Neocarzinostatin: Selective Tryptophan Oxidation and Neocarzinostatin-Chromophore Binding to Apo-Neocarzinostatin

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Neocarzinostatin (NCS), an antitumor protein antibiotic, is composed of apo-neocarzinostatin (apo-NCS) and neocarzinostatin-chromophore (NCS-chr), the principle of the biological activities of NCS. Apo-NCS having two tryptophan (Trp) residues at positions (39 and 83) was chemically modified by *N*-bromosuccinimide in a study on the correlation of the binding site(s) of NCS-chr. Selective oxidation of Trp residues was observed when NCS was titrated with *N*-bromosuccinimide. In contrast, non-selective oxidation of the two Trps on apo-NCS was observed and both Trp (39 and 83) of apo-NCS were titrated with *N*-bromosuccinimide. After selective oxidization, the remaining Trp residue of NCS was assigned as Trp (83). These results clearly indicate that the Trp (83) residue of apo-NCS changed from the "reactive type" to the "non-reactive type" after the binding of NCS-chr with apo-NCS. The fluorescence emission intensity of apo-NCS generated from the Trp (39) residue was quenched by NCS-chr. These data suggest that NCS-chr directly interacts with the Trp (39) residue and that a β -sheeted loop containing the Trp (83) residue of apo-NCS changes the high-order structure upon binding with NCS-chr.

Keywords neocarzinostatin; apo-neocarzinostatin; neocarzinostatin-chromophore; selective tryptophan oxidation; apoprotein-chromophore interaction

Neocarzinostatin (NCS) with an antitumor activity has been isolated from the culture filtrate of *Streptomyces carzinostaticus* var. F-41.¹⁾ NCS consists of two components, an acidic polypeptide (apo-NCS) (Fig. 1), and a labile nonprotein chromophore (NCS-chr) (Fig. 2). Clinical studies showed that NCS is a potent anticancer reagent useful for treatment of bladder cancer²⁾ or, in the form of a new derivative, for the treatment of liver cancer.³⁾ Much evidence has suggested that NCS-chr is primarily responsible for the biological activity of NCS and its interaction with deoxyribonucleic acid (DNA) while the apo-NCS is essential for its stability and transport.⁴⁻⁶⁾ The total chemical structure and the absolute configuration of NCS-chr were elucidated.^{7,8)} On the other hand, the primary sequence of apo-NCS, first elucidated by Meienhofer *et al.*,⁹⁾ was revised by several groups¹⁰⁻¹³⁾ and the secondary structure of apo-NCS was characterized by us.¹⁴⁾

It is reported that binding between apo-NCS and

NCS-chr is specific and no other proteins are exchangeable for apo-NCS.¹⁵⁾ Recently, we suggested that carboxyl-terminal-43-peptide residue could be bound to NCS-chr.¹⁶⁾ This complex retains its antimicrobial activity and there is a strong hydrophobic interaction between apo-NCS and NCS-chr as well as ionic interaction.^{17,18)}

In this paper we studied the selective Trp oxidation of NCS and apo-NCS to further understand the mode of reversible binding between apo-NCS and NCS-chr. It is suggested that the Trp (39) residue of apo-NCS directly interacts with NCS-chr whereas a loop containing the Trp (89) residue changes the high-order structure upon binding to NCS-chr and that *N*-bromosuccinimide oxidation of the Trp residues on apo-NCS changes the high-order structure of apo-NCS to reduce the affinity with NCS-chr to apo-NCS and consequently monotryptophan-39 oxidized NCS (MTO-NCS) and ditryptophan-39 and 83 oxidized NCS (DTO-NCS) reduce those biological activities.

Fig. 1. Primary Structure of Neocarzinostatin Apoprotein

The tryptic peptides T1, T2, T3, T4, and T5 correspond to positions 1—20, 21—70, 71, 72—82, and 83—113, respectively.

Fig. 2. The Absolute Chemical Structure of Neocarzinostatin-Chromophore

Experimental

Materials Purified NCS and apo-NCS were obtained from Kayaku Antibiotic Research Co., Ltd., Tokyo, Japan. NCS-chr was prepared by the previously reported method.¹⁹⁾ Other reagents were of analytically high grade and were obtained commercially. *N*-Bromosuccinimide was recrystallized from deionized warm water before use and had a mp at 173 °C. Urea was recrystallized from 50% aqueous ethanol before use.

Spectral Measurement Absorption spectra were recorded on a Hitachi 557 double wavelength double beam spectrometer at room temperature. Spectrofluorometric studies were carried out in 0.1 M acetate buffer (pH 4.0) at room temperature with a Hitachi 650-60 fluorescence spectrophotometer. The excitation wavelength was 280 nm, and the emission wavelength was between 250 and 500 nm. Circular dichroic (CD) spectra were carried out at room temperature under constant nitrogen flush, using a JASCO spectropolarimeter J-400X equipped with a data processor. The data are expressed in terms of molar ellipticity, $[\theta]$, in $\text{deg} \cdot \text{cm}^2/\text{dmol}^{-1}$.

High Performance Liquid Chromatography (HPLC) (Pharmacia Fast Protein Liquid Chromatography (FPLC) System) The system consists of two P-500 pumps, a 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 $-\text{CH}_2\text{N}^+(\text{CH}_3)_3$ on the monodisperse gel particles, was used. Samples were eluted from the column at a flow rate of 2 ml/min maintaining a pressure of 20 bar (2 MPa). The separation time was 16 min. The solvent A (start buffer) was 0.1 M Tris-HCl buffer pH 7.2 and solvent B (limit buffer) was a start buffer containing 0.5 M NaCl. The gradient programmer was set as follows: a linear gradient of 0% B for 3.0 min, followed by 0–50% B for 10 min, and finally maintaining by 100% B for 3 min.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Ten percent SDS-PAGE was carried out as described by Laemmli.²⁰⁾ The gel (1 mm thick) was fixed in 5% trichloroacetic acid for 1 h and stained with Coomassie brilliant blue. Molecular weight markers were used as the standard.

Isoelectric Focusing on Polyacrylamide Gel Polyacrylamide (5%) gel containing 2% carrier Ampholytes (LKB-producter, Bromma, Sweden) pH 2.5–4.5 was prepared as specified in the LKB manual. Riboflavin was added as a photochemical polymerizing agent. After applying the sample (1 mg/ml, 40 μl), isoelectric focusing was carried out at 4 °C. The voltage was increased from 220 to 700 V for the initial 30 min and then kept constant at 700 V for the following 3 h. The protein bands were stained with Coomassie brilliant blue R-250.

***N*-Bromosuccinimide Titration** The *N*-bromosuccinimide oxidation of NCS (M.W. 11752.7) and apo-NCS (M.W. 11093.1) was performed according to the method of Patchornik *et al.*²¹⁾ and Spande & Witkop.²²⁾ Ultraviolet (UV) photometric titration was conducted by adding 0.01 M *N*-bromosuccinimide dropwise to NCS (0.84 mg (7.14×10^{-8} mol)/2 ml) solutions in 0.1 M acetate buffer (pH 4.0) in the presence and absence of 8 M urea. The same titration was done to apo-NCS (1.65 mg (1.49×10^{-7} mol)/2 ml). After adding each increment of *N*-bromosuccinimide, a spectrometric reading was taken at 280 nm at room temperature.

The number of Trp residues per mole²²⁾ is calculated by the following expression:

$$n = \mu\text{mol Trp}/\mu\text{mol protein}$$

$$= (\Delta\text{OD} \times 1.31 \times V/5.50)/(\text{OD} \times \text{a.f.} \times V \times 1000/\text{M.W.})$$

$$= (\Delta\text{OD} \times 1.31 \times \text{M.W.})/(\text{OD} \times \text{a.f.} \times 5500)$$

where n = number of Trp residues per mole of protein, OD = initial optical density at 280 nm, ΔOD = corrected optical density decrease at 280 nm, a.f. = absorptivity factor to convert optical density at 280 nm to mg/ml of protein (0.466 for NCS, 0.929 for apo-NCS), V = initial volume of titrated solution (ml), M.W. = molecular weight of protein, 5500 = molar extinction coefficient at 280 nm for Trp.

Preparation of MTO-NCS and DTO-Apo MTO-NCS: All operations were performed in dark conditions at 4 °C. The reaction mixture of 60 mg of NCS and 0.84 mg of *N*-bromosuccinimide in 60 ml of 0.1 M acetate buffer (pH 4.0) was left at 4 °C for 30 min and then was lyophilized. Lyophilizate was applied to Bio-gel P-4 column chromatography (1.3 $\times$ 20 cm) previously equilibrated with deionized water. The optical density was monitored by absorbance at 214 nm. The main peak pooled was lyophilized (yield, 52 mg (87%)).

DTO-Apo: Thirty mg of apo-NCS in 30 ml of 0.1 M acetate buffer (pH 4.0) was added to 1.68 mg of *N*-bromosuccinimide and then the reaction mixture was left for 30 min at 4 °C. The same treatment method used for the reaction mixture was applied to the MTO-NCS preparation (yield, 23 mg (77%)).

Preparation and Purification of MTO-Apo After 25 mg of MTO-NCS in 25 ml of deionized water was left at 4 °C for 24 h in the presence of 8 M urea, this MTO-NCS solution was dialyzed overnight against deionized water and lyophilized. This crude MTO-apo solution was re-lyophilized and purified with FPLC followed by desalting with a Bio-gel P-4 column (1.3 $\times$ 20 cm) and lyophilization (yield, 20 mg (85%)).

Preparation and Purification of DTO-NCS The mixture of NCS-chr (10 mg/ml, 0.5 ml) in methanol and DTO-apo (0.53 mg/ml, 9.5 ml) in 0.1 M acetate buffer (pH 4.0) was incubated at 37 °C for 12 h. The reconstituted mixture was lyophilized and purified by FPLC under the conditions previously described. Purified DTO-NCS was desalted with a Bio-gel P-4 column (1.3 $\times$ 20 cm) and then main peak monitoring at 214 nm was lyophilized (yield, 1.62 mg (31%)).

Chemical Modification of Disulfide Bonds of MTO-Apo To prepare the four-cysteines-modified derivatives of MTO-apo for proteolytic digestion, 10 mg of purified MTO-apo was dissolved in 1.2 ml of 0.5 M Tris-HCl buffer (pH 8.0) containing 8 M urea and 0.2% ethylenediaminetetraacetic acid disodium salt (EDTA-2Na). After the addition of 300 μmol of dithiothreitol, the mixture was introduced with nitrogen gas for 10 min and heated for 3 h at 50 °C. To this mixture, 20 mg of iodoacetic acid in 0.12 ml of 1 M NaOH was added. Alkylation was conducted at pH 8.0 by adding 1 M NaOH for 30 min. The mixture was dialyzed against 0.2 M acetic acid and lyophilized. The lyophilizate was desalted by gel filtration on a Bio-Gel P-4 column (1.3 $\times$ 20 cm) eluted with deionized water and re-lyophilized (yield, 9 mg (90%)).

Tryptic Digestion of Tetra-S-carboxymethyl MTO-Apo and Separation of Trp-Containing Peptide For proteolytic digestion of reduced MTO-apo, tetra-S-carboxymethyl apo-NCS (10 mg) in 1.5 ml of 0.1 M Tris-HCl buffer (pH 7.3) was incubated with trypsin (0.13 mg) at 37 °C. The time course of the digestion was continuously monitored by FPLC at 280 nm absorbance. After the digestion was completed (about 8 h), the reaction mixture was immediately frozen and lyophilized. The separation and purification of tryptic peptide of tetra-S-carboxymethyl-MTO-apo was employed by FPLC (yield, 0.5 mg (18%)).

Amino Acid Analysis Amino acid analyses were performed with a JASCO model TRI-ROTAR VI instrument for *o*-phthalaldehyde detection. The samples were hydrolyzed with *p*-toluenesulfonic acid in sealed ampoules at 110 °C for 24 h. The NH_2 -terminal amino acids were determined by using 5-dimethylaminonaphthalene-1-sulfonyl chloride.²³⁾

Reconstitution Experiment of NCSs The mixture of NCS-chr in methanol (10 mg/ml, 50 μl) and each Trp-oxidized apo-NCSs (0.53 mg/ml, 950 μl) in 0.01 M acetate buffer (pH 5.0) were incubated at 37 °C for various time intervals. Reconstitution was monitored by the isoelectric focusing technique on polyacrylamide gel using a pH gradient of 2.5 to 4.5. The protein bands were stained with Coomassie brilliant blue R-250. The reconstitution ratios were calculated by scanning at 570 nm with a densitometer (Shimadzu Model CS-920).²⁴⁾

Biological Activities Native NCS and apo-NCS and those Trp oxidized derivatives were assayed for both antibacterial and antileukemic activities. Minimum inhibitory concentrations (MIC) against *Micrococcus luteus* (*M. luteus*) (1×10^7 cells/ml) were determined using the agar plate dilution method with nutrient agar. The cytotoxic activity of purified NCS, apo-NCS and the Trp modified derivatives were assayed using an established cell line of mouse leukemic cells (L1210): Cell suspensions of

L1210 leukemia (5×10^4 cells/ml), which contained various amounts of NCS, apo-NCS and the Trp modified derivatives in RPMI 1640 medium supplemented with 5% fetal calf serum (Gibco), were incubated at 37 °C for 2 d in a CO₂ incubator. Cell numbers were counted by the dye exclusion method with trypan blue to calculate the 50% growth-inhibitory concentration (IC₅₀) values.

Results

N-Bromosuccinimide Titration of Trp Residues in NCS and Apo-NCS Oxidation of NCS and apo-NCS with 0.01 M *N*-bromosuccinimide in 0.1 M acetate buffer (pH 4.0) in the presence and absence of 8 M urea were spectrometrically monitored. The addition of *N*-bromosuccinimide in small increments to the NCS solution in the presence of 8 M urea and to the apo-NCS solution in the presence or absence of 8 M urea led to a similar proportional and instantaneous decrease in Trp UV absorption at 280 nm, as shown in Fig. 3A and B. However, this decrease of NCS was halved in the absence of 8 M urea (Fig. 3A). These results showed that both Trp residues of apo-NCS in the absence of 8 M urea were oxidized in almost the same manner as in the presence of 8 M urea, and the consumption of *N*-bromosuccinimide amounted to 2.1 mol (Table I). However, NCS in the absence of 8 M urea was oxidized only at one Trp residue with 2.2 mol of *N*-bromosuccinimide. These results may indicate that one Trp residue on NCS is reactive and accessible to *N*-bromosuccinimide in the absence of 8 M urea and must be located at or near the surface of the molecule and the other Trp is resistant to oxidation by *N*-bromosuccinimide since this Trp changes from the reactive type to the non-reactive type due to the NCS-chr binding of apo-NCS.

The Preparation and Physicochemical Properties of DTO-Apo, MTO-NCS and MTO-Apo

Fig. 3. Ultraviolet Spectrophotometrically Monitoring of *N*-Bromosuccinimide Oxidation of Tryptophan Residues of NCS and Apo-NCS

The oxidation of NCS (7.14×10^{-8} mol/2 ml, A) and apo-NCS (1.49×10^{-7} mol/2 ml, B) were monitored at 280 nm in 0.1 M acetate buffer (pH 4.0) in the presence (---) or in the absence (—) of 8 M urea, adding aliquots (10 μl) of 0.01 M *N*-bromosuccinimide. Plotted absorbances are corrected for dilution (<5%).

TABLE I. Tryptophan Oxidation of NCS and Apo-NCS by NBS in the Presence and Absence of 8 M Urea

	8 M urea	Oxidized tryptophan (M)	Consumed NBS (M)/Trp
NCS	—	0.97 (1)	2.2
	+	1.74 (2)	2.8
Apo-NCS	—	1.81 (2)	2.1
	+	1.88 (2)	1.6

method of Trp oxidation for apo-NCS and NCS by *N*-bromosuccinimide was described in Materials and Methods. The UV spectra of NCS, MTO-NCS, apo-NCS, MTO-apo and DTO-apo in 0.1 M acetate buffer (pH 4.0) at 0.25 mg/ml concentration were shown in Fig. 4. NCS and MTO-NCS, which retain chromophore, exhibit UV absorption at around 300–400 nm but non-associated chro-

Fig. 4. UV Spectrum of NCS, MTO-NCS, Apo-NCS, MTO-Apo and DTO-Apo

The spectra were measured to 1 cm quartz cell at 0.25 mg/ml in 0.1 M acetate buffer (pH 4.0). A: NCS, B: MTO-NCS, C: apo-NCS, D: MTO-apo, E: DTO-apo.

TABLE II. Amino Acid Composition (in Residue) of NCS, MTO-NCS, MTO-Apo, DTO-Apo and Apo-NCS

Amino acid	NCS	MTO-NCS	MTO-apo	DTD-apo	Apo-NCS
Asp	12	11.80	12.66	11.55	12.33
Thr	13	12.08	12.92	11.96	13.03
Ser	11	10.20	10.50	9.23	11.39
Glu	5	5.08	5.57	5.25	5.14
Pro	4	4.33	4.61	5.02	3.93
Gly	15	14.83	14.57	15.02	15.51
Ala	18	17.93	17.74	17.14	18.60
Cys/2	4	ND	ND	ND	ND
Val	12	10.33	12.06	12.66	11.47
Ile	1	1.00	1.10	1.50	0.89
Leu	6	5.79	5.39	5.74	6.00
Tyr	1	1.01	0.99	0.96	0.76
Phe	5	4.99	4.86	5.07	5.01
Lys	1	0.96	0.81	1.14	1.11
Arg	3	2.89	2.75	2.94	3.14
Trp	2	1.85	0.95	1.02	0.0

Analyses were conducted on a JASCO model TRI-ROTAR VI instrument after hydrolysis of samples in *p*-toluenesulfonic acid for 24 h at 110 °C. ND, not determined.

TABLE III. Physicochemical Properties of NCS, Apo-NCS and These Tryptophan-Oxidized Derivatives

	NCS	MTO-NCS	MTO-apo	DTO-apo	Apo-NCS
Tryptophan number	2	1	1	0	2
Isoelectric point (pI)	3.3	3.3	3.2	3.2	3.2
M.W. by SDS-PAGE	12000	12000	12000	12000	12000
N-Terminal	Ala	Ala	Ala	Ala	Ala

mophore proteins, apo-NCS, MTO-apo and DTO-apo, exhibited no UV absorption at this wavelength range. Amino acid analysis of those proteins are indicated in Table II. These data suggest that DTO-apo was oxidized at the two Trp residues, whereas MTO-NCS was oxidized only at one Trp residue under the reaction condition described above. The data of SDS-PAGE, isoelectric focusing and N-terminal analysis by the dansyl method of NCS and

apo-NCS derivatives are summarized in Table III.

The Elucidation of Tryptophan Residue Being Resistant against *N*-Bromosuccinimide Oxidation of NCS To elucidate the position of the Trp residue remaining after selective *N*-bromosuccinimide oxidation in NCS, MTO-apo was reduced by dithiothreitol and then alkylated by iodoacetic acid for proteolytic digestion. This tetra-*S*-carboxymethyl MTO-apo was hydrolyzed with trypsin at 37°C for 8 h by the method of Hirayama.¹²⁾ When this hydrolyzate was analyzed by HPLC, only one peak possessing high 280 nm absorbance was detected (Fig. 5). This isolated Trp containing fragment, which exhibited further positive reaction by *N*-bromosuccinimide oxidation (data not shown), was analyzed by an amino acid analyzer (Table IV). The amino acid compositions of this fragment were in agreement with the T5 peptide (position 83—113) designed by Kuromizu *et al.*¹³⁾ and had 0.71 mol per 1 mol of peptide. Next, the amino acid composition of a tryptic peptide corresponding to the T2 (position 21—70) was analyzed. This peptide had 0.14 mol of Trp per 1 mol of peptide. This indicates that Trp-83 was resistant to *N*-bromosuccinimide oxidation five times as much as Trp-39.

Fluorescence Analysis of Apo-NCS-NCS-chr Interaction

In order to confirm the interaction of Trp on apo-NCS with NCS-chr, the fluorescence emission spectra of apo-NCS, NCS, MTO-apo, and MTO-NCS were measured (Fig. 6). When excited at 280 nm, the solution of apo-NCS in 0.1 M acetate buffer (pH 4.0) demonstrated maximum fluorescence at 352 nm. The 352 nm emission band is attributable to Trp residues. This Trp emission intensity of NCS is only 30% of that of apo-NCS. It is reported that on analysis of the molecular interaction of protein and fluorescence active compounds, fluorescence emission intensity was generally decreased by the

Fig. 5. Purification of Trypsin Digest of Tetra-*S*-carboxymethyl Mono-tryptophan-Oxidized Apo-NCS by FPLC

A 50 μ l sample (10 mg/ml) was chromatographed on mono Q HR 5/5 by elution at 2 ml/min with 0.1 M Tris-HCl buffer (pH 7.2) by a NaCl gradient (---) as shown: pump pressure, 2 MPa; sample injection time, zero; tryptophan-contained peptide (▨▨▨).

TABLE IV. Amino Acid Composition of Tryptic Peptide Obtained from MTO-Apo

	Calculated ^{a)}			Analysis			
	NCS	T2 ^{b)}	T5	Tryptic peptide having tryptophan residue		Tryptic peptide corresponding to T2 residue ^{c)}	
				Found	Estimated	Found	Estimated
Asp	9	5	2	4.09	(4)	6.05	(6)
Asn	3	1	2				
Thr	13	5	3	2.88	(3)	5.35	(5)
Ser	11	5	2	2.14	(2)	5.03	(5)
Glu	2		1	2.03	(2)	1.82	(2)
Gln	3	2	1				
Pro	4	1	1	1.05	(1)	1.72	(1)
Gly	15	6	5	4.95	(5)	6.04	(6)
Ala	18	10	5	4.95	(5)	9.58	(10)
Cys/2	4	2	2	ND	(2)	ND	(2)
Val	12	6	3	3.44	(3)	4.87	(6)
Ile	1		1	0.95	(1)	0	(0)
Leu	6	3	1	1.10	(1)	2.76	(3)
Tyr	1	1		0	(0)	1.02	(1)
Phe	5	1	1	0.99	(1)	1.05	(1)
Lys	1			0	(0)	0	(0)
Arg	3	1		0	(0)	0.84	(1)
Trp	2	1	1	0.71	(1)	0.14	(0)
Total	113	50	31		(31)		(50)

a) The amino acid composition of apo-NCS (Kuromizu *et al.*, 1986) was used. b) The tryptic peptides T2 and T5 correspond to positions 21—70 and 83—113, respectively. c) T2 residue was prepared from MTO-apo according to Kuromizu's method.¹³⁾ ND, not determined.

Fig. 6. Fluorescence Emission Spectra of Apo-NCS, NCS, MTO-Apo, MTO-NCS, and DTO-Apo

Uncorrected fluorescence emission spectra of protein (5×10^{-6} M) in 0.1 M acetate buffer (pH 4.0) were recorded at room temperature. Excitation wavelength was 280 nm. A: native apo-NCS, B: native NCS, C: MTO-apo, D: MTO-NCS, E: DTO-apo.

Fig. 7. Fluorescence Spectra of Apo-NCS in the Presence of NCS-chr

Fifty microliters volume of NCS-chr in methanol at various concentrations were added to 2 ml in a 0.1 M acetate buffer solution (pH 4.0) containing apo-NCS (5×10^{-6} M) and after incubation at 37°C under dark conditions for 2 h the fluorescence emission was recorded. The uncorrected fluorescence spectrum from top to bottom, correspond to varying final concentrations of NCS-chr, as follows: 0, 3.2×10^{-7} , 6.3×10^{-7} , 1.25×10^{-6} , 2.5×10^{-6} , and 5×10^{-6} M were measured at room temperature. The highest curve shows the fluorescence spectrum of free NCS-chr (5×10^{-6} M). The excitation wavelength was 280 nm.

participation of the Trp emission of protein.^{25,26}) This reduced emission of native NCS might be due to quenching resulting from the association of NCS-chr to apo-NCS. In

Fig. 8. CD Spectra of Apo-NCS, MTO-Apo and DTO-Apo

Spectra were recorded with apo-NCS, MTO-apo, and DTO-apo concentrations of 0.5 mg/ml (in the wavelength region of 260–400 nm) or 0.2 mg/ml (in the wavelength region of 190–270 nm) in 0.01 M phosphate buffer (pH 7.4). For measurement of near-UV and far-UV spectra, 1 and 0.1 cm cells were used, respectively. Apo-NCS (—), MTO-apo (---), DTO-apo (· · ·).

fact, the intensity of the emission spectrum of apo-NCS at 352 nm is quenched in the presence of NCS-chr parallel with the increase of the NCS-chr concentration with blue shift. A typical example of fluorescence titration is shown in Fig. 7 for the apo-NCS–NCS-chr system. These data suggest that the interaction of NCS-chr occurred at least one of the Trp residues of apo-NCS and the environment of this Trp changed the hydrophobic circumstance. On the other hand, the fluorescence intensity of MTO-apo actually found is only 7% of that of apo-NCS, nevertheless MTO-apo contains half as much Trp as apo-NCS. Furthermore, the quenching of the fluorescence emission intensity of MTO-NCS is 44% of that of MTO-apo and the fluorescence emission intensity of DTO-apo is almost negligible. The quenching of the fluorescence emission generated from apo-NCS by NCS-chr can be reasonably assumed to be due to the NCS-chr interaction with apo-NCS. This assumption is in good agreement with the X-ray and three-dimensional computed graphical data discussed later.

Extensive Conformational Change during *N*-Bromosuccinimide Modification of Apo-NCS The CD spectrum of native apo-NCS was characterized by two negative peaks at about 214 and 275 nm, and two positive peaks at about 198 and 226 nm (Fig. 8). After the Trp oxidation of apo-NCS, the positive peaks at 198 and 226 nm decreased in intensity with blue shift and the negative peaks at 214 and 275 nm increased. It is assumed that the spectrum of MTO-apo contains the contribution of the intact Trp at position 89 whereas the spectrum of DTO-apo is free of the Trp contribution. According to the curve-fitting analysis (data not shown), these spectral changes suggest that the contents of α -helix and random coil decreased and those of β -sheet structure increased. These observations suggested that the *N*-bromosuccinimide modified apo-

Fig. 9. The Time Course of the Reconstitution to NCS, MTO-NCS and DTO-NCS

The mixture of NCS-chr (10 mg/ml, 50 μ l) in methanol with apo-NCS ($\blacktriangle$ — $\blacktriangle$), MTO-apo ($\square$ — $\square$), and DTO-apo ($\blacksquare$ — $\blacksquare$) (0.53 mg/ml, 950 μ l) in 0.01 M acetate buffer (pH 5.0) was incubated at 37°C for 24 h, respectively. Reconstitution ratio (%) was calculated on the integral value of densitometry: % of NCS reconstitution = $[\text{NCS}] \times 100 / ([\text{NCS}] + [\text{apo-NCS}])$.

TABLE V. Biological Activities of NCS, Apo-NCS and These Tryptophan-Oxidized Derivatives

	Number of Trp oxidized	Position of oxidized Trp	Biological activities	
			<i>M. luteus</i> MIC (μ g/ml) ^{a)}	L1210 cells IC ₅₀ (μ g/ml) ^{b)}
NCS	0		0.31	0.0088
Apo-NCS	0		> 100	> 5
MTO-NCS	1	39	0.78	0.038
MTO-apo	1	39	> 100	> 5
DTO-NCS	2	39 and 83	1.25	0.073
DTO-apo	2	39 and 83	> 100	> 5

a) MIC, minimum inhibitory concentration. b) IC₅₀, concentration which caused 50% inhibition of growth.

NCSs changed their fine high-order structure.

Reconstitution of NCS-chr and *N*-Bromosuccinimide Modified Apo-NCSs In order to show the requirement of Trp residues of apo-NCS–NCS-chr binding, apo-NCS, MTO-apo, and DTO-apo were reconstituted with NCS-chr by a previously reported method.²⁴⁾ The mixture of NCS-chr and apo-NCS (MTO-apo or DTO-apo) were incubated at 37°C (Fig. 9). The reconstitution ratio of those mixtures reached a maximum of about 90% of the theoretical value for apo-NCS, 75% for MTO-apo and 40% for DTO-apo after 5 h under identical experimental conditions.

Biological Activities of *N*-Bromosuccinimide Modified NCSs and Apo-NCS The antibacterial and antitumor activities of Trp modified NCS and apo-NCS, as well as NCS and apo-NCS, are summarized in Table V. The MICs of MTO-NCS and DTO-NCS against *M. luteus* were 40 and 25% of NCS, respectively, and the antitumor activities (IC₅₀) of MTO-NCS and DTO-NCS were 23 and 12% of NCS, respectively. On the other hand, MTO-apo and DTO-apo at several 100-fold high concentrations of NCS had none of these biological activities because these apo-NCS derivatives were not associated with NCS-chr. In this experiment, the biological activities of Trp-oxidized NCSs were reduced to 12–40% of that of the native NCS.

These data suggest that MTO-apo and DTO-apo, especially DTO-apo, were not able to tightly bind with NCS-chr because of the change of the high-order structure of apo-NCS by the oxidation of Trps on apo-NCS.

Discussion

NCS has been clinically used as a cancer chemotherapeutic agent in Japan since 1977.²⁾ This antibiotic was the first antitumor protein in which an amino acid sequence was proposed⁹⁾ but recently its sequence has been revised by a few researchers.^{10–13)} We¹⁹⁾ and others^{27,28)} reported that NCS is composed of apo-NCS and NCS-chr. Many reports concerning the interactions between NCS-chr and DNA were published by Goldberg *et al.*⁵⁾ and Myers,^{6,8)} but there are few reports on the specific binding of NCS-chr and apo-NCS. We focused our study on the specific binding between apo-NCS and NCS-chr because NCS-chr, due to the biological activity of NCS, is very labile, and it is protected from the loss of its activities by apo-NCS.

The selective oxidation of Trp residues in NCS and its conformational effect and biological activities have been reported by Samy *et al.*²⁹⁾ Namely, the two Trp residues exhibited the differential behavior of striking selectively toward oxidation by *N*-bromosuccinimide. Since Samy's paper, however, was published before the discovery of NCS-chr in the NCS molecule, there are many equivocal interpretations in this paper.

We show that the two Trp residues of NCS exhibited a differential reaction toward oxidation by *N*-bromosuccinimide while those of apo-NCS exhibited a similar effect (Fig. 3). These results are related to the selective modification of the two Trp residues by *o*-nitrophenyl sulfonyl chloride and the selective oxidation of the two Trp residues by *N*-bromosuccinimide.^{30,31)} Purified MTO-apo, DTO-apo and MTO-NCS were homogeneous, respectively, as shown in Table II. The NH₂-amino terminals of all the peptides were identical to Ala. These results indicate that during the oxidation process of NCS or apo-NCS by *N*-bromosuccinimide, the cleavage of the tryptophyl C-terminal was negligible. Samy *et al.* reported that when *N*-bromosuccinimide oxidation was carried out in the presence of 8 M urea, Asp and Gly were obtained as new NH₂-terminal amino acids, showing that in the denaturated state both the tryptophyl–glycyl bond (positions 79 and 80) and tryptophyl–aspartyl bond (positions 46 and 47) of NCS were cleaved (position numbers referred from Meienhofer *et al.*⁹⁾) but in the absence of 8 M urea no new NH₂-terminal was observed.²⁹⁾ Our experimental results clearly showed that Trp-39 of NCS was easily oxidized by *N*-bromosuccinimide and the Trp residue, being resistant to *N*-bromosuccinimide oxidation, was at position 83 of NCS (Table IV). These results mean that the Trp (83) residue of NCS must be located in the interior of the protein molecule while that of apo-NCS must be located in the exterior of the protein molecule. Denaturation by 8 M urea was required to make this non-reactive type Trp (83) residue accessible to *N*-bromosuccinimide. Accordingly, the high-order structure of NCS might be destroyed by 8 M urea to convert apo-NCS from NCS after the release of NCS-chr.¹⁸⁾

It is reported that on analysis of the molecular interaction of protein and fluorescence active compounds, fluorescence intensity was generally decreased by the participation of the

Trp residue of the protein.^{25,26)} The 352 nm emission of apo-NCS was quenched in the presence of NCS-chr in a dose-response manner (Fig. 7). NCS-chr may quench the emission generated from one of the Trp on apo-NCS by competitively absorbing the excitation energy and serving as an energy acceptor as mentioned by Napier.²⁷⁾ As MTO-apo exhibited only about 10% of the fluorescence emission intensity of apo-NCS, it would appear that this remaining Trp (83) residue has a relatively low quantum yield and does not contribute greatly to the total fluorescence spectrum of apo-NCS, whereas the Trp (39) residue greatly contributes. The much greater loss of fluorescence intensity (70%) of apo-NCS binding with NCS-chr must be due to the loss of fluorescence emission generated from Trp (39), suggesting the possibility that the interaction of NCS-chr directly occurred with the Trp (39) residue of apo-NCS.

The reconstitution ratio of apo-NCS, MTO-apo, and DTO-apo with NCS-chr decreased in this order (Fig. 9). As the number of oxidized Trp in apo-NCS increased, the ability of reconstitution decreased in that order and the reconstitution ratio of DTO-apo with NCS-chr was reduced to 40%. Purified MTO-NCS and purified DTO-NCS prepared by the reconstitution of NCS-chr and DTO-apo, exhibited lower activities than NCS itself. These findings mean that tryptophan oxidation on apo-NCS changes the high-order structure of peptide moiety contained tryptophan to reduce the ability of stabilization and transport of NCS-chr. In fact, it is observed that the CD spectral data of MTO-apo and DTO-apo is different from that of apo-NCS.

Sieker reported according to X-ray crystallography at 3.5 Å of resolution that NCS was composed of twisted 7-strand β -sandwich for the major portion but NCS-chr was not delineated from the course of the peptide chain at this resolution.³²⁾ However, only an incomplete stereo diagram of NCS was indicated in this paper because it was believed that NCS consisted of 109 amino acids. It appeared that the cavity was formed by three β -sheet loops, i.e. position 73—90 contained hydrophobic residues (Phe—Glu—Gly—Phe—Leu—Phe (position 73—78)), position 97—112 contained hydrophobic residues (Val—Ala—Ile—Ser—Phe (position 108—112)) and position 37—50 contained hydrophobic residues (Cys—Ala—Trp—Val (position 37—40)). Recently, Ishiguro *et al.*³³⁾ reported the modeling of the three dimensional structure of apo-NCS based on the actinoxanthin crystal structure.^{34,35)} These reports indicate that Trp (83) of apo-NCS locates at far from a cleft binding to NCS-chr, whereas Trp (39) locates at a cleft surface. On the basis of these reports and our experimental results we conclude that Trp (39) residue located in the exterior of apo-NCS directly interacts with NCS-chr in the binding of NCS-chr to apo-NCS, and that a β -sheeted loop structure containing Trp (83) residue may change the high-order structure and then hold NCS-chr.

A complete X-ray analysis of NCS may elucidate the structural changes of apo-NCS produced upon binding with NCS-chr.

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