Identification of the Active Species Responsible for Holo-Neocarzinostatin-Induced DNA Cleavage

Der-Hang Chin and Irving H. Goldberg*

Department of Biological Chemistry and Molecular Pharmacology Harvard Medical School Boston, Massachusetts 02115

Received July 15, 1993

The non-protein chromophore (NCS-Chrom) of the enediyne anticancer antibiotic neocarzinostatin cleaves duplex DNA following its activation by thiol addition to C12 (Scheme I, pathway A).[1,2] The proposed 2,6-diradical **2** presumably abstracts hydogens from sites on the deoxyribose moieties, mainly from C5′, in the minor groove of DNA to form the stable, reduced tetrahyroindacene **3**. In the absence of DNA, thiol inactivates NCS-Chrom by the same mechanism;[3] hydrogen is abstracted by the radical centers from solvent or from some other available source.[4] Recently, Sugiyama et al.[5] reported thatn when holo-NCS (NCS-Chrom bound to its apoprotein) is treated with 2-mercaptoethanol (BME) in aqueous solution, another pathway of cyclization is the major one (Scheme I, pathway B), leading to the formation of a novel product (**5**, 59%) via a proposed 3,7-zwitterion intermediate (**4**); only a small amount (9.5%) of **3** is formed under these conditions. Since thiol-activated holo-NCS cleaves DNA,[1] it is important to determine if **4** (or its diradical tautomeric form) participates in this reaction. Our findings indicate that it does not.

The activation of holo-NCS by several thiols was studied at their optimal concentrations.[6] In the absence of DNA, both dithiothreitol (DTT) (5 mM) and L-cysteine (L-Cys) (10 mM) react with the drug in a similar way as does BME (10 mM). HPLC analysis using a system similar to that of Sugiyama et al.[5a] showed that, as reported, **5** was the major product and **3** was a minor product[7] following incubation of holo-NCS with thiol either at 37 °C for 0.5 h or at 0 °C for 16 h in an aqueopus solution at pH 8. When the incubation mixture was made 80% in 2-propanol to facilitate the release of chromophore from the protein,[8] the major product shifted from **5** to **3**, in accord with the results of Sugiyama et al.[5a] By contrast, glutathione (Glu) (5–70 mM), which is very effective in activating both holo-NCS and isolated NCS-Chrom to the DNA-cleaving species,[1] failed to produce any detectable **5**; somewhat less than 5% of the starting chromophore was converted into **3**, and most of the remainder was unmodified. It appears that the protein-bound chromophore is shielded from Glu and that it is the free NCS-Chrom in equilibrium with the bound form that undergoes conversion to **3**. These data suggest that **2** rather than **4** is the DNA-cleaving species. Further, since NCS-Chrom, whether or not bound to its apoprotein, shows the same cleavage pattern (chemistry and sequence) when activated by BME,[9] its seems unlikely that the DNA-cleaving species would be different.

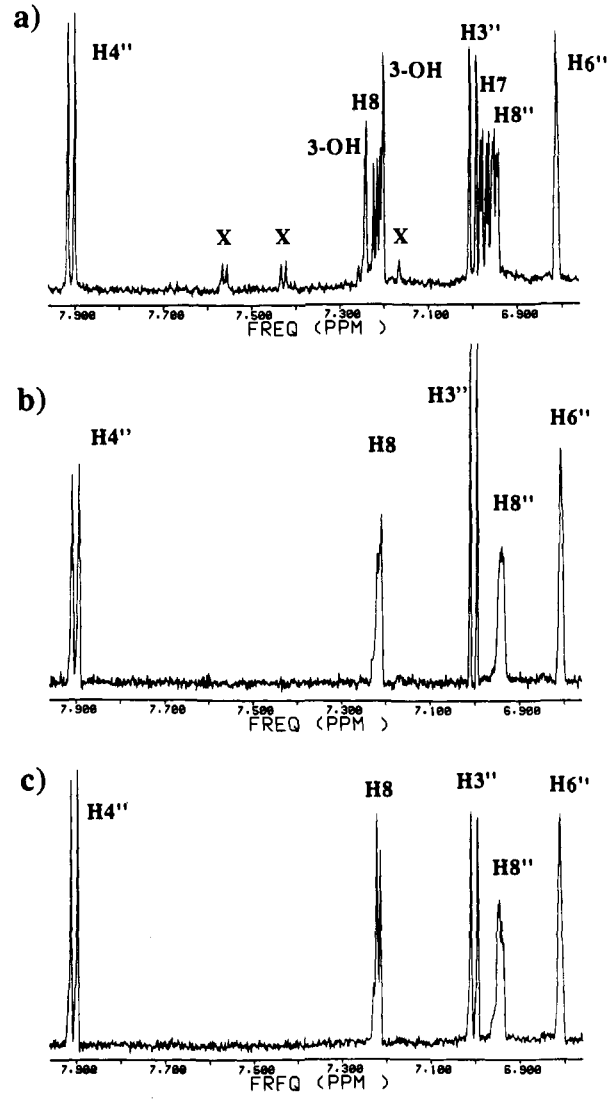
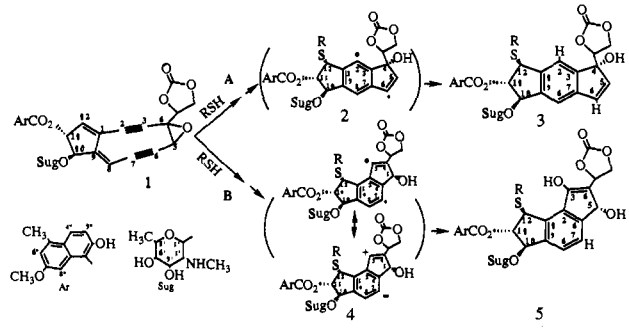


Figure 1. (a) Partial 600-Mhz ^{1}H NMR spectra of **5** in DMSO-d_6. **5** was isolated from a reaction of 0.108 mM holo-NCS, 10 mM BME, 4.32 mM calf thymus DNA, and 50 mM Tris–HCl, pH 8, after incubation at 0 °C for 16 h. (b) **5** was isolated from the same reaction in D_2O, except for the absence of DNA. (c) **5** was isolated from the same reaction in D_2O and with DNA. X in spectrum a represents decomposition products generated during the accumulation of the NMR data. They can be eliminated by adding 1 μL of acetic acid-d_4 to the NMR solvent, as demonstrated in spectra b and c. The assignments of the ^{1}H resonance signals were based on those of Sugiyama et al.[5a]

Scheme I



* Author to whom correspondence should be addressed.

(1) Goldberg, I. H. *Acc. Chem. Res.* **1991**, *24*, 191 and references therein.
(2) Myers, A. G. *Tetrahedron Lett.* **1987**, *28*, 4493.
(3) Chin, D.-H.; Zeng, H. H.; Costello, C. E.; Goldberg, I. H. *Biochemistry* **1988**, *27* 8106.
(4) (a) Chin, D.-H.; Goldberg, I. H. *J. Am. Chem. Soc.* **1992**, *114*, 1914. (b) Chin. D.-H.; Goldberg, I. H. *Biochemistry* **1993**, *32*, 3611.
(5) (a) Sugiyama, H.; Yamashita, K.; Nishi, M.; Saito, I. *Tetrahedron Lett.* **1992**, *33*, 515. (b) Sugiyama, H.; Fujiwara, T.; Kawabata, H.; Yoda, N.; Hirayama, N.; Saito, I. *J. Am. Chem. Soc.* **1992**, *114*, 5573.
(6) Holo-NCS was obtained from Kayaku Antibiotics, and its purity and stability wree assessed by UV–visible spectroscopy and isoelectric focusing electrophoresis. (a) Kappen, L. S.; Goldberg, I. H. *Nucleic Acids Res.* **1978**, *9*, 2959. (b) Dedon, P. C.; Goldberg, I. H. *Biochemistry* **1992**, *31*, 1909.
(7) Based on total fluorescence emission (excitation at 340 nm), the relative ratios of **5** to **3** were 11.3 (10 mM BME), 3.9 (10 mM L-Cys), and 3.8 (5 mM DTT). In 80% 2-propanol, the ratio was 0.19 for both BME and L-Cys.
(8) Kappen, L. S.; Goldberg, I. H. *Biochemistry* **1979**, *18*, 5647.
(9) Takeshita, M.; Kappen, L. S.; Grollman, P.; Eisenberg, M.; Goldberg, I. H. *Biochemistry* **1981**, *20*, 7599.

0002-7863/93/1515-9341$04.00/0

Table I. Incorporation of ^3H from [5-^3H]Thymidine-Labeled λ DNA into Products Generated by Activation of Holo-NCS (3 nmol) by Different Thiols

thiol	P/D[a]	conditions	^3H incorporation (cpm × 10^{-2}) 3	5
BME (10 mM)	4	0 °C, 16 h	2.8	N.D.[a]
BME (10 mM)	4	37 °C, 30 min	2.9	N.D.
BME (10 mM)	20	0 °C, 16 h	9.0	N.D.
DTT (5 mM)	4	0 °C, 16 h	3.8	N.D.
L-Cys (10 mM)	4	0 °C, 16 h	3.6	N.D.
Glu (5 mM)	4	37 °C, 30 min	8.2	

[a] Molar ratio of DNA phosphate to drug. [b] N.D., not detected.

In earlier work we identified the DNA-cleaving species on HPLC by its having abstracted ^3H from DNA labeled at C-5′ of the target thymidine residue.[10] Here, we have analogously followed the incorporation of ^3H from DNA [5′-H]thymidine-labeled λ DNA into the drug products generated by treatment of holo-NCS with thiol (Table I).[11] All the thiols tested except for Glu induced significant amounts of **5**, but the abstracted ^3H was associated with **3**, clearly identifying it as the main DNA-active form of the drug.

This conclusion was further strengthened by ^1H NMR analysis of **5**, isolated from a reaction of holo-NCS with 10 mM BME with or without calf thymus DNA. In each case, **5** predominates over **3** as the product and exhibits identical ^1H resonance signals as reported values.[5a] Applying a reverse deuterium incorporation strategy previously used to identify **2** as the active form of NCS-Chrom,[3] we determined that **5** had not incorporated any hydrogen from DNA into the C7 (or C3) position. The ^1H NMR spectrum of **5**, isolated from the reaction with DNA in H_2O, shows the resonance signals of the two diastereomeric doublets of H8 (separated by 0.008 ppm, J = 8.3 Hz) (between two singlets of 3-OH) and two doublets of H7 (separated by 0.004 ppm, J = 8.3 Hz) (Figure 1a). When D_2O was used as solvent for the reaction without DNA, deuterium was incorporated at C7 to an extent of almost 100%, and H8 was converted into two diasteromeric singlets (Figure 1b). Deuterium was also incorporated into C3 (100%), but inconsistent with the assignment of an OH at this position was the finding that there was no exchange with hydrogen from solvent (unlike the hydrogen of 5-OH on the same indacene ring).[12] When D_2O was used as solvent for the drug–DNA reaction, no hydrogen from DNA replaced the deuterium at C7 (or at C3) (Figure 1c), showing that **5** incorporated deuterium from solvent but not from DNA and was not involved in the DNA-cleavage reaction.

Although **5** is the major inactivation product resulting from thiol (other than Glu) treatment of holo-NCS in the presence or absence of DNA, the precursor of **3**, not **5**, is the species responsible for DNA damage. Since **5** is formed only when NCS-Chrom is bound to its apoprotein, this cyclization pathway probably occurs only within the apoprotein cleft. With some thiols the chromophore is converted to **4** and **5** before it can interact with the DNA. The fact that Glu, the most abundant cellular thiol, is shielded from reaction with the protein-bound chromophore is significant, since this protection may be important in preserving the drug activity until it reacts with the target DNA.

Acknowledgment. This work was supported by NIH Grant CA 44257. The ^1H NMR experiments at 600 MHz were performed at the Comprehensive NMR Center for for Biomedical Research (supported by NIH Grant RR-00995) of the Francis Bitter National Magnet Laboratory at the Massachusetts Institute of Technology.

(10) (a) Charnas, R. L.; Goldberg, I. H. *Biochem. Biophys. Res. Commun.* **1984**, *122*, 642. (b) Kappen, L. S.; Goldberg, I. H. *Nucleic Acids Res.* **1985**, *13*, 1637.

(11) [5′-^3H]Thymidine-labeled λ DNA (0.240 mM for P/D = 4 and 0.725 mM for P/D = 20, 9.2 cpm/pmol), prepared as previously described,[10b] was incubated under the designated conditions in Tris–HCl, pH 8, with the indicated thiols. The supernatant after DNA precipitation was analyzed by reverse-phase HPLC on an analytical C18 colmn using a gradient of methanolic 5 mM ammonium acetate, pH 4 buffer.[10b]

(12) These findings suggest that **5** has a hydrogen at C3 rather than a hydroxyl group, in accord with an earlier proposed structure (Fujiwara, K.; Kurisaki, A.; Hirama, M. *Tetrahedron Lett.* **1990**, *31*, 4329).