Vol. 89, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 27, 1979 Pages 635-642

NEOCARZINOSTATIN: SPECTRAL CHARACTERIZATION AND SEPARATION OF A NON-PROTEIN CHROMOPHORE

M.A. Napier*, B. Holmquist†, D.J. Strydom†, and I.H. Goldberg*

*Department of Pharmacology, Harvard Medical School and
†Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and The Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts 02115

Received June 12,1979

Summary

Chromatographically purified neocarzinostatin exhibits absorption, fluorescence, magnetic circular dichroic and circular dichroic spectral characteristics above and below 300 nm atypical for a protein with its reported amino-acid composition, indicating the presence of a non-protein chromophore. The drug complex, stable at acidic pH, can be dissociated by treatment with reducing or denaturing agents at neutral or basic pH. Chromatography of the dissociated complex, or more conveniently, methanol extraction of the lyophilized drug, separates a protein with an amino-acid composition identical to neocarzinostatin and a highly fluorescent chromophore free of amino-acids.

The antitumor antibiotic neocarzinostatin (NCS)‡ is a single chain acidic protein of MW 10,700 with 2 disulfide bonds (1,2). Evidence indicates that the cytotoxic action of NCS is closely related to its ability to damage DNA. NCS selectively inhibits DNA synthesis in sensitive cells (3-6) and induces both DNA strand breakage (5-11) and DNA repair synthesis in whole cells (12-14) and isolated nuclei (13). NCS introduces single-strand breaks in helical and linear duplex DNA *in vitro*, almost entirely at deoxythymidylic and deoxyadenylic acid residues (15-17). Mercaptans greatly stimulate this reaction (7,11,15). Pre-incubation with mercaptans in the absence of DNA rapidly inactivates the drug (18).

The isolation, purification and chemical characterization of NCS have been described (1,2,19-21). In addition to the 2 disulfides, NCS contains 2 tryptophan, one tyrosine, and 5 phenylalanine residues. Optically active absorption

‡Abbreviations: NCS, neocarzinostatin; CM-NCS, S-carboxymethylated neocarzinostatin; CD, circular dichroism; MCD, magnetic circular dichroism; Tris, Tris(hydroxymethyl)aminomethane.

0006-291X/79/140635-08$01.00/0

bands have been reported above 300 nm (19), and inactivation of NCS by irradiation with 300 nm-400 nm light has been found (22,23, M. Napier and I.H. Goldberg, unpublished). These spectral characteristics are inconsistent with those expected of a purified protein and have led us to further characterize native NCS spectroscopically and to separate a previously unidentified highly fluorescent chromophore from the protein.

MATERIALS AND METHODS

NCS (clinical form, lot #730859) was generously provided by Dr. W.T. Bradner of Bristol Laboratories. Purified NCS and S-carboxymethylated NCS (CM-NCS) were gifts from Dr. T.S.A. Samy of the Sidney Farber Cancer Institute. The original source of NCS is Kayaku Antibiotics Research Laboratories, Tokyo, Japan. Preliminary experiments were carried out with the clinical drug, but all data reported here were obtained with highly purified NCS. All chemicals were of reagent grade. Absorption spectra were recorded on a Cary 118 or 219 spectrophotometer, and uncorrected fluorescence spectra were obtained on a Perkin-Elmer 512 or MPF-3 spectrofluorometer. All spectra were recorded at room temperature. Magnetic circular dichroism (MCD) spectra were measured on a Cary Model 61 circular dichroism (CD) instrument equipped with a Varian Model V4145 superconducting solenoid at a field strength of 40 kgauss. The slit width was adjusted to permit spectral dispersion of 2 nm or less. CD spectra were measured under identical experimental conditions in the absence of a magnetic field. All samples were corrected for appropriate solvent blanks. Protein concentrations were determined by amino-acid analysis. Methanol extraction was done by addition of methanol to lyophilized NCS (1 ml per mg). The mixture was vortexed and allowed to stand at room temperature in the dark for 1 hour, centrifuged and the supernatant, fraction A, removed. The precipitate was washed with methanol and dissolved in 0.01 M aqueous ammonium acetate, fraction B. In all steps, care was taken to reduce the exposure of the drug to light.

RESULTS AND DISCUSSION

NCS purified by CM-cellulose, Sephadex G-50, and DEAE-Sephadex A-25 (19) is homogeneous on acrylamide gel electrophoresis and exhibits an absorption maximum at 270 nm (ε 23,000) and a broad shoulder tailing into the visible between 300 nm and 400 nm ($\varepsilon_{340} \sim 8{,}000$) (Fig. 1). The spectrum is unchanged in 0.1 N HCl after 24 hours at room temperature, or after extensive dialysis against 0.1 N acetic acid. In alkaline solution a maximum is observed at 250 nm - 255 nm, as previously reported (21). The presence of an abnormally high extinction coefficient, the location of λmax below 277 nm, and the increase in absorbance at 250 nm at alkaline pH is not expected based on the amino-acid composition of NCS

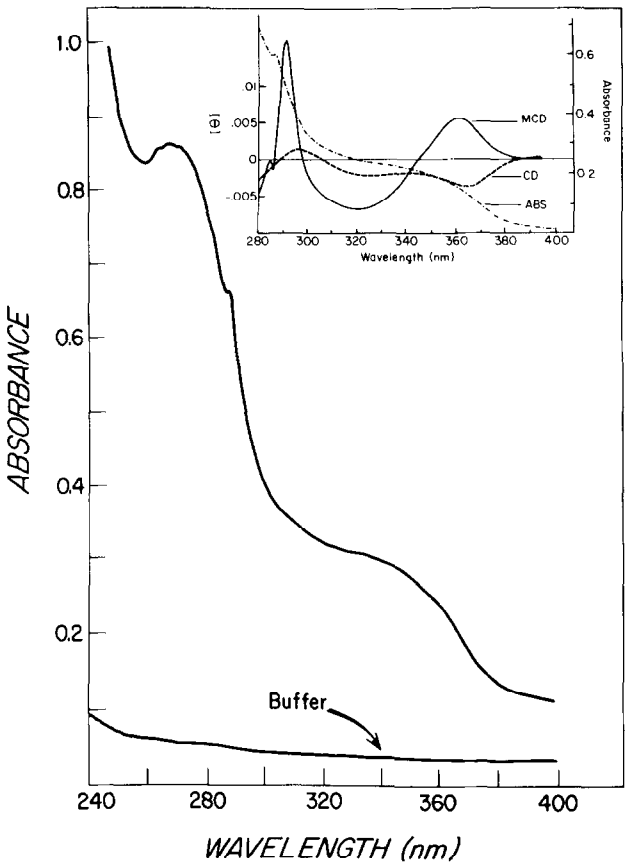

Figure 1. Ultraviolet-visible absorption spectrum of neocarzinostatin, (3.5 x 10^{-5} M, 0.015 M sodium acetate, pH 4.5). Inset: Magnetic circular dichroism (——), circular dichroism (---), and absorption (-·-·-) spectra of neocarzinostatin (3.5 x 10^{-4}M, 0.015 M sodium acetate, pH 4.5).

alone. The atypical absorbances above and below 300 nm indicate the presence of a chromophore other than simple amino-acids.

The MCD and CD (Fig. 1, inset) of NCS both exhibit strong activity above 300 nm, with extremes in the MCD at 320 nm and 365 nm corresponding to the CD minima at these wavelengths (19). The positive maximum in the MCD near 290 nm and 283 nm is due to two tryptophan residues (24).

The fluorescence spectrum of NCS (Fig. 2) has major excitation maxima at 285 nm, 340 nm and 380 nm with emission maxima at 345 nm, 420 nm, and 490 nm, respectively. The 345 nm emission band is attributable to tryptophan residues,

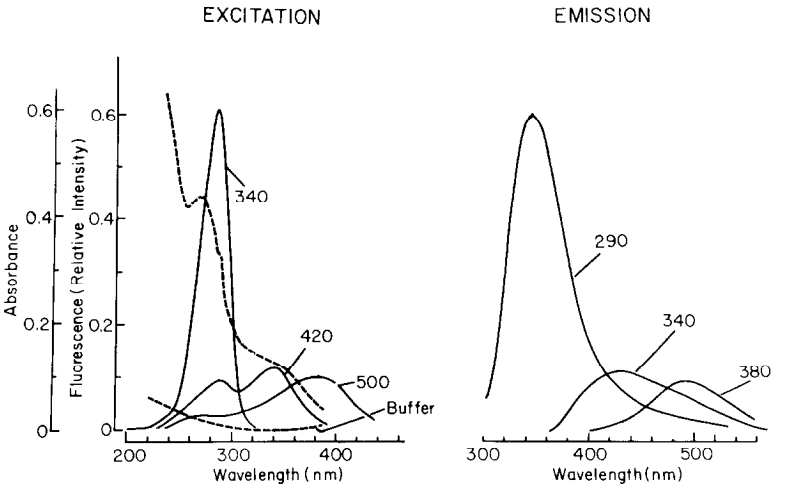

Figure 2. Fluorescence excitation (emission wavelength = 345 nm, 420 nm, 500 nm) and emission (excitation wavelength = 290 nm, 340 nm, 380 nm) of neocarzinostatin (2 x 10^{-5} M, 0.05 M Tris, pH 8.0). Absorption spectrum (---) of neocarzinostatin.

but the intensity is low for the two tryptophans present, 45% of the intensity of an equivalent concentration of N-acetyltryptophanamide. The tryptophan emission is enhanced by treatment with reducing or denaturing agents. In the presence of 0.01 M 2-mercaptoethanol or 4 M guanidine-HCl, in 0.05 M Tris, pH 8.0, for 24 hours, the 345 nm emission is increased 1.4-fold and 2.0-fold respectively. CM-NCS, which has virtually no absorption or fluorescence excitation above 300 nm, exhibits normal tryptophan emission. Guanidine-HCl-treated NCS has increased absorption below 270 nm and a broad maximum at 380 nm. The fluorescence emission intensity is increased 1.4-fold at 420 nm and 8-fold at 490 nm. NCS treated with 2-mercaptoethanol (or sodium borohydride) exhibits no apparent change in the absorption above 300 nm. However, the intensity of the 420 nm and 490 nm emission bands is increased 2.4-fold and 1.3-fold, respectively. The reduced emission of native NCS might be due to quenching resulting from the tightly folded structure of NCS, which is relaxed by these treatments, as measured by CD (19, M. Napier and I. Goldberg, unpublished). It is more likely that the altered emission is due to the dissociation of a non-protein

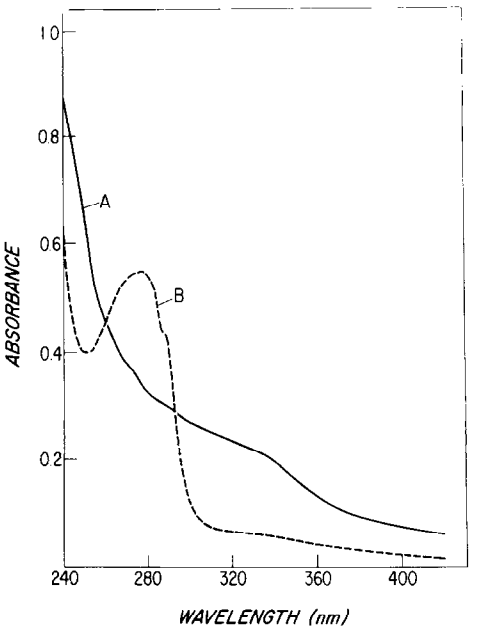

Figure 3. Methanol extraction of neocarzinostatin. Absorption spectra of methanol-soluble fraction A, in methanol (——) and methanol-insoluble fraction B in water (---). The fractions were diluted to a volume approximately equivalent to 1 mg/ml neocarzinostatin.

chromophore, which quenches the tryptophan emission by competitively absorbing excitation energy and by serving as an energy acceptor.

Separation of a non-protein UV/visible absorbing material from the NCS protein is possible by several procedures. Sephadex G-50 chromatography of guanidine-treated, of β-mercaptoethanol-treated, or of base-treated (0.1 N NaOH), but not of acid-treated (0.1 N HCl), NCS, separates the protein and non-protein components. The most convenient separation procedure, however, is methanol extraction of the lyophilized drug. The methanol fraction (A) (Fig. 3), free of common amino-acids, has two broad absorption shoulders near 270 nm and 340 nm, CD and MCD activity above and below 300 nm, and fluorescence emission at 420 nm and 490 nm (Fig. 4A). The methanol-insoluble fraction (B) with an amino-acid composition identical to NCS, exhibits typical protein absorbance (Fig. 3), with λmax at 277 nm (ε 14,000). The absorption at 250 nm, seen for NCS, is reduced. Absorption, fluorescence excitation, and optical activity

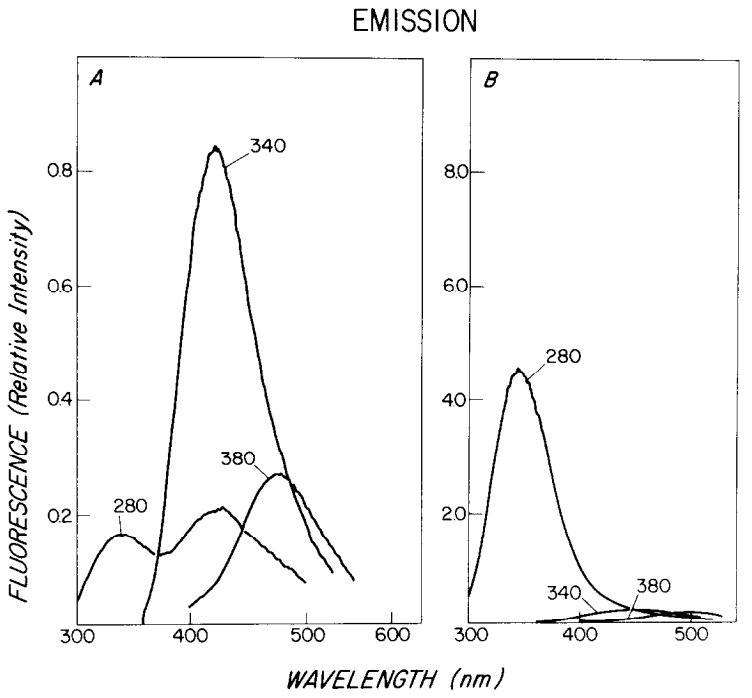

Figure 4. Fluorescence emission spectra (excitation wavelength = 280 nm, 340 nm, 380 nm) of neocarzinostatin fraction A and fraction B.

above 300 nm are virtually absent. Also, there are no changes in the tryptophan residues as determined by MCD. The only significant fluorescence remaining is the tryptophan emission at 345 nm (Fig. 4B). The spectral properties of fraction B are similar to those found for CM-NCS which has lost the non-protein chromophore probably due to reduction and methanol extraction procedures used in its preparation (20).

Thus, NCS purified by chromatography is an acid-stable complex of a protein and a non-protein chromophore which can be dissociated and separated by a number of procedures. It is of interest that carzinostatin, an uncharacterized antibiotic also isolated from S. carzinostaticus, has been described as a complex consisting of a methanol-soluble, low molecular weight fraction and a methanol-insoluble, water-soluble, high molecular weight fraction (25). Neither fraction had significant biological activity alone, but recombination restored activity.

NCS is a more stable antibiotic isolated from variant 41 of S. carzinostatin, with biological properties similar to carzinostatin.

Experiments are in progress to purify, identify and determine the biological activity of the non-protein chromophore in NCS, and to determine its contribution to the biological activity of NCS.

Acknowledgements

The authors wish to thank Dr. T.S.A. Samy for the gift of the purified NCS and Dr. W.T. Bradner for the gift of clinical NCS. This work was supported by U.S. Public Health Service Research Grant GM 12573 from the National Institutes of Health (MAN and IHG) and by the Grant-in-Aid GM 15003 from the Department of Health, Education and Welfare of the National Institutes of Health (BH and DJS).

References

1. Ishida, N., Miyazaki, K., Kumagai, K. and Rikimaru, M. (1965) J. Antibiot. 18, 68-76.
2. Meienhofer, J., Maeda, H., Glaser, C.B., Czombos, J. and Kuromizu, K. (1972) Science 178, 875-876.
3. Ono, Y., Watanabe, Y. and Ishida, N. (1966) Biochim. Biophys. Acta 119, 46-58.
4. Homma, M., Koida, T., Saito-Koide, T., Kamo, I., Seto, M., Kumagai, K. and Ishida, N. (1970) Progress in Antimicrobial and Anticancer Chemotherapy, Proceedings of the VI International Congress of Chemotherapy 2, 410-415.
5. Sawada, H., Tatsumi, K., Sasada, M., Shirakawa, S., Nakamura, T. and Wahishaka, G. (1974) Cancer Res. 34, 3341-3346.
6. Beerman, T.A. and Goldberg, I.H. (1977) Biochim. Biophys. Acta 475, 281-293.
7. Beerman, T.A. and Goldberg, I.H. (1974) Biochem. Biophys. Res. Commun. 59, 1254-1261.
8. Tatsumi, K., Nakamura, T. and Wakisaka, G. (1974) Gann 65, 459-461.
9. Ohtsuki, K. and Ishida, N. (1975) J. Antibiot. 28, 143-148.
10. Ohtsuki, K. and Ishida, N. (1975) J. Antibiot. 28, 229-236.
11. Beerman, T.A., Poon, R. and Goldberg, I.H. (1977) Biochim. Biophys. Acta 475, 294-306.
12. Tatsumi, K., Sakane, T., Sawada, H., Shirakawa, S., Nakamura, T. and Wakisaka, G. (1975) Gann 66, 441-444.
13. Kappen, L.S. and Goldberg, I.H. (1978) Biochim. Biophys. Acta 520, 481-489.
14. Hatayama, T. and Goldberg, I.H. (1979) Biochim. Biophys. Acta, in press.
15. Poon, R., Beerman, T.A. and Goldberg, I.H. (1977) Biochemistry 16, 486-492.
16. Hatayama, T., Goldberg, I.H., Takeshita, M. and Grollman, A.P. (1978) Proc. Natl. Acad. Sci. USA 75, 3603-3607.
17. D'Andrea, A.D. and Haseltine, W.A. (1978) Proc. Natl. Acad. Sci. USA 75, 3608-3612.

18. Kappen, L.S. and Goldberg, I.H. (1978) Nucleic Acids Res. 5, 2959-2967.
19. Samy, T.S.A., Atreyi, M., Maeda, H. and Meienhofer, J. (1974) Biochemistry 13, 1007-1013.
20. Maeda, H., Glaser, C.B., Czombos, J., Meienhofer, J. (1974) Arch. Biochem. Biophys. 164, 369-378.
21. Maeda, H., Kumagai, K., Ishida, N. (1966) J. Antibiot. 19, 253-259.
22. Kohno, M., Haneda, I., Koyama, Y. and Kikuchi, M. (1974) Jap. J. Antibiot. 27, 707-714.
23. Burger, R.M., Peisach, J. and Horwitz, S.B. (1978) J. Biol. Chem. 253, 4830-4832.
24. Holmquist, B. and Vallee, B.L. (1978) in Methods in Enzymology, 49, 149-179.
25. Shoji, J. (1961) J. Antibiot. 14, 27-33.