Solution Structure of the Antitumour Antibiotic Neocarzinostatin, a Chromophore–Protein Complex

Toshiyuki Tanaka,[a] Masahiro Hirama,*[a] Ken-ichi Fujita,[b] Seiichi Imajo[c] and Masaji Ishiguro[c]

[a] *Department of Chemistry, Faculty of Science, Tohoku University, Sendai 980, Japan*
[b] *Analytical Instruments Division, JEOL Ltd., Akishima 196, Japan*
[c] *Suntory Institute for Biomedical Research, Shimamoto, Osaka 618, Japan*

The chromophore-binding structure of the neocarzinostatin complex is determined for the first time by two-dimensional ^{1}H NMR spectroscopy, and reveals the elements of the specific binding and the stabilization interactions of the unstable dienediyne chromophore by the apoprotein.

The extremely potent enediyne anticancer antibiotics possess unprecedented and highly unusual structures as well as intriguing modes of action.[1] Neocarzinostatin chromophore[2] (NCS-chr, **1**) as such[3] is very labile to heat, light and pH above 6, but is stabilized substantially by the specific[4] and tight[5] binding to its apoprotein[6] (apo-NCS, 113 amino acids) secreted simultaneously. However, neither the tertiary structure of the NCS complex nor the stabilization interactions preventing NCS-chr from decomposing has been determined, although this information is of great interest in connection with molecular recognition and protein function. Only the computer modelling of the NCS structure based on the known X-ray crystallographic structure of actinoxanthin (AXN) apoprotein has been reported.[7,8] Knowledge concerning all other so-called chromoprotein antibiotics, auromomycin (AUR),[9] AXN,[10] C-1027[11] and kedarcidin,[12] is at a similar status. We report herein the fully NMR-derived three-dimensional structure of the NCS complex, which reveals the elements of the specific binding and the stabilization interactions of the unstable NCS-chr by apo-NCS. This is the first time that the chromophore-binding structure has been determined in a member of the chromoprotein antibiotic family.

The three-dimensional structure of the NCS complex was calculated in two steps.† The structure of the apo-NCS portion was computed first using the DADAS90 program (MolSkop system; JEOL Ltd., Akishima, Japan)[13] on a Titan 750 computer and the constraints for apo-NCS.‡ Starting with 100 randomly generated structures, 15 final structures with the lowest target function values were obtained. The average of the root-mean-square deviations (RMSDs) of the heavy backbone atoms for all possible pairs among the 15 structures is 0.60 ± 0.18 Å. None of the structures have NOE (nuclear Overhauser effect) violations over 0.42 Å or van der Waals distance violations larger than 0.40 Å. The DGEOM (E. I. du Pont de Nemours and Co., Wilmington, Delaware)[14] calculation was then performed to elucidate the complete structure of the NCS complex on a Power IRIS computer: Ten initial complex structures were generated by docking NCS-chr onto the best DADAS90 structure with the smallest sum of NOE and van der Waals violations (14.0 Å) so as to minimize the violation of the intermolecular NOE distance constraints as less as possible. The subsequent minimization of these initial structures was performed with the DGEOM program to satisfy all the constraints for the complex. The average of all 45

† The DADAS90 program is appropriate for rapid determination of a protein solution structure because it adopts only dihedral angles as variables, as opposed to the DGEOM program which uses Cartesian coordinates of constituent atoms. However, the former cannot deal with two molecules simultaneously.

‡ Homonuclear two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiments based on straightforward resonance assignments produced 934 interproton distance restraints: 849 for apo-NCS, 24 for NCS-chr, and 61 intermolecular restraints between apo-NCS and NCS-chr. These restraints were supplemented by 65 torsion angle constraints composed of 55 ϕ, 3 Ψ and 7 χ_1 restraints, 6 constraints for 2 disulfide bridges, and 68 constraints for 34 backbone NH-CO hydrogen bonds. Thus, 1073 NMR restraints were obtained.

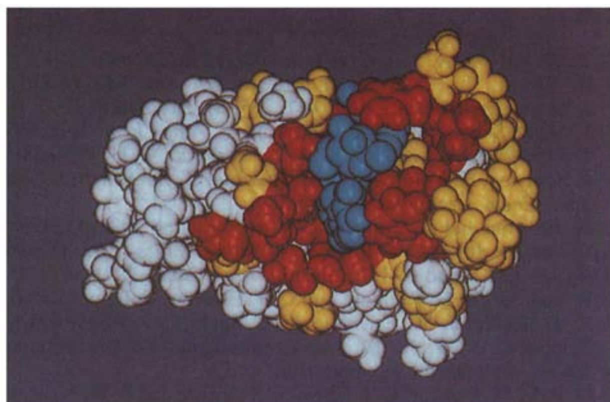


Fig. 1 Space-filling model of the best refined structure of the NCS complex. Apo-NCS residues with significant backbone and/or side chain chemical shift changes ($\geqslant$0.1 ppm) upon chromophore binding are yellow, residues with intermolecular NOE with NCS-chr are red, and the remaining residues are white. NCS-chr is shown in blue. Only heavy atoms are shown for NCS-chr.

pairwise RMSD values of the heavy backbone atoms among the 10 refined structures is 0.30 ± 0.06 Å, and none have NOE or van der Waals distance violations larger than 0.32 Å.

The holoprotein structure with the smallest violation is shown in Figs. 1 and 2. A hydrophobic pocket with approximate dimensions of 12 × 9 × 9 Å is formed by the four-stranded β-sheet (residues 44–47, 53–56; 31–40; 93–97; 107–109) and two loops (residues 77–80; 99–102). This pocket is the binding site of NCS-chr (Fig. 1). Apo-NCS accepts the naphthoate moiety of NCS-chr deeply into the pocket. The C(5″)–C(7″) site of the naphthoate is in proximity to the bottom of the pocket where the main chains of three strands (Val34-Gly35-Gln36, Leu97-Gly96-Val95 and Gly107-Val108) are exposed. The carbocyclic core is located on the Cys37-Cys47§ disulfide bond, and the aminosugar and the carbonate moieties are facing outwards. There seems to be no aromatic stacking interaction with the naphthalene ring, such as that predicted by computer modelling.[7] Instead, several other principal interactions are indicated (Fig. 2).¶ The oxygen atoms of C(7″)-O and C(2″)-O of the naphthoate are close enough to OγH of Ser98 and NεlH of Trp39, respectively, to produce a hydrogen bond. The C(5″)-methyl is approximately in van der Waals contact with the β-methylene of Gln94. The upfield shift of the C(7″)OMe resonance by 1 ppm upon binding is most likely due to the diamagnetic anisotropy of the nearby aromatic ring of Phe52, which supports a possible CH-π interaction.[15] CH···O type hydrogen bonding[16] is likely between C(6″)H and the backbone carbonyl of Gly96. On the other hand, the C(2)–C(3) triple bond of the core is just above the sulfur atom of Cys47, and its side is covered perpendicularly by the aromatic ring of Phe52. The aromatic ring of Phe78 is surrounding the other side of the core, in van der Waals contact with the C(6)–C(7) triple bond. The Leu45 side chain is close to the C(8)–C(9) double bond of the core. The core epoxide is facing down towards the hydrophobic bottom of the pocket. Thus, the hydrophobic interactions are mainly observed with the nine-membered core. Another key interaction is the salt bridge between the protonated aminosugar methylamino group (2′-NHMe) and the Asp33 carboxylate. The carbonate carbonyl group appears to interact with the hydroxy of Ser53 by hydrogen bonding. Though the overall shape of the binding pocket is similar to the related chromoproteins, AUR[9] and AXN,[10] the side chains of the residues along the binding pocket are very different in size and functionality except the Cys37-Cys47 disulfide. Since NCS-chr is a poor ligand for AUR apoprotein,[4] it is very likely that the residues described above are crucial for chromophore binding specificity.[4,9]

Since the naphthoate group is located most deeply in the pocket, it may be essential for the specific and strong binding. This is consistent with the binding of β-naphthol[5] and a synthetic compound **2** to apo-NCS. The naphthoate's role is also supported by the NCS-chr analogue **3** with low solubility in water, which exhibits antitumour and DNA-cleaving activities only when incubated with apo-NCS solution.[17]

The conformation of bound NCS-chr in the complex shows some interesting features. The sugar portion hangs over the

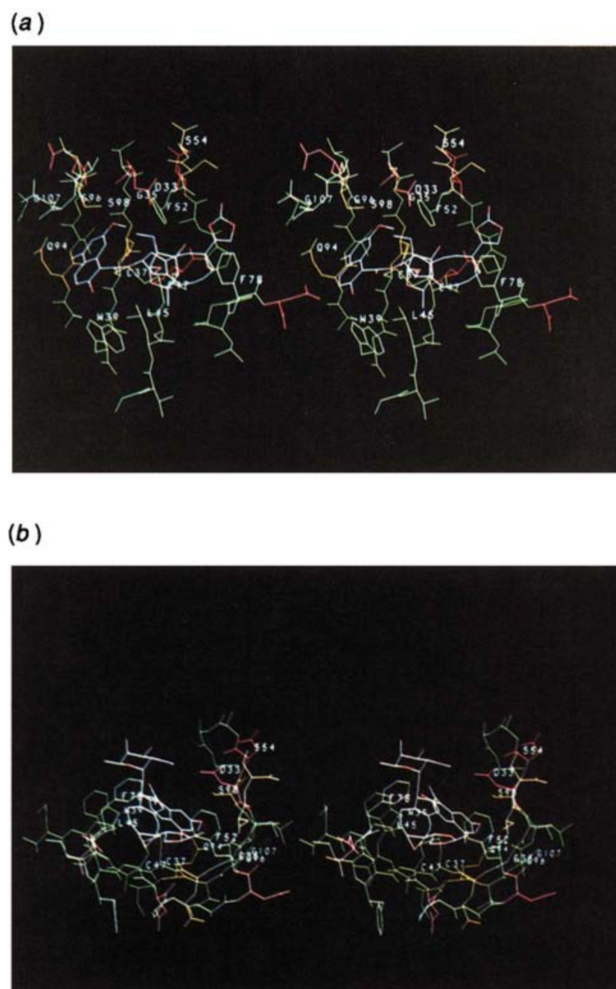
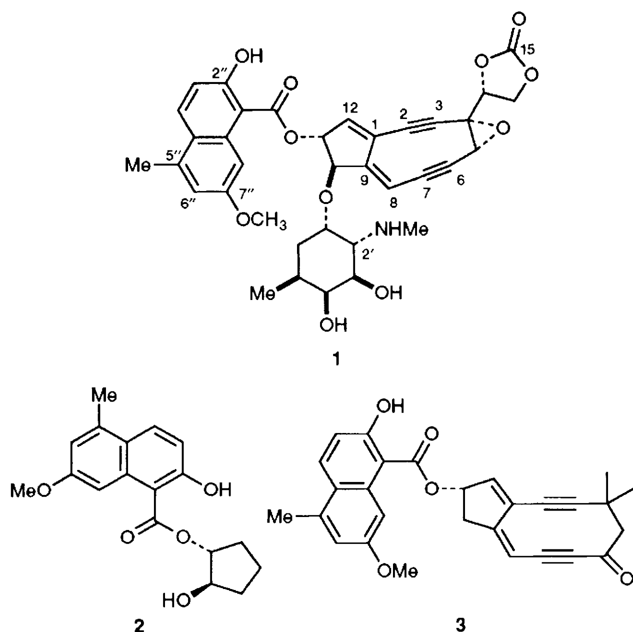


Fig. 2 Stereoviews of the binding site of the NCS complex. All heavy atoms of NCS-chr are shown: carbon atoms are white, the nitrogen atom is blue, and oxygen atoms are red. Heavy atoms of the apo-NCS residues that are within 5 Å from NCS-chr are included. Acidic, hydrophilic, and hydrophobic amino acid residues are red, yellow and green, respectively. Residues with side chains projecting into the pocket are indicated by one-letter codes.

§ The amino acid residues with side chains that project into the pocket are underlined.

¶ Selected atomic distances (Å) between NCS-chr and apo-NCS residues are: (C1, Cys37 Sγ), 3.84; (C1, Cys47 Sγ), 3.46; (C1, Phe52 Cε1), 3.45; (C1, Phe52 Cζ), 4.27; (C2, Cys47 Sγ), 3.97; (C2, Phe52 Cδ1), 3.59; (C2, Phe52 Cε1), 3.00; (C3, Cys47 Sγ), 4.69; (C3, Phe52 Cδ1), 3.60; (C3, Phe52 Cε1), 3.34; (C6, Phe78 Cγ), 3.30; (C6, Phe78 Cδ2), 3.60; (C7, Phe78 Cγ), 3.59; (C7, Phe78 Cδ2), 3.42; (C7, Phe78 Cε2), 3.77; (C8, Leu45 Cδ2), 4.08; (C8, Cys47 Sγ), 4.41; (C8, Phe78 Cδ2), 3.82; (C8, Phe78 Cε2), 3.93; (C9, Leu45 Cδ2), 3.64; (C9, Cys37 Sγ), 4.47; (C9, Cys47 Sγ), 3.74; (C12, Cys37 Sγ), 3.30; (C12, Cys47 Sγ), 3.70; (C12, Phe52 Cδ1), 4.17; (C12, Phe52 Cε1), 3.14; (C12, Phe52 Cζ), 3.59; (C15=O, Ser53 Oγ), 3.06; (N2′, Asp33 Oδ2), 3.08; (C2″-O, Trp39 Nε1), 2.86; (C5″-C, Gln94 Cβ), 3.34; (C6″, Gly96 O), 3.95; (C7″-O, Ser98 Oγ), 2.95; (C7″-OC, Phe52 Cγ), 3.52; (C7″-OC, Phe52 Cζ), 3.44.



five-membered ring of the core with the hydrophobic side down, thus satisfying the *exo*-anomeric effect (Fig. 2). The aminomethyl group is forced to come closer to C(12) (4.3 Å) due to salt bridge with Asp33 compared with the free form (5.4 Å).|| The naphthoate stays away from the core to fit the pocket, thus allowing the core to lie on the disulfide bridge, while in the free form it lies just below the core to become more compact.

A nucleophile[3] or radical[18] addition to C(12) of the core, and a concomitant epoxide opening, initiate the aromatization of NCS-chr. As shown in Fig. 2, the side chains of Ser98, Asp33, and Phe52, as well as the protonated methylamino group of the aminosugar, cover the reactive centre C(12). These steric hindrances to C(12) may be the major stabilizing factor of NCS-chr. Moreover, facing down towards the hydrophobic bottom of the pocket protects the epoxide from an acid catalyst. In addition, the core lies on the disulfide bond, which might stabilize the strained unsaturated system of NCS-chr probably through their orbital HOMO (the diene-diyne π-system)–LUMO (disulfide σ^* or vacant d orbital) interaction. Since the disulfide bridge is conserved in all chromoprotein antibiotics,[9–12] such an interaction with the strained enediyne chromophore[11,12] may be common in this family.

|| The solution structure of free NCS-chr in protic media was elucidated by DGEOM calculation using 19 useful NOE constraints that were obtained through one-dimensional NOE experiments performed at −5 °C in CD_3OD/D_2O = 2/1.

This work was supported by the Ministry of Education, Science and Culture, Japan and also by the Mitsubishi, CIBA-GEIGY, Shorai, TERUMO, Takeda, and Fujisawa Science Foundations.

Received, 11th May 1993; Com. 3/02687F

References

1 K. C. Nicolaou and W.-M. Dai, *Angew. Chem., Int. Ed. Engl.*, 1991, **30**, 1387.
2 K. Edo, M. Mizugaki, Y. Koide, H. Seto, K. Furihata, N. Otake and N. Ishida, *Tetrahedron Lett.*, 1985, **26**, 331.
3 I. H. Goldberg, *Acc. Chem. Res.*, 1991, **24**, 191, and references therein.
4 L. S. Kappen and I. H. Goldberg, *Biochemistry*, 1980, **19**, 4786.
5 K. Edo, K. Saito, Y. Akiyama-Murai, M. Mizugaki, Y. Koide and N. Ishida, *J. Antibiot.*, 1988, **41**, 554.
6 K. Kuromizu, S. Tsunasawa, H. Maeda, O. Abe and F. Sakiyama, *Arch. Biochem. Biophys.*, 1986, **246**, 199; K. Hirayama, T. Ando, R. Takahashi and A. Murai, *Bull. Chem. Soc. Jpn.*, 1986, **59**, 1371.
7 M. Ishiguro, S. Imajo, M. Hirama, *J. Med. Chem.*, 1991, **34**, 2366.
8 T. Tanaka, M. Hirama, M. Ueno, S. Imajo, M. Ishiguro, M. Mizugaki, K. Edo and H. Komatsu, *Tetrahedron Lett.*, 1991, **32**, 3175.
9 P. V. Roey and T. A. Beerman, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 6587.
10 V. Z. Pletnev, A. P. Kuzin, S. D. Trakhanov and P. V. Kostetsky, *Biopolymers*, 1982, **21**, 287.
11 T. Otani, T. Yasuhara, Y. Minami, T. Shimazu, R. Zhang and M.-Y. Xie, *Agric. Biol. Chem.*, 1991, **55**, 407; K. Yoshida, Y. Minami, R. Azuma, M. Saeki and T. Otani, *Tetrahedron Lett.*, 1993, **34**, 2637.
12 S. J. Hofstead, J. A. Matson, A. R. Malacko and H. Marquardt, *J. Antibiot.*, 1992, **45**, 1250; J. E. Leet, D. R. Schroeder, S. J. Hofstead, J. Golik, K. L. Colson, S. Huang, S. E. Klohr, T. W. Doyle and J. A. Matson, *J. Am. Chem. Soc.*, 1992, **114**, 7946.
13 S. Endo, H. Wako, K. Nagayama and N. Go, in *Computational Aspects of the Study of Biological Macromolecules by Nuclear Magnetic Resonance Spectroscopy*, ed. J. C. Hoch, F. M. Poulsen, C. Redfield, Plenum Press, New York, 1991, p. 233.
14 J. M. Blaney, G. M. Crippen, A. Dearing and J. S. Dixon, DGEOM, QCPE No. 590, 1990.
15 M. Nishio and M. Hirota, *Tetrahedron*, 1989, **45**, 7201.
16 K. A. Thomas, G. M. Smith, T. B. Thomas and R. J. Feldmann, *Proc. Natl. Acad. Sci. USA*, 1982, **79**, 4843.
17 M. Hirama, T. Gomibuchi, K. Fujiwara, Y. Sugiura and M. Uesugi, *J. Am. Chem. Soc.*, 1991, **113**, 9851.
18 T. Tanaka, K. Fujiwara and M. Hirama, *Tetrahedron Lett.*, 1990, **31**, 5947.