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Molecular recognition in neocarzinostatin complex: how does the apoprotein bind specifically and stabilize the chromophore?

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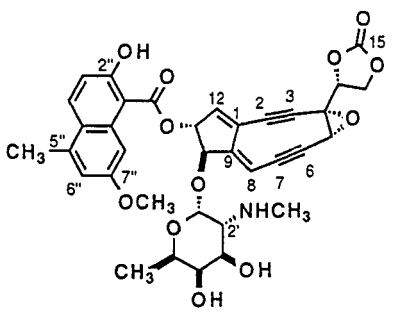
The chromophore-binding structure of the neocarzinostatin complex has been determined by two-dimensional proton nuclear magnetic resonance method, and reveals the elements of the specific binding and the stabilization interactions of the unstable dienediyne chromophore (**1**) with the apoprotein.

INTRODUCTION

The extremely potent enediyne anticancer antibiotics possess unprecedented and highly unusual structures as well as intriguing modes of action (ref. 1). These molecules have elicited extensive research in the frontiers of chemistry, biology, and medicine (refs. 1 & 2). They exert their biological actions mainly by virtue of their common abilities to generate carbon-diradicals upon suitable activation, leading to DNA cleavage. Some are very labile and yet are substantially stabilized by noncovalent complexation with a specific protein secreted simultaneously. These so-called chromoprotein antibiotics include neocarzinostatin (NCS) (ref. 3), auromomycin (AUR) (ref. 4), actinoxanthin (AXN) (ref. 5), C-1027 (ref. 6), and kedarcidin (ref. 7). Neither the tertiary structures of these chromophore-apoprotein complexes nor the stabilization interactions preventing the chromophore from decomposing have been determined, although this information is of great interest in connection with molecular recognition and protein function (refs. 8-12).



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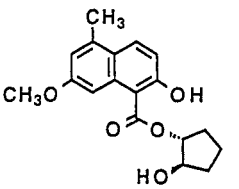

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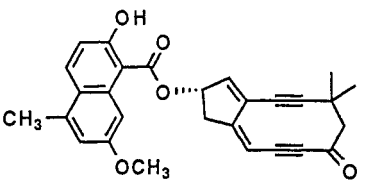

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Ala- Ala- Pro- Thr- Ala- Thr- Val- Thr- Pro- Ser- Ser- Gly- Leu- Ser- Asp- Gly- Thr- Val- Val- Lys-
Val- Ala- Gly- Ala- Gly- Leu- Gln- Ala- Gly- Thr- Ala- Tyr- Asp- Val- Gly- Gln- Cys- Ala- Trp- Val-
Asp- Thr- Gly- Val- Leu- Ala- Cys- Asp- Pro- Ala- Asn- Phe- Ser- Ser- Val- Thr- Ala- Asp- Ala- Asn-
Gly- Ser- Ala- Ser- Thr- Ser- Leu- Thr- Val- Arg- Arg- Ser- Phe- Glu- Gly- Phe- Leu- Phe- Asp- Gly-
Thr- Arg- Trp- Gly- Thr- Val- Asn- Cys- Thr- Thr- Ala- Ala- Cys- Gln- Val- Gly- Leu- Ser- Asp- Ala-
Ala- Gly- Asp- Gly- Pro- Glu- Gly- Val- Ala- Ile- Ser- Phe- Asn

apo-NCS

THREE-DIMENSIONAL STRUCTURE OF NEOCARZINOSTATIN (NCS)

NCS is a 1:1 complex of a nonprotein chromophore (NCS-chr, **1**) (ref. 13) and an apoprotein (apo-NCS, 113 amino acids) (ref. 14) isolated from the culture filtrates of *Streptomyces carzinostaticus* var. F-41 (ref. 3). NCS-chr is very labile to heat, light and pH above 6, but is stabilized substantially by the specific (ref. 15) and tight (ref. 16) binding to apo-NCS. Although the three-dimensional structure of NCS has been studied by X-ray crystallographic analysis at a resolution of 3.5 Å (ref. 8), the precise binding structure has not been defined because of the instability of NCS-chr. Several groups have recently reported the results of nuclear magnetic resonance (NMR) studies on free apo-NCS (refs. 9 & 10). Knowledge concerning all other chromoprotein antibiotics is at a similar status. We previously predicted the three-dimensional structures of apo-NCS and the complex by computer modeling based on the known X-ray crystallographic structure of AXN apoprotein, which shows high sequence-homology (ref. 17). Its binding orientation, however, differed from that which had been deduced semiempirically from the above AXN-derived apo-NCS structure and the preliminary intermolecular proton nuclear Overhauser effect (NOE) data between NCS-chr and apo-NCS (ref. 18). Here we report the fully NMR-derived three-dimensional structure of the NCS complex. This is the first time that the chromophore-binding structure has been determined in a member of the chromoprotein antibiotic family.

NMR measurements and distance geometry calculation

Homonuclear two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiments based on straightforward resonance assignments (refs. 18 & 19) produced 934 interproton distance restraints: 849 for apo-NCS, 24 for NCS-chr, and 61 intermolecular restraints between apo-NCS and NCS-chr (ref. 20). Sixty-five 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 (ref. 21) were also used as supplement data for calculation. 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) (ref. 22) 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 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) calculation (ref. 23) 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. The average of all 45 pairwise RMSD values of the heavy backbone atoms among the 10 refined structures is 0.30 ± 0.06 Å, and none have NOE and van der Waals distance violations larger than 0.32 Å.

Three-dimensional structure of apo-NCS

The apo-NCS portion consists of three antiparallel β-sheet domains, an external three-stranded β-sheet (residues 5-8; 18-24; 62-68), an internal four-stranded β-sheet (residues 44-47, 53-56; 31-40; 93-97; 107-109), and a small two-stranded β-sheet (residues 72-76; 82-87) (ref. 18). The seven β-strands of the external and internal β-sheets form a β barrel (Fig. 1). The folding is identical to that of free apo-NCS (refs. 8 & 9) and very similar to those reported for the related protein antibiotics, AUR (ref. 11), AXN (ref. 12), and C-1027 (ref. 24). The internal β-sheet and loops (residues 77-80; 99-102) form a hydrophobic cleft with approximate dimensions of 12 x 9 x 9 Å. This cleft is the binding site of NCS-chr based on the intermolecular NOEs with NCS-chr (ref. 18). The architecture of this cleft is characterized by three features (Fig. 2): First, the bottom of the cleft is formed primarily by the main chains of three strands, Val^{34}-Gly^{35}-Gln^{36}, Leu^{97}-Gly^{96}-Val^{95}, and Gly^{107}-Val^{108}. Second, the cleft contains a Cys^{37}-Cys^{47} disulfide bridge. Third, the side chains of Trp^{39}, Leu^{45}, Phe^{52} Phe^{78} and Gln^{94} are aligned to form a hydrophobic wall, while Ser^{54}, Asp^{33}, and Ser^{98} form a hydrophilic small wall.

Chromophore binding structure of NCS complex and elements of the specific binding

The holoprotein structure with the smallest violation is shown in Fig. 3. Apo-NCS accepts the naphthoate moiety deeply into the cleft. The C5"-C7" site of the naphthoate is in close proximity to the bottom of the cleft where the backbones of three strands are exposed. The carbocyclic core is located on the Cys^{37}-Cys^{47} disulfide bridge, and the aminosugar and the carbonate groups are facing outwards. No aromatic stacking

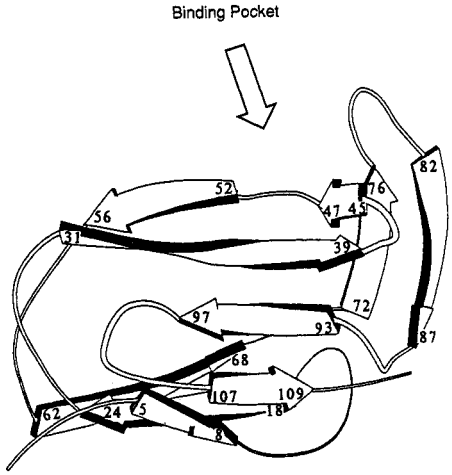

Fig. 1. Motif of apo-NCS tertiary structure.

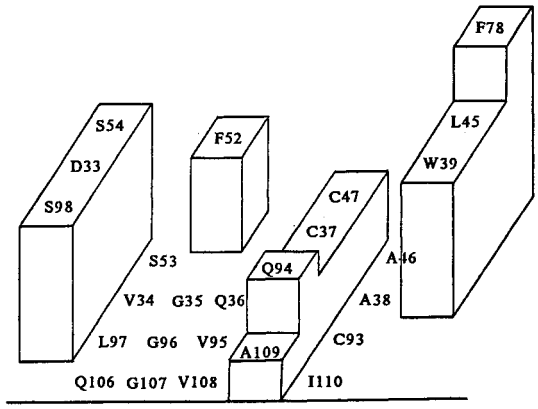

Fig. 2. Schematic drawing of the residues of the hydrophobic cleft of apo-NCS.

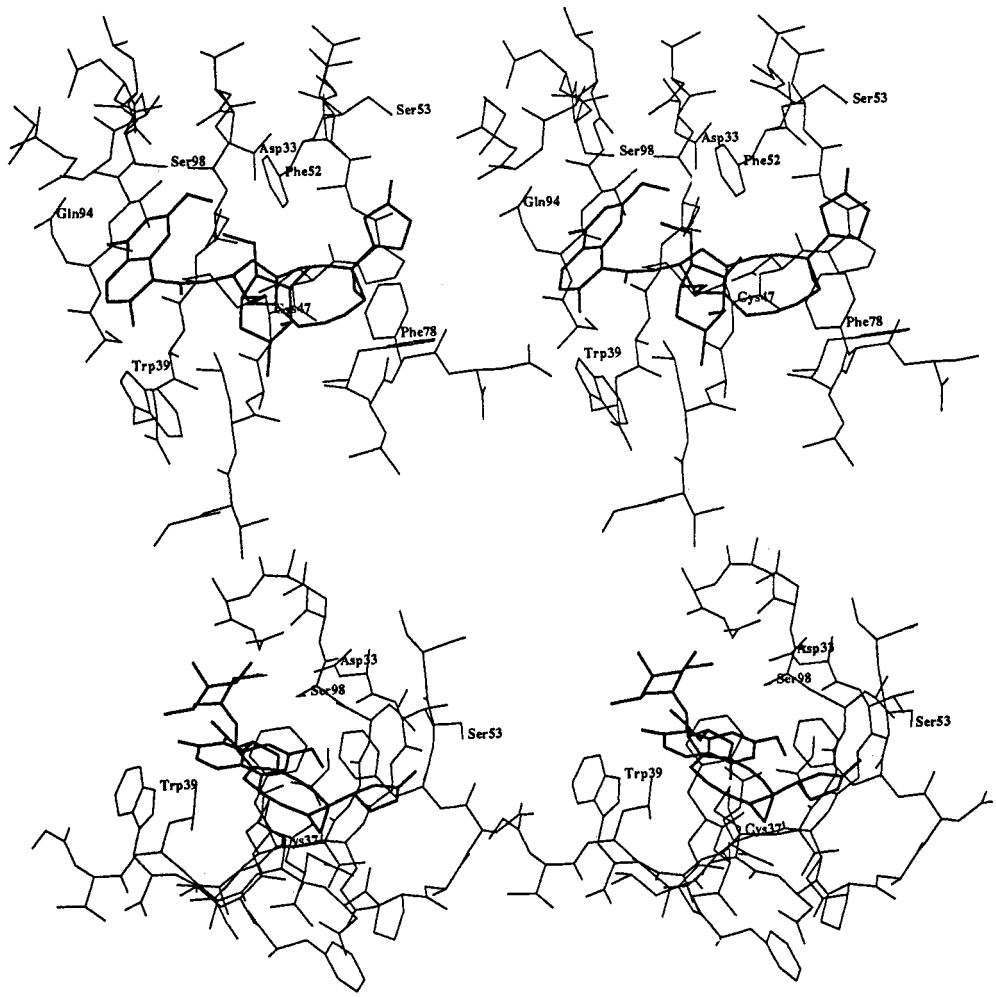

Fig. 3. Stereoview of the binding site of the NCS complex.

interaction with the naphthalene ring, such as that predicted by computer modeling (ref. 17) are observed. Instead, several other principal interactions are indicated (Fig. 3). The oxygen atoms of C7"-O and C2"-O of the naphthoate are close enough to OγH of Ser^{98} and Nε1H of Trp^{39}, respectively, to produce a hydrogen bond. The upfield shift of the C7"-OCH_3 resonance by 1 ppm upon binding to apo-NCS is most likely due to the diamagnetic anisotropy of the nearby aromatic ring of Phe^{52}, which supports a possible CH-π interaction (ref. 25). The CH···O type hydrogen bonding (ref. 26) is likely between C6"-H and the backbone carbonyl of Gly^{96}. The C5"-methyl is approximately in van der Waals contact with the β-methylene of Gln^{94}. On the other hand, the C2-C3 triple bond of the core is just above the sulfur atom of Cys^{47} (refs. 17 & 18), and its side is covered perpendicularly by the aromatic ring of Phe^{52}. The aromatic ring of Phe^{78} is covering the other side of the core, in van der Waals contact with the C6-C7 triple bond. One of the Leu^{45} methyls is close to the C8-C9 double bond of the core. The core epoxide is facing down towards the bottom of the cleft. Another key interaction is the salt bridge between the protonated 2'-$NHCH_3$ of the aminosugar and the Asp^{33} carboxylate. The carbonate carbonyl group appears to interact with the hydroxyl of Ser^{53} by hydrogen bonding. The lack of the conservation of the above residues, except Gly^{96}, Cys^{37}, and Cys^{47}, in the related chromoproteins, AUR (ref. 4) AXN (ref. 5) and C-1027 (ref. 6), indicates that these residues are crucial for chromophore binding specificity (refs. 11 & 15).

Role of the naphthoate moiety of NCS-chr. Since the naphthoate group of NCS-chr (**1**) is located most deeply in the cleft, it may be essential for specific and strong binding. This is consistent with the binding of β-naphthol (ref. 16) and a synthetic compound (**2**) (ref. 27) to apo-NCS. Naphthoate's role is also supported by the NCS-chr analogue (**3**), which exhibits antitumor and DNA-cleaving activities only when incubated with apo-NCS solution (ref. 28).

Conformation of NCS-chr. The conformation of NCS-chr (**1**) in the complex shows some interesting features. The sugar portion hangs over the 5-membered ring of the core with the hydrophobic side down, thus satisfying the exo-anomeric effect (ref. 29) (Fig. 3). The aminomethyl group is forced to come closer to C12 (4.3 Å) due to salt bridge with Asp^{33} compared with the free form (5.4 Å) (ref. 30). The naphthoate stays away from the core to fit the cleft, 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 (ref. 30).

Stabilization interactions

A nucleophile (refs. 1 & 13) or radical (ref. 31) addition to C12 of the core, and a concomitant opening of the epoxide, initiate the cycloaromatization of NCS-chr (**1**). The side chains of Ser^{98}, Asp^{33}, and Phe^{52}, as well as the protonated methylamino group of the aminosugar, cover the reactive center C12 (Fig. 3). These steric hindrances may be the major stabilizing element of NCS-chr (**1**). Moreover, facing down towards the bottom of the cleft protects the epoxide from an acid catalyst. In addition, the core lies on the disulfide bridge, which might stabilize the strained unsaturated system of NCS-chr (**1**) (ref. 17) probably through their orbital HOMO (the dienediyne π-system) - LUMO (disulfide σ* or vacant d orbital) interaction. Since the disulfide bridge is conserved in all chromoprotein antibiotics (refs. 4-7), such an interaction with the strained enediyne chromophore (refs. 6 & 7) may be common in this family (ref. 32).

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