2D NMR STUDY OF DRUG-PROTEIN INTERACTIONS : ETHIDIUM BROMIDE - NEOCARZINOSTATIN COMPLEX

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Introduction

Neocarzinostatin (NCS) is a small acidic holo-protein isolated from the culture broth of ***Streptomyces Carzinostaticus*** [1]. It has a protein component (apo-NCS) of 113 amino acid residues and a non-covalently bound heat and light sensitive chromophore (NCS-chr) (Fig.1). This protein possesses antibiotic activity against organisms such as ***Sarcina Lutea*** and antitumor activity against the experimental tumors ***Ascitic Sarcoma 180, Ascitic Leukemia SN-36, Leukemia L-210*** [1-3]. It is known that the chromophore is responsible for all the biological activities and the apo-protein stabilizes and acts as a carrier for this UV sensitive component of the antitumor drug [4].

Though the secondary and tertiary structure of the apo-protein is well understood from X-ray and NMR studies, little is known about the binding region and the amino acid residues involved in the drug - protein interactions in the holo-protein.

Both the crystal structure at 2.8 Å [5] and the 2-D NMR work done on apo-NCS [6-8] indicate that a major part of the protein is composed of a seven strand antiparallel ß-sandwich formed by a three strand ß-sheet and a four strand ß-sheet. The rest of the protein is composed of two loops oriented somewhat perpendicularly to the ß - sandwich, thus forming a distinct U-shaped cleft between the four strand face of the sandwich and one of the loops of the molecule (Fig. 2). The crystal structure of holo-NCS at 2.0 Å resolution indicates that the chromophore is located in this cleft. But a detailed knowledge in the region of the chromophore could not be obtained from the 2.0Å map. Preliminary NMR studies also indicate that the chromophore binds within the cleft and interacts with amino acid residues in the region C37-D48 [9, 10]. Although NCS does not bind the chromophore of other streptomyces derived anti-tumor proteins (e.g. Auromomycin) [11] it is known from X-ray studies [12] to strongly bind a number of drugs including ethidium bromide (Fig. 3) and daunomycin. In order to further elucidate the nature of drug-NCS interactions, we have initiated a study of both holo-NCS and the complex between ethidium bromide and NCS.

Materials and Methods

Apo-NCS solution used in our experiments was prepared from holo-NCS. The chromophore was extracted

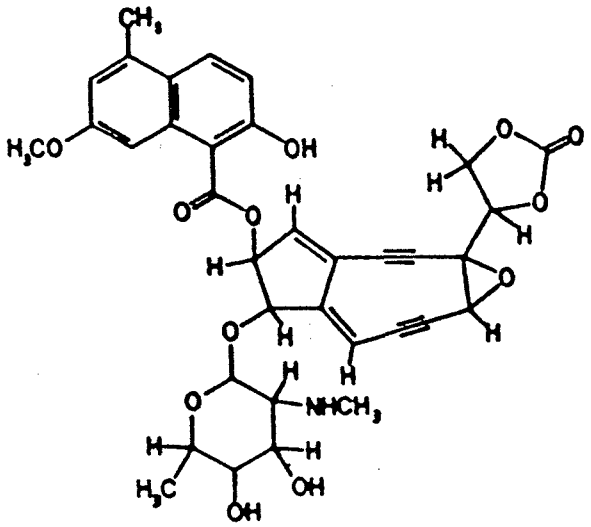

Fig. 1: NCS-Chromophore

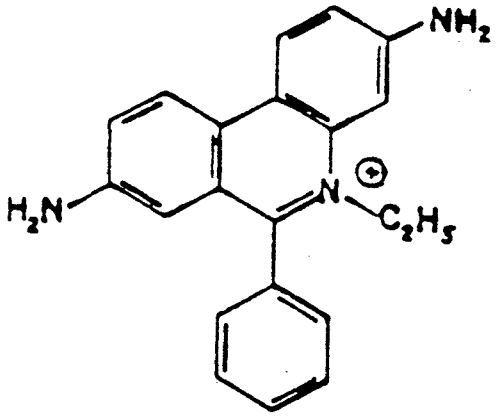

Fig. 3: Ethidium Bromide

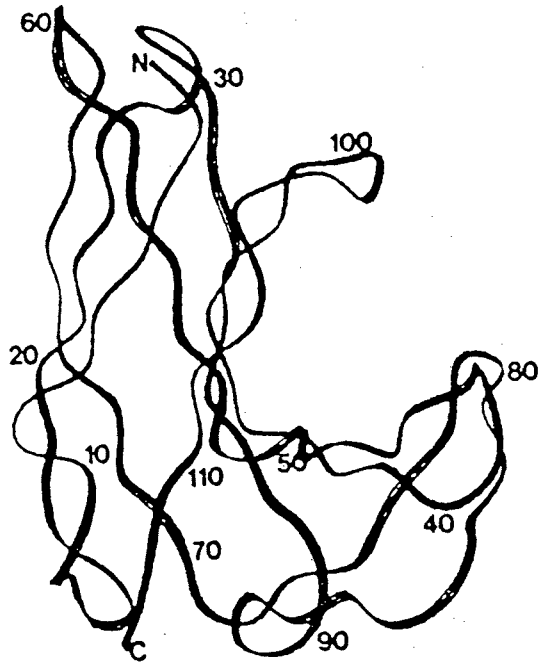

Fig. 2: Ribbon picture of NCS by X-ray

the procedure of Napier et.al.[13]. Purified lyophilized holo-NCS was a gift from Kayaku Co. Ltd. The 1:1 Ethidium Bromide-NCS complex was prepared by adding the protein solution in to vials containing the drug. The final solution was purified by passing through sephadex G-25 column followed by lyophilization. The lyophilized complex was brought up in 10mM acetate buffer (pH 5, 90% H_2O/ 10% D_2O for non exchanged protein sample and 99.98% D_2O for exchanged protein sample) and 10mM EDTA. The concentration was adjusted between 2.0 mM to 2.5 mM for 500µl sample.

All NMR experiments were performed on a Bruker AM-500 Spectrometer at 313 K. DQF- and TQF-COSY [14], RELAY [15], TOCSY [16] and NOESY [17] were acquired in TPPI mode with standard phase cycling schemes. The water resonance was presaturated by selective irradiation between 1.5 s to 2 s. RELAY spectra were performed with mixing times of 30 ms (90%H_2O) and 25 ms (for 99.98%D_2O). TOCSY spectra were performed with a variety of mixing times ranging between 40 to 80 ms. NOESY spectra were recorded with 150 ms mixing time, randomly varied by 10%. The data were processed with FTNMR software of Dr. Dennis Hare [18].

Results and Discussion

Our ^{1}H-NMR assignments of ethidium bromide - NCS complex in solution indicates the drug to be located in the cleft region. There are two lines of evidence which support this conclusion. First, the chemical shifts of numerous residue protons within the cleft are strongly perturbed upon binding to ethidium bromide. Such chemical shifts changes would be expected to arise due to the ring currents from ethidium bromide's extensive aromatic system. The double quantum filter cosy (DQF COSY) of the complex in water shows significant shifts of the methyl proton resonance lines of Leu-45 (Fig. 4a and 4b) and of residues Gln-36, Cys-37, Ala-38, Trp-39, Leu-45, Cys-47, Asp-48, Cys-93, Gln-94, Leu-97 in the fingerprint region (Fig. 5 and Fig. 6).

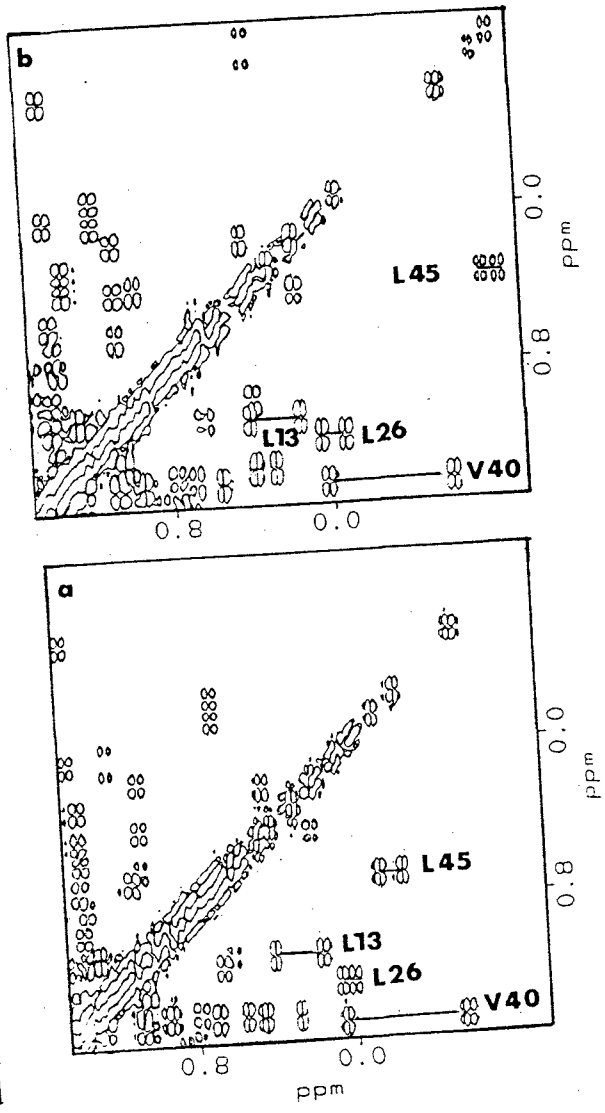

Fig. 4: DQF-COSY spectrum in D_2O showing upfield shift of Leu-45 methyl protons chemical shifts (a): apo-NCS; (b): complex

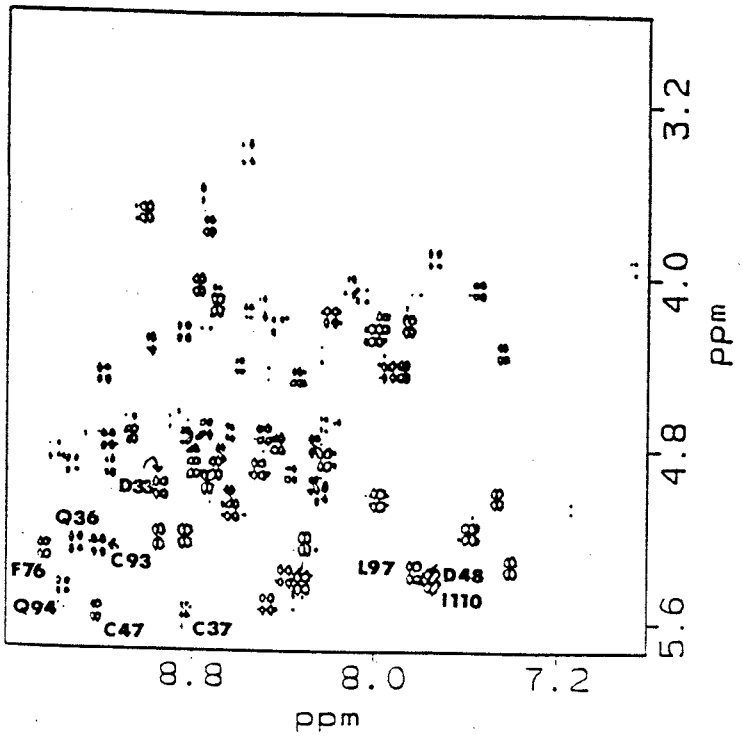

Fig. 5: DQF-COSY fingerprint region of apo-NCS recorded at 40°C .

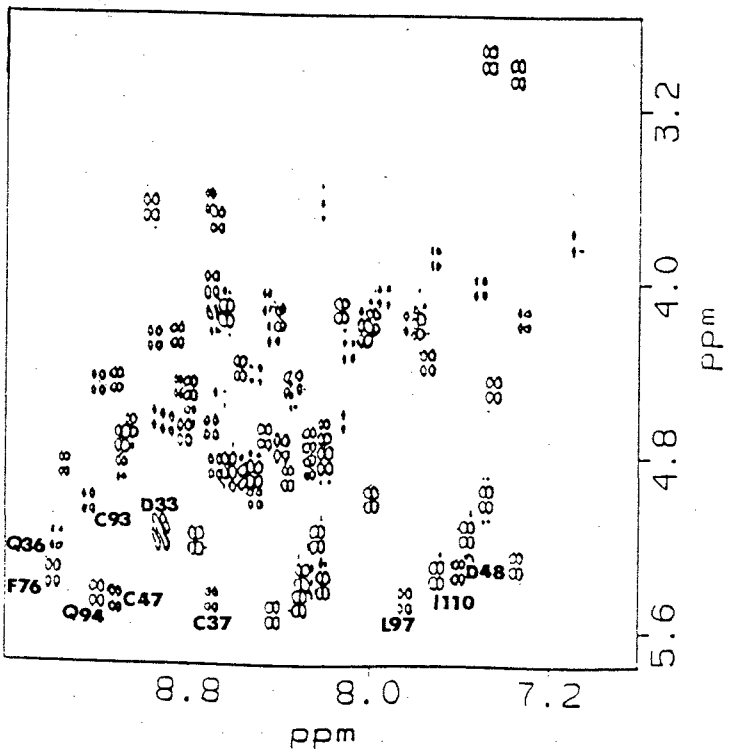

Fig.6: DQF-COSY fingerprint region of ethidium bromide - NCS complex recorded under similar conditions as above.

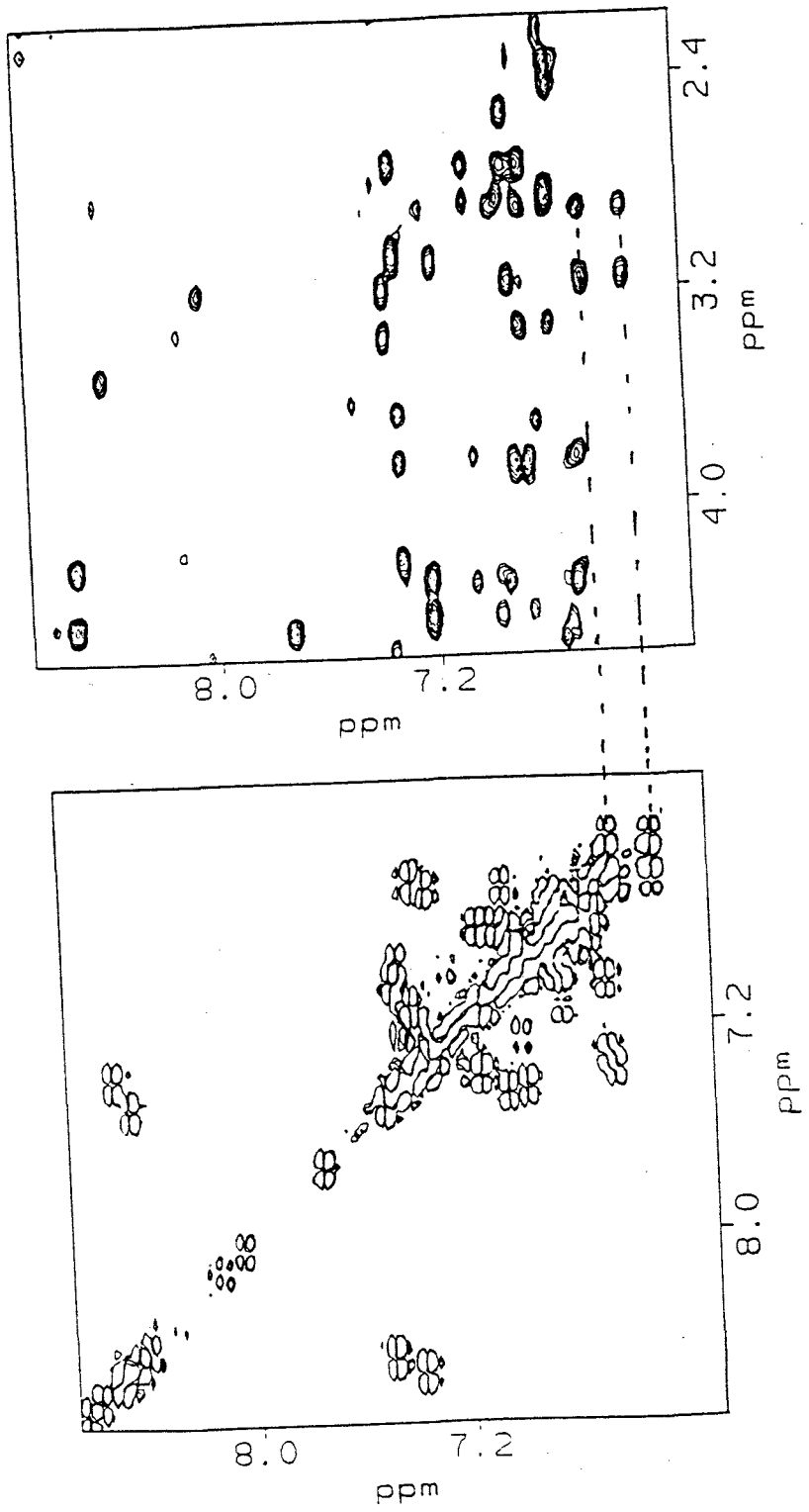

Fig.7: DQF-COSY and NOESY spectra of the complex in D_2O showing some of the intermolecular NOE observed between the aromatic protons of ethidium bromide (shown by dotted lines) and ß-protons of certain residues within the cleft.

While residues Cys-93, Gln-94, Leu-97 are in the four strand face of the ß-sandwich, which forms one side of the cleft, residues Gln-36, Cys-37, Ala-38, Trp-39, Leu-45, Cys-47, Asp-48 are in one of the loops that forms the other side of the cleft. Second, a number of NOEs have been observed to occur between protons on ethidium bromide and residue protons within the cleft. The Leu-45 methyl group is ring current shifted and shows NOEs to the methylene protons and aromatic protons of ethidium bromide. Intermolecular NOEs are also observed between the aromatic protons of ethidium bromide to the aromatic proton of Trp-39 and to the ß protons of Ser-98, Cys-37 and Gly-96 (Fig. 7). Additional intermolecular NOEs are observed but have not been unambiguously assigned.

Conclusion

NMR assignment based on coherence transfer experiments and nuclear overhauser studies done so far indicates that ethidium bromide binds to a single site within the chromophore binding cleft of NCS. Ultimately a knowledge of the binding of ethidium bromide will be doubly useful : as a probe of drug -NCS interactions but also as a probe of molecular dynamics. Ethidium bromide is a dye with a fluorescent life time long enough (10 ns) to give direct information on the degree anisotropy in the overall tumbling of NCS. Coupled with NMR relaxation studies of ethidium bromide - NCS complex, fluorescence studies should furnish a very complete view of the internal dynamics of the protein and its complexes to a number of drugs.

References

[1] N. Ishida, K. Miyazaki, K. Kumgai, and M. Rikimaru, *J. Antibiot.*, **A18**, 68. (1965).

[2] Y. Ono, Y. Watanable, and N. Ishida, *Biochem. Biophys. Acta.*, **119**, 46 (1966).

[3] W. T. Bradner and D. J. Hutchinson, *Cancer chemothr. Rep.*, **50**, 79. (1966).

[4] L. S. Kappen, M. A. Napier, and I. H. Golgberg, *Proc. Natl. Acad.Sci. , USA.*, **77**, 1970. (1980).

[5] L. C. Sieker, 1981, University of Washington, Seattle, WA:

[6] M. L. Remerowski, S. J. Glaser, L. C. Sieker, T. S. A. Samy, and G. P. Drobny, *Biochemistry.*, **29**, 8401. (1990).

[7] E. Adjadj, *et al.*, *Eur. J. Biochem.*, **190**, 263.(1990).

[8] X. Gao and W. Burkhart, *Biochemistry.*, **30**, 7730.(1991).

[9] S. Mohanty and G. P. Drobny. . in *Experimental Nuclear Magnetic Resonance Conference.*, **WP-18**, 181 Asilomer, CA (1992).

[10] H. Takashima, S. Amiya, and Y. Kobayashi, *J. Biochem.*, **109**, 807. (1991).

[11] L. S. Kappen, M. A. Napier, I. H. Goldberg, and T. S. A. Samy, *Biochemistry.*, **19**, 4780.(1980).

[12] L. C. Sieker, Personal communication(1991).

[13] M. A. Napier et.al., *Biochem. Biophys. Res. Commun.*, **89**, 635. (1979).

[14] U. Piantini, O. W. Sørensen, and R. R. Ernst, *J. Am. Chem. Soc.*, **104**, 6800. (1982).

[15] G. Eich, G. Bodenhausen, and R. R. Ernst, *J. Am. Chem. Soc.*, **104**, 3732. (1982).

[16] L. Braunschweiler and R.R. Ernst, *J. Magn. Reson.*, **53**, 521. (1983).

[17] Anil Kumar, R. R. Ernst, and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, **95**, 1. (1980).

[18] D. Hare, Hare Research: Woodinville, WA.