (Figure 2A). Irradiation of frozen samples produced a much stronger signal (~10 X) which exhibited little hyperfine structure (Figure 2B). When the sample was thawed to room temperature for 3 minutes and refrozen, the EPR signal was much reduced in intensity (Figure 2C). These observations are consistent with a rapid decay of radicals at room temperature and their stability in the frozen state. The loss of hyperfine structure in the samples irradiated while frozen could arise from spin-exchange due to the higher density of radicals in the frozen state.

Our observation that the presence of BME does not generate radicals in apo-NCS or in previously inactivated NCS, together with the fact that the time-course of radical decay in BME-treated samples is similar to the time-course previously reported for the irreversible inactivation of NCS by preincubation with BME (3), shows that the EPR-detectable radicals are dependent on native chromophore and are activity-related. It is reasonable from the hyperfine structure of the radicals to infer that they are located on the chromophore. Mercaptans activate NCS in the presence of DNA but irreversibly inactivate it in the absence of DNA. Moreover, the time-course for the release of bases by the cleavage reaction is similar to the time-course for the inactivation of NCS in the absence of DNA at similar NCS and BME concentrations. Partly denaturing conditions enhance both the rate of DNA-cleavage and the inactivation rate (3) in the absence of DNA. These observations can be simply explained by assuming that mercaptan-activation involves generating reactive chromophoric radicals which decay spontaneously. DNA, if present, reacts with some of the reactive chromophoric radicals before their decay. Mercaptan-inactivation of NCS in the absence of DNA would then be similar to unproductive mercaptan-activation. We believe that the BME-induced radical we observe is one of the intermediates in the DNA-cleavage reaction. The fact that DNA seems to have no effect on the EPR signal of the radical is not inconsistent with this. Under our conditions the overall reaction rate of DNA with EPR detectable radicals may be low compared to the spontaneous decay rate. Alternately, it may be that the EPR-detectable species decays into or causes the generation of an undetectable radical (e.g. reduced oxygen radical) which is the ultimate species reacting with DNA. The sequence of the steps thought to be necessary for cleavage (including reduction of the chromophore, release of the chromophore from apo-NCS, binding of chromophore to DNA, oxidation of the reduced chromophore by O_2, etc. (10)) remains to be elucidated.

The ^1H NMR spectrum of native NCS (Figure 3) shows a number of resonances which are affected by UV-irradiation (12) and BME-treatment. The native

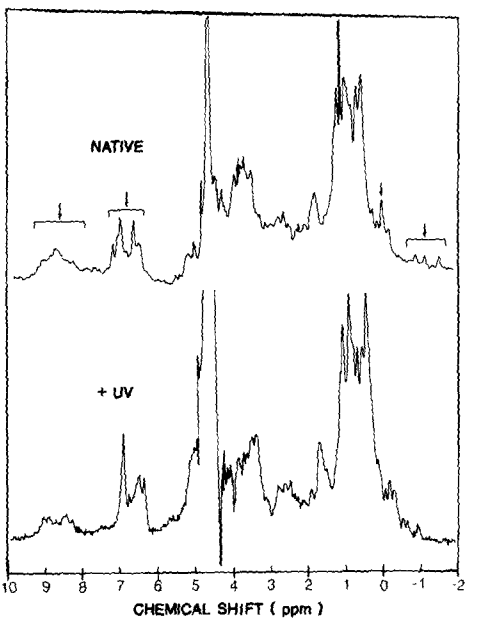

Figure 3. ^{1}H-NMR spectra of native and UV-irradiated NCS (pH 8.0) at 220 MHz. Arrows indicate resonances or groups of resonances which show large changes upon UV-irradiation or treatment with β-mercaptoethanol. Chemical shift is measured relative to external $(CH_3)_3Si$. The strong resonance at ~4.8 ppm is the residual HDO signal. Samples contained ~4 x 10^{-3} M NCS in D_2O. The spectra of apo-NCS and β-mercaptoethanol-treated NCS are qualitatively similar to that of UV-inactivated NCS.

spectrum shows three upfield shifted one proton resonances at -0.8, -1.0 and -1.4 ppm, which are not seen in apo-NCS, UV-inactivated NCS or BME-treated NCS. These resonances, then, are dependent on native chromophore. Further, the envelope of aromatic protein resonances at 6.5 - 7.5 ppm is much sharper in apo-NCS, BME-treated NCS or UV-inactivated NCS than in native NCS. It appears that the spectra of BME-treated and UV-inactivated NCS resemble that of apo-NCS. A reasonable interpretation for the changes in the NMR spectrum is that upon BME-activation or UV-inactivation the chromophore unstacks from aromatic groups in the protein. This agrees with observations of fluorescence enhancement in BME-treated (6) and irradiated NCS and with observations of reduced affinity of apo-NCS for BME-treated (10) and irradiated (7) chromophore relative to native chromophore, any change, activating or inactivating, in the native chromophore presumably disrupting the interactions with the protein.

Our interpretation is consistent with an earlier inference (11) which conversely related partial denaturation of the protein with an enhanced activation. Although both BME-treatment and UV-irradiation cause unstacking and induce radicals, the chromophore radicals induced by BME are different from those induced by UV-irradiation; the hyperfine splitting patterns of their EPR signals are different. Also, the optical and ^{1}H NMR spectra of the resulting chromophore products are different (Sheridan and Gupta, unpublished).

Albers-Schönberg et al. (13) have proposed a partial structure of the NCS chromophore based on ^{1}H NMR and mass spectroscopy. The structure (elemental composition $C_{35}H_{35}NO_{12}$) consists in part of a 2,6-dideoxy-2-methylamino-galactose moiety and a 2-hydroxy-5-methoxy-7-methyl-1-naphthoic acid moiety. The remaining portion of the molecule has the composition $C_{15}H_{10}O_4$. Either the napthoic acid moiety or the C_{15} substructure may be involved in the production of radicals. The degree of unsaturation in both groups would be consistent with the ability to stabilize radicals or with sensitivity to UV-irradiation. Although identification of the exact chemical nature of free radicals must await determination of the detailed structure of the chromophore, our study demonstrates their existence in the mercaptan-activation and UV-inactivation of neocarzinostatin. These radicals are activity-related and may represent a reactive intermediate in the antitumor action of this drug.

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