RECONSTITUTION OF NEOCARZINOSTATIN (NCS)

Sir:

Neocarzinostatin (NCS), an acidic antitumor protein which contains a non-protein chromophore, was isolated in 1965 from the culture filtrate of *Streptomyces carzinostaticus* var. F-41[1]. The chemotherapeutic uses of NCS for patients with gastric cancer, pancreatic cancer, acute leukemia, bladder cancer and hepatocellular carcinoma were comprehensively documented by MAEDA in 1981[2].

Recently, we[3] and others[4,5] demonstrated that the NCS chromophore can be separated from NCS. The isolated chromophore is responsible for the inhibition of bacterial and tumor cell growth[3] and for the inhibition of DNA synthesis in tumor cells[6]. The NCS chromophore is very unstable to UV irradiation and heat and contains 1-naphthalenecarboxylic acid[7], *N*-methylfucosamine[8] and peroxide[9] moieties. It is suggested that naphthalenecarboxylic acid and *N*-methylfucosamine moieties are covalently linked to a highly unsaturated part of the chromophore which possesses a cyclic carbonate ring[10]. Although no biological activity of the NCS apoprotein (protein moiety which carries the chromophore)[3,6,11] has been observed, the protein may nevertheless play an important role in enhancement[3] and stabilization[11] of the biological activities of the chromophore. Physicochemical and analytical data on the NCS apoprotein suggest that this protein is identical to pre-neocarzinostatin[12] which is a biosynthetic precursor of NCS[13]. Two distinct research groups reported that the NCS apoprotein can be reconstituted with the NCS chromophore to form a whole NCS molecule. KAPPEN *et al.*[12] demonstrated that NCS can be reconstituted when the chromophore is incubated with the NCS apoprotein both under diluted and concentrated conditions. Furthermore, JUNG *et al.*[14] reported that the NCS chromophore binds to apoprotein which coupled with monoclonal I_gG_1 antibody against mice myeloma cells. However, these authors did not mention the optimum conditions for reconstituting NCS.

In this communication, we will describe the optimum conditions for reconstituting whole NCS molecule from the chromophore and apoprotein of NCS detecting with acrylamide isoelectric focusing analysis. Also we discuss the application of this reconstituting method to modify the NCS molecule.

NCS preparation (clinical grade, 1,500 units/mg), which is produced by the Kayaku Antibiotics Research Laboratory (Tokyo, Japan), was used in this study. The NCS chromophore and NCS apoprotein were prepared from the powder according to the method previously reported[3,6,7]. The protein bands of NCS, NCS apoprotein and reconstituted NCS were detected at a concentration of 1 mg/ml by the isoelectric focusing technique, after staining with Coomassie brilliant blue R-250 and then decolorizing. Stained bands were quantified by scanning at 570 nm with a densitometer (Asuka Model OZ-802). The ratio of reconstituted NCS to unreconstituted NCS apoprotein was calculated from the integral value obtained by densitometry. In this experiment, all chemical preparations and reactions were carried out in the dark room.

The isoelectric focusing patterns of native NCS, NCS apoprotein and reconstituted NCS are shown in Fig. 1. The isoelectric point of the reconstituted NCS was found to be identical to that of native NCS (pI=3.3). Whereas, the NCS apoprotein had a isoelectric point of about 3.2, cle-

Fig. 1. Isoelectric focusing of reconstituted NCS on polyacrylamide gel.

Polyacrylamide (5%) gel contained 2% carrier Ampholite (LKB-produkter AB, Bromma, Sweden) pH 2.5~4.0, was prepared as per specification given in the LKB manual. Riboflavin was added as a photochemical polymerizing agent. After application of the sample (1 mg/ml, 40 μl), isoelectric focusing was done. The run was increased from 270 to 550 V after an initial 30 minutes run at a constant current of 50 mA. The run continued for 3 hours at 550 V. Focusing was carried out under running water at 10°C.

(1) NCS apoprotein plus chromophore (10 : 1, weight ratio), incubated at 37°C for 24 hours, (2) Native NCS, (3) NCS apoprotein.

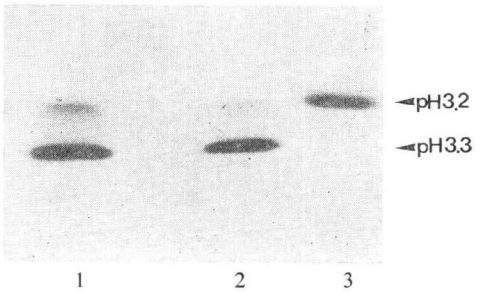
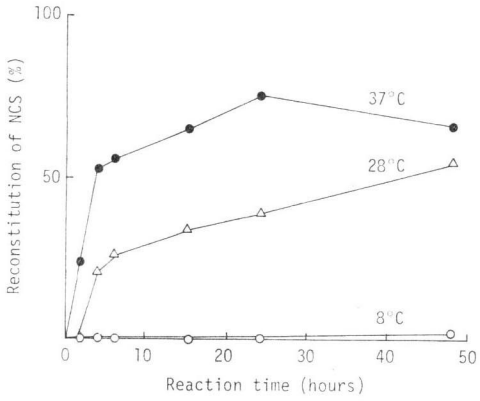


Fig. 2. Effect of temperature for the reconstitution of NCS.

Chromophore in methanol (10 mg/ml, 10 μl) and NCS apoprotein (1 mg/ml, 1 ml) in 0.01 M acetate buffer solution (pH 5.0) mixture was incubated for 24 hours. Reaction temperatures are at 8°C, at 28°C and at 37°C. Reconstitution ratio (%) was calculated on the integral value of densitometry:

$$\% \text{ of NCS reconstitution} = \frac{\text{NCS (pI 3.3)}}{\text{NCS (pI 3.3)} + \text{NCS apoprotein (pI 3.2)}} \times 100.$$



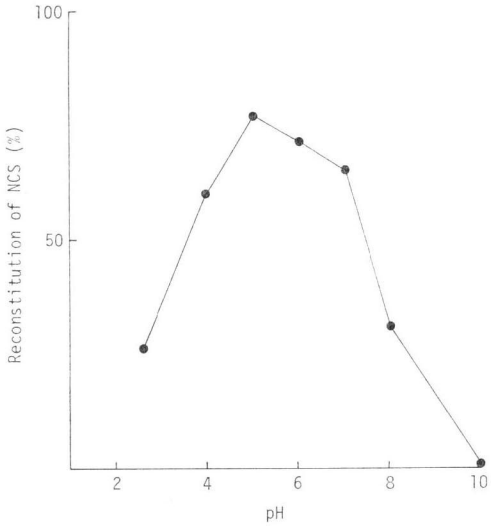
arly distinguishing it from reconstituted NCS. Under the same condition, the chromophore alone (1 mg/ml, 10 μl) did not show any bands (data not shown).

The process of reconstituting NCS from NCS apoprotein and chromophore was examined under various conditions.

First, in order to show the effects of temperature and reaction time on the NCS reconstitution, the chromophore and NCS apoprotein mixture was incubated for 48 hours at 8°C, 28°C and 37°C (Fig. 2). Reconstitution reached a maximum of 75% of the theoretical value at 37°C after 24 hours, whereas no reaction was observed at 8°C even after 48 hours. At 28°C, however, 50% reconstitution was obtained after 48 hours. By these experiments, it is clear that the reconstitution of NCS is highly dependent on the temperature. In general, the side chain of a protein is apt to change its three-dimensional conformation more ready at higher temperature. We think that the energy of high temperature may make the conformation of apoprotein suitable for reconstitution with chromophore. On the basis of these results, reconstitution of NCS was carried out at 37°C for 24 hours.

Fig. 3. Effect of pH for the reconstitution of NCS.

NCS apoprotein (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 solutions of NCS apoprotein at various pH's (1 ml) were incubated with a methanol solution of chromophore (10 mg/ml, 10 μl) at 37°C for 24 hours, respectively. Reconstitution ratio (%) of NCS was calculated by the same method as in Fig. 2.

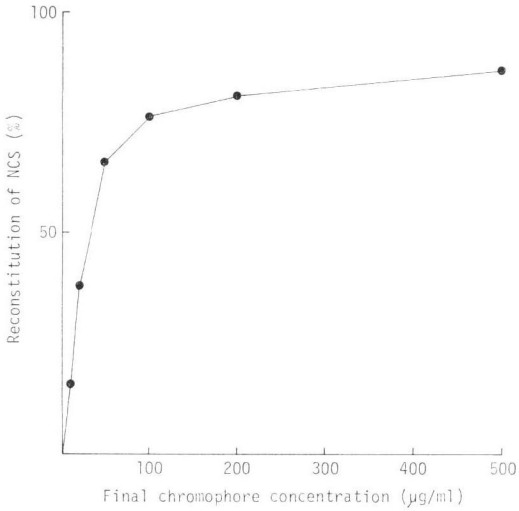


Second, the effect of pH on NCS reconstitution was examined. The NCS apoprotein in various buffer solutions at different pH levels (1 ml) were incubated with methanol solution of chromophore (10 mg/ml, 10 μl) at 37°C for 24 hours. Fig. 3 shows that the chromophore and apoprotein were reconstituted at a wide pH range between 3.0 and 8.0. However, the highest level of 75% reconstitution was obtained at pH 5.0. Coincidentally, this was equal to the pH at which the biological activities of NCS was most stable[15].

Third, the effect of chromophore concentration on NCS reconstitution was tested. As shown in Fig. 4, higher reconstitution was obained in proportion to the final chromophore concentration (10~500 μg/ml) when the concentration of NCS apoprotein (1 mg/ml at pH 5.0) was kept constant. However, at concentrations higher than 100 μg/ml, the reconstitution was equilibrated. These experimental results may indicate that at the weight ratio of 10: 1 of apoprotein to chromo-

Fig. 4. Effect of the chromophore concentration on the reconstitution of NCS.

Various concentrations of the chromophore were prepared in methanol at 1~50 mg/ml. The concentration of the NCS apoprotein was held constant at 1.0 mg/ml in 0.01 M acetate buffer solution (pH 5.0). Chromophore (10 μl) was added to the solution (1 ml) and incubated at 37°C for 24 hours. The reconstitution ratio (%) of NCS was calculated by the same method as in Fig. 2.



phore (molar ratio, 1: 1), the greatest amount of reconstituted NCS is obtained when they are incubated at 37°C for 24 hours. This ratio is almost the same as that found with native NCS. All of these results suggested that the chromophore and the NCS apoprotein combine stoichiometrically to form NCS. One hundred percent of reconstitution, however, did not occur, probably because the chromophore was released spontaneously from the reconstituted NCS. This hypothesis was supported by the experimental data in Fig. 2, where the reconstitution rate decreased after 48 hours at 37°C.

Through these experiments, the optimum condition for NCS reconstitution were determined to be incubation of chromophore and apoprotein at a molar ratio of 1: 1 at pH 5.0 and 37°C for 24 hours.

When the chromophore was inactivated beforehand by heating (65°C, 2 hours) or UV irradiation (germicidal lamp, 500 μW/cm^2 for a few minutes) treatment, it failed to reconstitute with NCS apoprotein. However, pretreatment of the NCS apoprotein by heating or UV irradiation under the same conditions as for chromophore did not impair the reconstitution of the apoprotein with the active chromophore (data not shown).

These results demonstrate that the apoprotein is stable but the chromophore is quite sensitive to such treatments. Heating or UV irradiation causes NCS to lose its biological activity and also reduces its active oxygen content[9]. These observations suggest that degradation of the peroxide moiety of the chromophore decreases its ability to bind itself to the NCS apoprotein.

Through this method of reconstitution, we could also verify that the 41 residue fragment (position 68~109) of NCS apoprotein is necessary for the chromophore to retain its antibacterial activity[10]. However, the chemical mechanism by which the chromophore binds with the NCS apoprotein is still unknown. We would like to speculate, however, that the chromophore may bind to the NCS apoprotein not only by an ionic bond between the aminosugar basic center of the chromophore and the acidic residue(s) of the NCS apoprotein residue (position 68~109) but also by a hydrophobic bond between the hydrophobic region of the chromophore and the two hydrophobic regions of the NCS apoprotein (position 69~74 and 102~108).

By fully understanding how NCS is reconstituted, researchers could be able to discover new types of NCS with different molar ratios of apoprotein to chromophore and to develop new hybrid molecules particularly with antitumor cell antibodies.

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References

1) ISHIDA, N.; K. MIYAZAKI, K. KUMAGAI & M. RIKIMARU: Neocarzinostatin, an antitumor antibiotic of high molecular weight. J. Antibiotics, Ser. A 18: 68~76, 1965
2) MAEDA, H.: Neocarzinostatin in cancer chemotherapy (review). Anticancer Res. 1: 175~186, 1981
3) KOIDE, Y.; F. ISHII, K. HASUDA, K. KOYAMA, K. EDO, S. KATAMINE, F. KITAME & N. ISHIDA: Isolation of a non-protein component and a protein component from neocarzinostatin (NCS) and their biological activities. J. Antibiotics 33: 342~346, 1980
4) NAPIER, M. A.; B. HOLMQUIST, D. J. STRYDAM & I. H. GOLDBERG: Neocarzinostatin: Spectral characterization and separation of a non-protein chromophore. Biochem. Biophys. Res. Commun. 89: 635~642, 1979
5) SUZUKI, H.; K. MIURA, Y. KUMADA, T. TAKEUCHI & N. TANAKA: Biological activities of non-protein chromophore of antitumor protein antibiotics: Auromomycin and neocarzinostatin. Biochem. Biophys. Res. Commun. 94: 255~261, 1980
6) OHTSUKI, K. & N. ISHIDA: The biological effect of a non-protein component removed from neocarzinostatin (NCS). J. Antibiotics 33: 744~750, 1980
7) EDO, K.; S. KATAMINE, F. KITAME, N. ISHIDA, Y. KOIDE, G. KUSANO & S. NOZOE: Naphthalenecarboxylic acid from neocarzinostatin (NCS). J. Antibiotics 33: 347~351, 1980
8) SCHÖNBERG, G. A.; R. S. DEWEY, O. D. HENSENS, J. M. LISCH, M. A. NAPIER & I. H. GOLDBERG: Neocarzinostatin: Chemical characterization and partial structure of non-protein chromophore. Biochem. Biophys. Res. Commun. 95: 1351~1356, 1980
9) EDO, K.; M. ITO, N. ISHIDA, Y. KOIDE, A. ITO, M. HAGA, T. TAKAHASHI, Y. SUZUKI & G. KUSANO: Peroxy acid as a partial structure of neocarzinostatin (NCS) chromophore. J. Antibiotics 35: 106~110, 1982
10) NAPIER, M. A.; I. H. GOLDBERG, O. D. HENSENS, R. S. DEWEY, J. M. LIESCH & G. A. SCHÖNBERG: Neocarzinostatin chromophore: Presence of acyclic carbonate subunit and its modification in the structure of other biological active forms. Biochem. Biophys. Res. Commun. 100: 1703~1712, 1981
11) KAPPEN, L. S. & I. H. GOLDBERG: Stabilization of neocarzinostatin nonprotein chromophore activity by interaction with apoprotein and with HeLa cells. Biochemistry 19: 4786~4790, 1980
12) KAPPEN, L. S.; M. A. NAPIER & I. H. GOLDBERG: Roles of chromophore and apo-protein in neocarzinostatin action. Proc. Natl. Acad. Sci. USA 77: 1970~1974, 1980
13) KIKUCHI, M.; M. SHOJI & N. ISHIDA: Preneocarzinostatin, a specific antagonist of neocarzinostatin. J. Antibiotics 27: 766~774, 1974
14) JUNG, G.; W. KÖHLEIN & G. LÜNDER: Biological activity of the antitumor protein neocarzinostatin coupled to a monoclonal antibody by *N*-succinimidyl 3-(2-pyridylthio)propionate. Biochem. Biophys. Res. Commun. 101: 599~606, 1981
15) KOHNO, M.; I. HANEDA, Y. KOYAMA & M. KIKUCHI: Studies on the stability of antitumor protein neocarzinostatin. I. Stability of solution of neocarzinostatin. Jap. J. Antibiotics 27: 707~713, 1974 (in Japanese)
16) EDO, K.; M. ITO, N. ISHIDA & Y. KOIDE: Characterization of peptide fragments from tripsin-treated neocarzinostatin protein. The 40th Annual Meeting of the Japanese Cancer Association: p-220, 1981 (in Japanese)