NOTES

CHEMICAL REACTIVITY AND STATE OF TRYPTOPHAN RESIDUES IN NEOCARZINOSTATIN UNDER PHYSIOLOGICAL CONDITION

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Neocarzinostatin (NCS)[a] is an antitumor antibiotic protein of which primary structure was reported by this author *et al.* recently.[1,2] It possesses 109 amino acid residues (molecular weight; about 11,000) including two tryptophan, one tyrosine, one lysine, three arginine and two disulfide bridges. But no methionine nor histidine residue was found. The conformational study of NCS in a physiological condition using ORD and CD showed strong COTTON effects in 240~300 nm area, which were assigned to be due to the contributions of such residues as tyrosine, phenylalanine and possibly disulfide chromophores. The contribution of the tryptophan residues which located at the positions 46 and 79, however, was not clear.[3] Furthermore, NCS molecule has been shown to have unusually tight gross structure and predominantly antiparallel β-structure.[3]

In the present communication, I want to report the state of tryptophan residues in the physiological condition (isotonic and near neutral pH) as judged by the chemical reactivity of tryptophan residue to *o*-nitrophenyl sulfenyl chloride (NPS-Cl)[a].[4] NPS-Cl was chosen because of its high selectivity toward the tryptophan residue.[4,5] Other tryptophan modifying reagents often react less selectively or less quantitatively.[5] For example, widely used N-bromosuccinimide is known to result in peptide bond cleavage and / or react less selectively other than tryptophan residue (*eg.* reaction with tyrosine *etc.*[5,6,7]).

The reactivity of two tryptophans in NCS in the unfolded state (using 50 % acetic acid or 8 M urea) has been shown previously that they reacted with NPS-Cl quantitatively[8] within one hour as expected. The present study was carried out under a mild condition[b] at pH 4.0~7.0 where NCS was stable and as closely physiological as possible, using the buffer solution of DULBECCO's[9] in 0.01 M Na^{+}-K^{+}- phosphate-buffered 0.15 M NaCl solution. But Ca^{++} and Mg^{++} were eliminated from the buffer. NPS-Cl, three times crystallized from chloroform, was a generous gift of Dr. KAWAUCHI of Hormone Research Laboratory, University of California, San Francisco. NCS, also a generous gift from Dr. Y. KOYAMA of Kayaku Antibiotic Research Laboratory, Tokyo, was used after chromatography with CM-cellulose as described in a previous report,[10] with a slight modification. Since NPS-Cl was not freely soluble in the buffer solutions used, the crystals of NPS-Cl were finely powdered before adding to the solutions containing NCS where NCS had an approximate concentration of 0.1 ~ 0.05 μ mole/ml. NPS-Cl was added to NCS solution at 53~100 molar excess[c] over NCS. The reaction mixture was kept under reciprocal shaking for 3 and 24 hours at 16~18°C and at pHs 4.0, 5.2 6.1 and 7.0 respectively. The pH of each solution was readjusted with 0.15 M HCl or 0.2 M NaH_2PO_4 when it was necessary. The reaction was terminated by applying the supernatant of the reaction mixture, after centrifugation (2,700 rpm, 20 min), on a column of Sephadex G-50 when biological activity should be assayed, or terminated by precipitation with a mixture of acetone and 1 M HCl (39:1) followed by further washing as described in a previous paper[8]. The quantitation of NPS-chromophore in NCS was determined by spectrophotometry using E=4,000 at 365 nm[4].

The present results in duplicates revealed that one tryptophan residue in NCS reacted readily with NPS-Cl at all pHs tested within 3 hours at 16~18°C. This can be interpreted that this

a) Abbreviations used, NPS: *o*-nitrophenylsulfenyl, NCS: neocarzinostatin.

b) Since NCS is stable in the acidic pH (<7), unfolding of the protein molecule should not be expected to occur.

c) Since NPS-Cl has not been dissolved completely in these buffers, the intrinsic concentrations of NPS-Cl were not known, but it should be lower than the values given here.

residue is accessible to the reagent and thus can be considered being exposed outward on the surface of the molecule of NCS. The second tryptophan residue reacted with NPS-Cl gradually within 24 hours[d] at 16~18°C. The numbers of NPS-chromophore after the reaction were 1.6~2.0/mole of NCS in all pHs tested. These results indicate that one of the tryptophan residues is exposed freely and the other to the lesser degree. The previous results of ORD and CD in which no tryptophan residue seemed to show typical COTTON effect in NCS[8] may be explained by the good to fair chemical reactivity of two tryptophan residues in physiological condition which was observed in the present experiments. Tyrosine residue on the contrary was chemically inert and exist as buried state[8].

The mono-NPS-derivative[e] of NCS showed bacteriocidal activity against *Sarcina lutea* at 0.4 μg/ml. This fact indicates at least one tryptophan is not involved in the function of antibiotic activity. The *bis*-NPS-derivative of NCS was devoid of inhibitory activity to the growth of *S. lutea*. This indicates the important role of the second tryptophan residue for the structural integrity of active molecular conformation of NCS. Furthermore the biologically active mono-NPS-derivative may be expected to exert a different pharmacological properties such as increased permeability on cell membrane due to incorporation of a hydrophobic aromatic ring. Such modification of amino groups in NCS, *bis*-succinyl-NCS for instance, resulted in loss of cytocidal activity but retained cytostatic property[11].

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Note added in proof:

A similar result was obtained in our separate study[12] and the tryptophan residue 79 was found selectively oxidized with N-bromosuccinimide in 0.1 M Na phasphate buffer at pH 6.1

d) As given in the Foot Note *c*), the time required for the completion of the reaction could be shorter, when given NPS-Cl could have been dissolved completely.

e) The bacteria, *Sarcina lutea*, is one of the most susceptible organism to NCS. A preliminary examination of the reaction product obtained after 3 hr reaction at pH 7.0 showed only one spot on electrophoresis at pH 8.2 and thus it is considered as a single principle.

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