AN ANTITUMOR POLYPEPTIDE ANTIBIOTIC NEOCARZINOSTATIN:
THE MODE OF APO-PROTEIN — CHROMOPHORE INTERACTION

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The mode of neocarzinostatin-chromophore (NCS-chr) — apo-neocarzinostatin (apo-NCS) interaction in neocarzinostatin (NCS) complex has been described. The NCS-chr release from the NCS complex in the presence of various reagents, which destroy the high-order structure of the protein under various pH conditions, was examined. We found that (i) sodium dodecylsulfate, Nonidet P 40, 8 M urea, and 2-propanol did release NCS-chr from NCS, (ii) no NCS-chr release is detected below pH 7, but it is enhanced at high pH and (iii) β-naphthol as a model of naphthalenecarboxylic acid derivative and D-galactosamine as a model of N-methylfucosamine of NCS-chr did release NCS-chr from NCS. These observations indicate that the binding of NCS-chr to apo-NCS may be due to not only ionic interaction between the acidic side chain of apo-NCS and the basic center of an aminosugar moiety of NCS-chr but also hydrophobic interaction between the hydrophobic amino acids of apo-NCS and hydrophobic moieties of NCS-chr.

Apo-NCS is a very hydrophilic protein, since it has an high hydrophilic amino acid content. So, local hydrophobicity, local hydrophilicity and secondary structure of apo-NCS were predicted. Hydrophobic residues of apo-NCS predominantly located in β-sheet structures near the carboxyl-terminus. These predictions are in good agreement with the results suggesting that NCS-chr bound carboxyl-terminal-43-peptide of apo-NCS in our previous result.

Neocarzinostatin (NCS), an antitumor antibiotic, isolated from a culture filtrate of Streptomyces carzinostaticus var. F-41, is composed of a protein moiety (apo-neocarzinostatin (apo-NCS), MW 11,000) and a non-protein chromophore (neocarzinostatin-chromophore (NCS-chr), MW 659) in the molar ratio of 1 : 12.2. Apo-NCS (Scheme 1), whose primary structure was recently revised, is composed of many hydrophilic amino acids. This hydrophilic apo-NCS stabilizes the highly hydrophobic and very labile chromophore against natural light and heating and serves as a carrier for NCS-chr.4,5 The total chemical structure of NCS-chr has been proposed to be a bicyclo[7,3,0]dodecadiyne derivative having 2-hydroxy-7-methoxy-5-methyl-1-naphthalenecarboxylic acid (NA)3 and α-D-N-methylfucosamine (MF)9 moieties (Fig. 1). NCS-chr binds to apo-NCS non-covalently and it can be reconstituted with apo-NCS to form NCS5.8. The binding of NCS-chr to apo-NCS is of very high affinity, since other proteins, such as bovine serum albumin and the apoprotein of macromomycin, one
of NCS like antitumor polypeptide antibiotic\(^5,9,10\) do not compete. Recently, we suggested that the polypeptide fragment after limited proteolysis of apo-NCS by protease, carboxyl-terminal-43-peptide, forms a complex with NCS-chr and exhibits antimicrobial activity\(^1\). However, experimental evidence for the molecular interaction between hydrophobic NCS-chr and hydrophilic apo-NCS remains to be elucidated.

In this paper, we show that the specific NCS-chr – apo-NCS interaction in NCS complex is due to not only ionic interaction between the acidic amino acid residue of apo-NCS and the basic center of amino sugar of NCS-chr but also hydrophobic interaction between the hydrophobic amino acids of apo-NCS and the hydrophobic moieties of NCS-chr by analysis of the competitive release of NCS-chr from NCS and determination of quenching fluorescence of apo-NCS with NCS-chr.

**Materials and Methods**

**Chemicals**
NCS and apo-NCS, which are produced by Kayaku Co., Ltd., (Tokyo, Japan), were used in this study. All the other chemicals were of highest grade commercially available.

**Competitive Release of NCS-chr from NCS**
NCS (1 mg) was dissolved in 1 ml of 0.1 \(\text{m}\) acetate buffer (pH 5.0) containing various reagents just before the start of incubation at 37°C for 24 hours in the dark condition. The final pH was measured by Horiba pH meter 7LC.

**Quantitative Analysis of NCS and Apo-NCS by Liquid Chromatography**
A HPLC system (Pharmacia FPLC) was employed for the quantitative analysis of NCS and apo-NCS. The system consists of two P-500 pumps, the gradient programmer GP-250, a fixed wavelength detector UV-1 (280 nm), and a two channel recorder REC-482 for the simultaneous recording of salt gradient and optical density. A Mono Q HR 5/5 anion exchange column (Pharmacia), charged \(\text{CH}_2\text{N}^+\text{(CH}_3)\text{)}_2\) on the monodisperse gel particles, was used. Samples were eluted from the column at a flow rate of 2 ml/minute maintaining a pressure of 20 bar (20.4 kg/cm\(^2\)). The separation time was 16 minutes. The solvent A (start buffer) was 0.1 \(\text{m}\) Tris-HCl buffer pH 7.2 and solvent B (limit buffer) was start buffer containing 0.5 \(\text{m}\) NaCl.
The gradient programmer was set as follows: A linear gradient of 0% B in 3.0 minutes, followed by 0~100% B in 10 minutes, and finally maintaining by 100% B in 3 minutes.

**Fluorescence Studies**

All measurements were performed on a Hitachi 650-60 spectrofluorometer. NCS-chr, NA and apo-NCS solutions were prepared fresh each day with 0.1 N acetate buffer pH 4.0 solution. The total fluorescence has been calculated from the digitized data. Kinetic measurements of fluorescence changes were made by adding NCS-chr in aqueous buffer solutions to buffer solution containing apo-NCS. Excitation of NCS-chr and NA were always at 340 nm for 440 nm emission and at 280 nm for 380 nm emission for apo-NCS. Kinetic constants were usually determined from the 1/\(JF\) vs. 1/(C), which could be described by linearity by the method of Kondo et al.12).

**Results**

**Quantitative Analysis of NCS and Apo-NCS**

Fifty \(\mu g\) of NCS and 50 \(\mu g\) of apo-NCS were separately applied onto a Mono Q column, previously equilibrated with 0.1 M Tris-HCl buffer pH 7.2. Elution was carried with salt gradient as described in Materials and Methods. NCS and apo-NCS were eluted at 5.3 minutes (Fig. 2a) and 6.6 minutes (Fig. 2b), respectively. The amounts of eluted NCS and apo-NCS were estimated from those absorbances at 280 nm from a molar extinction coefficient for NCS and apo-NCS in 0.1 M acetate buffer at pH 5.0. The calibration curves of NCS and apo-NCS gave a good straight line, respectively (data not shown).

Fig. 2. Quantitative analysis of NCS and apo-NCS using Mono Q HR 5/5 column.
(a) NCS, (b) apo-NCS.

![Fig. 2](image)

Each samples in 0.1 N acetate buffer pH 5.0 (1 mg/ml, 50 \(\mu l\)) were applied onto Mono Q column.

Fig. 3. The time course of NCS-chr release from NCS.

![Fig. 3](image)

NCS (1 mg/ml, 1 ml) in 0.1 M acetate buffer solution (pH 5.0) was incubated at 37°C (○) and 57°C (◇), respectively.

At specific time intervals, 50 \(\mu l\) of sample was withdrawn and applied to a Mono Q HR 5/5 column. The NCS-chr release ratio (% \(\Delta\)) was calculated on the integral of FPLC as follow:

\[
\% \text{ of NCS-chr release} = \frac{2 \times \text{apo-NCS}}{\text{NCS} + 2 \times \text{apo-NCS}} \times 100
\]

Retention time of apo-NCS: 6.6 minutes.
Retention time of NCS: 5.3 minutes.
Time Course of NCS-chr Release from NCS

The NCS-chr release from NCS at both 37°C and 56°C in 0.1 m acetate buffer (pH 5.0) were determined at the indicated periods. As shown in Fig. 3, at 37°C NCS-chr was gradually released from NCS over 48 hours. Less than 5% of total NCS-chr released from NCS after 48 hours incubation. At 56°C, the release of NCS-chr from NCS was relatively rapid and about 40% of NCS-chr was released after 48 hours incubation. No release of NCS-chr from NCS at 4°C was observed after 48 hours incubation (data not shown). From these results, experimental conditions of NCS-chr release from NCS were conducted at 37°C for 24 hours in 0.1 m acetate buffer (pH 5.0) in order to confirm the hydrophobic and ionic interactions of apo-NCS with NCS-chr.

The NCS-chr Release from NCS in the Presence of Protein Denaturants

In the presence of protein denaturants, such as sodium dodecyl sulfate (SDS), Nonidet P 40, urea, and 2-propanol, the NCS-chr release from NCS was tested. As summarized in Table 1, SDS, which is one of acidic detergents at the concentration of 0.1 m, could release 100% of NCS-chr and its release was decreased in proportion to the SDS concentration until 1 mM. Forty % of Nonidet P 40, one of polyether neutral detergents, and 8 m urea, released 90% and 95% NCS-chr from NCS, respectively. A 3-m 2-propanol also released approximately 70% NCS-chr from NCS. The final pH in the buffer solution after treatment of NCS with each of the four reagents were between 5.1 and 7.2. There was no pH dependency for the NCS-chr release in this experimental conditions as described next (Fig. 4).

Effect of pH for the NCS-chr Release from NCS

It is easily assumed that the chromophore-apoprotein interaction may be partially due to an ionic bond because apo-NCS is an acidic protein (pI 3.2) and NCS-chr is a basic compound having an amino sugar. In order to determine the ionic interaction between NCS-chr and apo-

Table 1. The NCS-chr release from NCS in the presence of protein denaturants.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration (M)</th>
<th>NCS-chr release (%)</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>0.1</td>
<td>100</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>75</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>55</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>0</td>
<td>5.1</td>
</tr>
<tr>
<td>Nonidet P 40*</td>
<td>40**</td>
<td>90</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>20**</td>
<td>21</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>10**</td>
<td>17</td>
<td>5.4</td>
</tr>
<tr>
<td>Urea</td>
<td>8</td>
<td>95</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>5.8</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>3</td>
<td>72</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>23</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>5.1</td>
</tr>
</tbody>
</table>

* Octylphenoxypolyethoxyethanol, ** %.

Fig. 4. Effect of pH for the NCS-chr release from NCS.

NCS (1 mg) was dissolved in 1 ml of 0.01 m tartarate (pH 2.6), 0.01 m acetate (pH 4.0), 0.01 m acetate (pH 5.0), 0.01 m phosphate (pH 6.0), 0.01 m phosphate (pH 7.0), 0.01 m phosphate (pH 8.0) or 0.01 m borate (pH 10.0) buffer solutions, respectively.

The solution of NCS at various pH's (1 ml) were incubated at 37°C for 24 hours, respectively in the dark conditions. The NCS-chr release ratio (%) was calculated by the same as Fig. 3.
Table 2. The effect of the moieties of NCS-chr for the NCS-chr release from NCS.

<table>
<thead>
<tr>
<th>Concentration (nm)</th>
<th>Release of NCS-chr (%)</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Naphthoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>8.0</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>8.0</td>
</tr>
<tr>
<td>β-Naphthol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>37</td>
<td>7.5</td>
</tr>
<tr>
<td>1</td>
<td>75</td>
<td>8.0</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>8.4</td>
</tr>
<tr>
<td>D-Galactosamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>5.2</td>
</tr>
<tr>
<td>50</td>
<td>21</td>
<td>6.2</td>
</tr>
<tr>
<td>100</td>
<td>32</td>
<td>7.2</td>
</tr>
<tr>
<td>500</td>
<td>96</td>
<td>9.0</td>
</tr>
<tr>
<td>D-Galactose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>17</td>
<td>5.0</td>
</tr>
</tbody>
</table>

NCS, the effect of pH on the NCS-chr release from NCS was tested. As illustrated in Fig. 4, NCS in various buffer solutions at a wide pH range between 2.6 and 10.0 was incubated for 24 hours at 37°C. About 50% of NCS-chr release from NCS at pH 10 were detected, whereas at lower than pH 6 little release of NCS-chr from NCS was detected.

Effect of Model Compounds of NCS-chr for the NCS-chr Release from NCS

In order to further confirm the interaction of NCS-chr with apo-NCS, the effect of the NCS-chr release from NCS by four model compounds of NCS-chr, α-naphthoic acid and β-naphthol as models of the naphthalene derivative of NCS-chr, and D-galactosamine and D-galactose as models of N-methylfucosamine of NCS-chr, were tested in 0.1 M acetate buffer solution (pH 4.0) at 37°C after 24 hours (Table 2). β-Naphthol (3 mm) completely released the NCS-chr from NCS, whereas α-naphthoic acid at the same concentration released only 3%. The NCS-chr release from NCS caused by addition of D-galactosamine was in proportion to its concentration between 10 and 500 mm. At 500 mm of D-galactosamine NCS-chr release from NCS reached about 100%, although D-galactose, which has no amino function, at even 500 mm was about only 20%.

Interaction between Apo-NCS and Methyl 2-Hydroxy-7-methoxy-5-methyl-1-naphthalencarboxylate

The fluorescence technique is an useful approach to determine the interaction of proteins and the protein-binding drugs. By using this technique, the interaction between apo-NCS and NCS-chr (or NA) was tested. The fluorescence of the mixture apo-NCS (final concentration for 5×10^-8 M) with NCS-chr (final concentration from 8×10^-6 to 5×10^-5 M) was immediately measured after both solutions were mixed. When NCS-chr was added to the solutions containing low concentration of apo-NCS, apo-NCS quenched the 448 nm fluorescence of NCS-chr excited at 340 nm and shifted the emission peak to 420 nm (Fig. 5). The spectrum of NCS was similar to that determined for NCS-chr,
which occupies a largely hydrophobic environment in a NCS complex. A maximum estimate of dissociation constant \( (K_d) \) was obtained from the fluorescence quenching curves by the method of Kondo et al.\(^\text{12}\). \( K_d \) of apo-NCS for NCS-chr under these experimental conditions was \( 7.0 \times 10^{-8} \text{ M} \), whereas apparent \( K_d \) of apo-NCS for NA was \( 1 \times 10^{-5} \text{ M} \).

**Discussion**

It is reported that many chromoproteins having antitumor activity such as macromomycin\(^\text{13}\), auromomycin\(^\text{14}\), actinoxanthin\(^\text{15}\), largomycin\(^\text{16,17}\) etc. are in general associated with highly acidic proteins in combination with basic chromophores. But the mode of apoprotein-chromophore interaction has not been clear yet because most of these chromoproteins have not necessarily been elucidated both the primary structure of apoprotein and the structure of its chromophore. NCS was elucidated both the primary structure of apo-NCS\(^\text{18,19}\) and total structure of its chromophore\(^\text{6}\). In this paper, authors described the chromophore-apoprotein interaction in NCS complex.

Free NCS-chr, which is very labile since it loses its biological activities quickly by light and heating, is stabilized by apo-NCS\(^\text{4}\). Recently, we proposed that most the unstable functionality of NCS-chr is a strained epoxide and this degradation process is due to the generation of singlet oxygen\(^\text{20}\). NCS-chr retains the biological activities of parent NCS and it is extractable from the NCS complex using organic solvents in the presence of hydrochloric acid. The NCS-chr can be reconstituted with apo-NCS and the complex was as effective as the native NCS\(^\text{9,10}\). These observations show that NCS-chr binds non-covalently to apo-NCS.

NCS-chr is efficiently released from NCS in the presence of protein denaturants such as SDS, Nonidet P 40 and 8 M urea (Table 1). These observations should contribute to understanding that NCS-chr may be hydrophobically bound with hydrophobic part of apo-NCS as described before\(^\text{9}\).

It was reported the hydrophobicity value of proteins\(^\text{21}\). Proteins have been included only if the content of all the amino acids is known. The average hydrophobicity of 620 selected proteins is 965 cal/mol. That of apo-NCS has been calculated 729 cal/mol. These results indicate that apo-NCS is one of the more hydrophilic proteins. Actually, NCS and apo-NCS are very highly solubilizable proteins in water. Therefore, these data are in conflict with the hydrophobic interaction of apo-NCS with NCS-chr.

Primary structure of apo-NCS\(^\text{18}\) (Scheme 1) was subjected to the local hydrophobicity\(^\text{21}\) (Fig. 6A) and hydrophilicity\(^\text{22}\) analysis (Fig. 6B). There are four hydrophobic regions in apo-NCS located to Val-Val-Lys-Val (position 18~21), Cys-Ala-Trp-Val (position 37~40), Phe-Glu-Gly-Phe-Leu-Phe (position 73~78) and Val-Ala-Ile-Ser-Phe (position 108~112). We found recently that the 43-carboxyl-terminal-peptide (C-terminal-43-peptide) residue fragment (position 71~113) having highly hydrophobic two clusters Phe-Glu-Gly-Phe-Leu-Phe (position 73~78) and Val-Ala-Ile-Ser-Phe (position 108~112), is necessary for the binding of NCS-chr and the resulted complex of this fragment and NCS-chr retains its antibacterial activity\(^\text{11}\). These two hydrophobic clusters of apo-NCS may play an important role in hydrophobic interaction with hydrophobic parts of NCS-chr.

It seems to be due to an extraordinary packed polypeptide folding that NCS is resistant to pronase digestion\(^\text{23}\). This assumption may be associated with preliminary X-ray analysis\(^\text{24}\). The secondary structure of the apo-NCS based on the sequence reported by Kuromizu et al.\(^\text{18}\) was predicted by the probabilistic method developed of CHOU and FASMAN\(^\text{25}\) (Fig. 7). These observations indicate that NCS possesses a very tightly folded conformation, which probably consists to a considerable extent in the \( \beta \)-pleated sheets and may be varied to a packed polypeptide folding containing \( \beta \)-sheet structure of the C-terminal-43-peptide.

The interaction between NCS-chr and apo-NCS was detected at very low concentration by fluorometric method (Fig. 5). The dissociation constant \( (K_d) \) of apo-NCS for NCS-chr was \( 7.0 \times 10^{-6} \text{ M} \) under these experimental conditions. The hydrophobic moiety, NA was similarly interacted with apo-NCS and the dissociation constant was more than that of NCS-chr itself. The wavelength shift on the UV spectrum of highly unsaturated five-nine fused ring of NCS-chr was observed to be proportional to apo-NCS concentration. These findings suggest that not only NA moiety but also highly
Fig. 6. Hydrophobicity and hydrophilicity profiles of apo-NCS.

(A) The average hydrophobicity value of pentapeptides were plotted at the center of amino acid number Nextumber21).

(B) The averaged hydrophilicity value of a hexapeptide composed of amino acid residues $i-2$ to $i+3$ has been plotted against $i$, where $i$ represents amino acid number22). The x axis contains 113 increments, each representing an amino acid in the sequence of apo-NCS. The y axis represents the range of hydrophobicity and hydrophilicity values. These data were calculated by using the biochemical soft of "Biochem, Okubo Micon, Tokyo"20).

Fig. 7. Schematic diagram of the predicted secondary structures in apo-NCS.

(a) 
(b) 
(c) 
(d) 

The secondary structure of apo-NCS was employed by the method of Chou and Fasman23) and predicted by personal computer commented in Fig. 6's legend. Positions of the predicted secondary structures are indicated by: (a), $\alpha$-helix; (b), $\beta$-sheet; (c), $\beta$-turn; (d) random coil.
unsaturated five-nine fused ring of NCS-chr contribute the hydrophobic interaction between apo-NCS to NCS-chr. β-Naphthol modeled as NA moiety of NCS-chr destroyed the apo-NCS – NCS-chr interactions although α-naphtoic acid did not (Table 2). β-Naphthol at 3 mM concentration completely released the NCS-chr from NCS. These observations suggest that the phenolic function of NA may play an important role in the apo-NCS – NCS-chr interaction but no detailed role could be understood. d-Galactosamine modeled of d-fucosamine at 500 mg/ml concentration released completely NCS-chr from NCS but the NCS-chr release of d-galactose was only 17% at the same concentration. It shows that the binding of apo-NCS with NCS-chr is also due to an ionic binding of NCS-chr at an basic center of amino sugar moiety and one of five acidic amino acid residues of apo-NCS (Asp: Position 79, 87 and 99, Glu: Position 74 and 106).

The inactivation of NCS-chr by autoxidation is due to a highly strained epoxide moiety. This epoxide moiety may be protected by apo-NCS. The specific structure of C-terminal-43-peptide of apo-NCS may be associated with NCS-chr and protect the degradation of highly unsaturated five-nine fused ring of NCS-chr linked a highly strained epoxide.

Acknowledgment

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