Neocarzinostatin: Effect of Modification of Side Chain Amino and Carboxyl Groups on Chemical and Biological Properties†

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ABSTRACT: The antitumor protein neocarzinostatin (NCS), isolated from *Streptomyces carzinostaticus*, is a single chain polypeptide with 109 amino acid residues. Complete acylation of the amino groups (alanine-1 and lysine-20) was observed when NCS was allowed to react with 3-(4-hydroxyphenyl)-propionic acid *N*-hydroxysuccinimide ester at pH 8.5. Since the ensuing bis[(alanine-1, lysine-20)-3-(4-hydroxyphenyl)]-propionamide NCS was fully active in antibacterial potency and in the inhibition of growth of leukemic (CCRF-CEM) cells in vitro, it appears that the two amino groups in the protein are not essential for biological activity. Radiolabeled NCS was prepared by using a tritiated or ^{125}I-labeled acylating agent. Since the CD spectra of native and bis(alanine-1, lysine-20)-amino modified NCS were indistinguishable, there is presumably no change in the native conformation of the protein due to acylation. Reaction of NCS with ammonium chloride in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at pH 4.75 converted all the 10 carboxyl groups into carboxamides and produced a protein derivative of basic character. This modification caused a change in the native conformation of the protein accompanied by a loss in biological inhibitory activities.

Neocarzinostatin (NCS),[1] an acidic protein (molecular weight 10 700) isolated from culture filtrates of *Streptomyces carzinostaticus* var. F41 (Ishida et al., 1960, 1965; Maeda et al., 1966), is active against experimental tumors in mice (Ishida et al., 1965; Kumagai et al., 1966; Samy et al., 1977b). In clinical studies carried out in Japan, NCS is reported to be effective in the treatment of certain solid tumors (Takahashi et al., 1969) and acute leukemia (Hiraki et al., 1973). The drug is now in Phase I clinical trials at several cancer centers in this country including the Sidney Farber Cancer Institute. In mammalian cells NCS inhibits DNA synthesis and induces DNA strand scissions, both in vivo and in vitro (Beerman and Goldberg, 1974; Tatsumi et al., 1974; Ohtsuki and Ishida, 1975; Poon et al., 1977).

The amino acid sequence of NCS was established by Meienhofer and his colleagues (Meienhofer et al., 1972; Maeda et al., 1974a,b) at this Institute. Although NCS has a particularly high content of alanine, glycine, serine, and threonine, it contains a number of residues that may be readily modified by site-specific reagents: i.e., one tyrosine, two tryptophan, one lysine, three arginine, and four half-cystine residues. We are engaged in chemical and enzymatic modification of NCS in order to arrive at structure-activity correlation and to prepare derivatives with improved chemotherapeutic properties. Our previous studies (Samy et al., 1974) showed that the single tyrosine residue in position 32 and one of the tryptophan residues in position 46 are buried in the interior of the molecule and are inaccessible to *N*-bromosuccinimide oxidation, whereas the tryptophan-79 residue can be selectively oxidized without appreciable loss of biological activity. In this paper, we report the ionization behavior of NCS and the effect of modification of amino and carboxyl groups on the inhibitory properties of the protein. Reaction of the protein with 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide (HPPoSu) ester resulted in complete acylation of the amino groups. Condensation of the carboxyl groups with NH_3 in the presence of carbodiimide resulted in amidation of all the free carboxyl residues. In growth inhibition assays against *Sarcina lutea* and human leukemic cells, the bis-N-acylated NCS showed the same activity as native NCS, whereas the carboxamide derivative was inactive.

Experimental Section

Materials. Crude NCS was obtained from Kayaku Antibiotics, Itubashi-Ku, Tokyo, Japan, and was purified according to Samy et al. (1977b). EDC, HPPoSu, and constant boiling HCl for protein hydrolysis were obtained from Pierce Chemical Co., Rockford, Ill. Ampholines (LKB-1809) were purchased from LKB Productor AB, Bromma, Sweden. Other reagents of analytical grade were purchased commercially.

Amino Acid Analysis. Amino acid analysis of the protein was performed according to Spackman et al. (1958) with a Beckman amino acid analyzer, Model 121C. The protein was hydrolyzed in constant boiling HCl at 105 °C in vacuo for 20 h. The ammonia content was corrected for contamination in the diluting buffer and introduction of ammonia during acid hydrolysis. The glassware used for acid hydrolysis was rinsed with distilled-demineralized water and dried immediately before use.

Potentiometric Titration. Electrometric titrations were carried out under a nitrogen atmosphere in a 5.0-mL protein solution using a water-jacketed cell maintained at 25 °C. A Radiometer automatic titration apparatus consisting of a pH meter (Model 28), titrator (TTT11), and recorder (CSBR) was used with a Radiometer semimicro combination electrode GK 2321C. The pH meter was standardized at 25 °C against the following standard buffers, prepared according to Bates (1964): 0.05 M potassium tetroxalate, pH 1.68; 0.08 M potassium acid phthalate, pH 4.01; 0.05 M phosphate buffer, pH

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[1] Abbreviations used are: NCS, neocarzinostatin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Tris, tris(hydroxymethyl)aminomethane; CM, carboxymethyl; DEAE, diethylaminoethyl; HPPoSu, 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide; BisHPP NCS, bis[(alanine-1, lysine-20)-3-(4-hydroxyphenyl)]propionamide neocarzinostatin; carboxamide-NCS, neocarzinostatin in which all the 10 carboxyl residues are converted into carboxamide residues; CD, circular dichroic.

6.86; and saturated $Ca(OH)_2$, pH 11.7. The isoionic point of NCS was determined as 4.8 by measuring the pH of the protein solution after passage through a mixed-bed (Dowex 1–Dowex 50) resin in hydroxide–hydrogen form. The protein solution (0.04%) was placed in the cell and equilibrated for 30 min while stirring under a nitrogen atmosphere. Titrations were conducted either with 0.12 N HCl or 0.12 N KOH, both in 0.1 M KCl solution. The procedure was to titrate from the isoionic point (pH 4.8) to pH 2.0, then to pH 13.0, and finally back to pH 4.8. The pH readings were recorded after the pH had stabilized, usually within 5 min, except during titrations in the pH region of 3.0–4.0, when about 15 min was necessary for equilibration. No pH drift was noted on back-titration with KOH from pH 2.0. Blank titrations of 0.1 M KCl with 0.12 N HCl and 0.12 N KOH were carried out in the same manner. Corrections for volume changes during titrations were made.

Gel-Electrophoresis and Isoelectric Focusing Analysis. Polyacrylamide gel electrophoresis was carried out in glycine–Tris buffer, pH 8.6, according to Reisfield et al. (1962) and Weber and Osborn (1969). Isoelectric focusing of proteins was carried out in 7% polyacrylamide columns (5 mm × 55 mm). Carrier ampholytes, in a final concentration of 1.6% (w/v), were selected to give pH gradients of 2.5 to 5.0 and 3.5 to 10.5. The electrode solutions consisted of cathode, 0.5% Ampholine (pH 5–7), and anode, 1 M H_3PO_4. A constant potential of 350 V was applied for 2.5 h. Gels were washed extensively with 10% trichloroacetic acid to remove carrier ampholytes and then stained with 0.2% Coomassie blue in ethanol, water, and acetic acid (3:8:1, v/v) solution for 1 h. Destaining of the gels was done in an E-C apparatus (E-C Apparatus Co., Swarthmore, Pa.) with the same solvent mixture. To determine the pH gradient, gels without protein were sliced into 5-mm sections, triturated with a glass rod, and allowed to stand overnight in 2 mL of deionized water. The pH of the supernatant solution was measured in a Beckman Research pH meter, Model 1019.

Spectral Measurements. The CD spectral measurements were made with a Cary 60 spectropolarimeter at 20 °C under a constant nitrogen flush. The data are expressed in terms of mean residue ellipticity $[\theta]$ as described in our earlier communication (Samy et al., 1974). Protein concentration was determined spectrophotometrically at 277 nm using a measured value, ϵ_{277} = 9500 mol^{-1} cm^{-1} (corrected for light scattering).

Determination of Primary Amino Groups. The primary amino groups in the protein were determined by the following two methods: (a) spectrophotometric analysis of the protein after reaction with 2,4,6-trinitrobenzene-1-sulfonic acid in the presence of 10% sodium dodecyl sulfate at 40 °C according to the modified procedure of Habeeb (1966). The extent of modification in the protein was calculated from absorbancy at 340 nm using a mean value of 13 100 ± 400 for the ϵ_{340} of trinitrophenyl amino acids; and (b) fluorescence analysis of the protein after labeling with fluorescamine. The procedure followed was similar to that of Bohlen et al. (1973). The fluorescence of the solution was measured in a Perkin-Elmer spectrofluorometer Model MPF4 (excitation 390 nm, emission 475 nm). Since a gradual decrease in fluorescence was observed after 30 min of reaction, the fluorescence of samples was measured within 15–30 min after addition of the reagent. Qualitative identification of amino groups was also made using 5-*N*,*N*-dimethylaminonaphthalene-1-sulfonyl chloride (Woods and Wang, 1967), or 1-fluoro-2,4-dinitrobenzene (Fraenkel-Conrat et al., 1960).

Determination of Carboxamide Groups. Amide content in the protein was determined on the amino acid analyzer. The ammonia values after blank correction were expressed as the number of carboxamide groups in both native and derivatized NCS based upon lysine, arginine, aspartic acid, and glutamic acid values.

Biological Inhibitory Activities. Native NCS and the chemically modified derivatives were assayed for growth inhibitory activities against *Sarcina lutea* and human leukemic (CCRF-CEM) cells in vitro according to procedures described earlier (Foley and Lazarus, 1967; Samy et al., 1974).

Results

Modification of Amino Groups. The amino groups in NCS were acylated with HPPoSu ester according to the procedure of Bolton and Hunter (1973). NCS (10 μmol) was dissolved in 50 mL of 0.1 M borate buffer, pH 8.5, at 4 °C and three 50-μmol portions of solid HPPoSu ester were added at 30-min intervals to the protein solution with continuous stirring. After a total of 90 min, 100 μmol of glycine was added to inactivate excess acylating reagent, and the solution was stirred for an additional 30 min. The reaction mixture was lyophilized and the protein was separated from the products of the conjugation reaction, i.e., 3-(4-hydroxyphenyl)propionic acid and its glycine conjugate, by passage through a Sephadex G-25 column (1.5 cm × 100 cm) in 0.2 M NaCl. The protein fraction eluted in the void volume was dialyzed extensively against deionized water at 4 °C and lyophilized. HPPoSu-reacted NCS was dissolved in 5 mL of 0.05 M acetic acid and applied to a CM-cellulose column (0.9 cm × 25 cm) equilibrated with 0.05 M acetic acid. The column was initially washed with 45 mL of 0.05 M acetic acid and chromatography was conducted by ionic gradient elution using 45 mL each of the following buffers: 0.05 M sodium acetate–acetic acid, pH 3.3, 3.8, and 4.3. Two protein fractions were obtained. The amino modified derivative eluted unadsorbed in the 0.05 M acetic acid wash, whereas the native protein eluted in buffer at pH 3.8. The fractions containing the modified protein were pooled and lyophilized.

^3H- or ^{125}I-labeled NCS was prepared using a ^3H- or ^{125}I-labeled HPPoSu ester by the above procedure. The radioactive HPPoSu ester was prepared by (a) tritiation of the active ester in acetic acid with ^3H gas (catalytic hydrogenation with platinum as a catalyst performed for us by New England Nuclear Inc., Boston, Mass.); or (b) iodination of the active ester with ^{125}I in the presence of Chloramine-T according to the method of Bolton and Hunter (1973). The radioactive protein was purified on Sephadex G-25 and CM-cellulose columns. Specific radioactivities were 150 mCi/mmol for the tritio and 30.0 Ci/mmol for the iodo derivatives.

Modification of Carboxyl Groups. The carboxyl groups in NCS were converted to amides in the presence of EDC. NCS (10 μmol) was dissolved in 30 mL of 5.5 M NH_4Cl and the pH of the solution was adjusted to 4.75. Three 1-mmol portions of solid EDC were added to the continuously stirred solution at 1-h intervals and the pH was maintained at 4.75 by addition of 0.5 N HCl. After a total of 3 h, 3 mL of 1.0 M sodium acetate buffer (pH 4.75) was added to the reaction mixture to inactivate excess carbodiimide. Stirring was continued for an additional 15 min. After removal of the insoluble material by centrifugation (5000 rpm for 15 min), the reaction mixture was extensively dialyzed at 4 °C against five 2-L portions of 0.001 M HCl. The contents of the dialysis bag were then lyophilized. The product was passed through a Sephadex G-15 column (1.5 cm × 30 cm) and eluted with 0.001 M HCl to remove any traces of noncovalently bound ammonia. The protein-rich fractions were pooled and lyophilized. In another experiment,

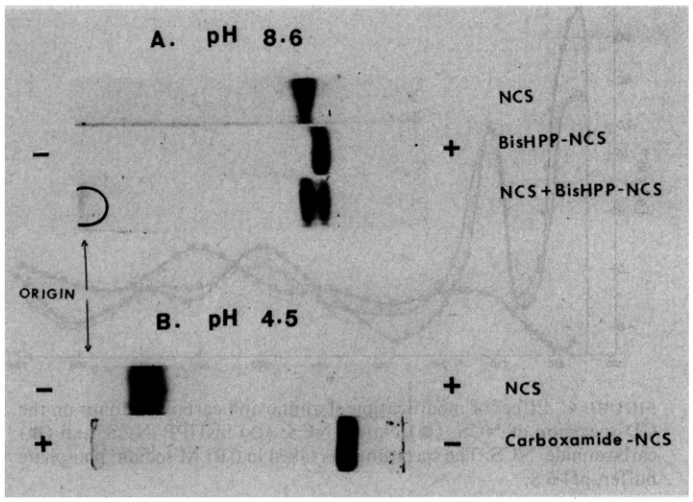

FIGURE 1: Polyacrylamide gel electrophoresis of native, bisHPP- and carboxamide-NCS. The electrode connections are reversed for carboxamide derivative at pH 4.5 (B). Time of electrophoresis was: 90 min in glycine-Tris buffer (pH 8.6); 60 min for native NCS and 10 min for carboxamide NCS in β-alanine-acetic acid buffer, pH 4.5. Protein concentration was 80 μg.

the native protein was treated similarly but without carbodiimide to obtain a control for the determination of carboxamide groups in carbodiimide-treated NCS.

The EDC-treated NCS that had been previously passed through Sephadex G-15 and dissolved in 5 mL of 0.05 M Tris-HCl buffer (pH 8.3) was put on a DEAE-Sephadex A-25 column (0.9 cm × 25 cm) and washed initially with 45 mL of the same buffer. Elution of the column was done by stepwise increase of ionic strength using 45 mL each of 0.1 M, 0.2 M, and 0.3 M NaCl. Two protein fractions were obtained under these conditions, native NCS eluting from the column in 0.3 M NaCl and the amide derivative eluting in the NaCl-free pH 8.3 wash. Fractions containing the amidated protein were dialyzed extensively against 0.001 M HCl and lyophilized.

Electrophoresis and Isoelectric Focusing of Native NCS and Its Chemically Modified Derivatives. Electrophoretic analysis of native NCS, bisHPP-NCS, and carboxamide-NCS was carried out on polyacrylamide gels and the results are shown in Figures 1A and 1B. Both bisHPP-NCS and carboxamide-NCS behaved as single fractions indicating the homogeneity of the preparations. The carboxamide derivative migrated toward the cathode in β-alanine-acetic acid buffer, pH 4.5, that is, in reversed direction compared with native NCS (Figure 1B). The charge reversal, faster mobility, and absence of multiple bands during electrophoresis indicated that the carboxamide derivative was homogeneous. On the other hand, the bisHPP-NCS (Figure 1A) migrated further toward the anode than the native protein in glycine-Tris buffer, pH 8.6, and a clear electrophoretic separation of a mixture of native NCS and bisHPP-NCS was obtained on polyacrylamide gels. The changed mobility of bisHPP-NCS was caused by the loss of charges on the protein molecule due to blocking of the free amino groups. Electrophoresis of NCS derivatives in 0.01 M phosphate buffer, pH 7.4, containing 0.1% sodium dodecyl sulfate and 0.01% β-mercaptoethanol resulted in the appearance of a single band corresponding to native NCS. This suggested no aggregation of the protein during both the reaction procedures.

The homogeneity of chemically modified preparations was also studied by analytical isoelectric focusing on polyacrylamide gel columns, with a pH gradient of 2.5–5.0 (Figure 2).

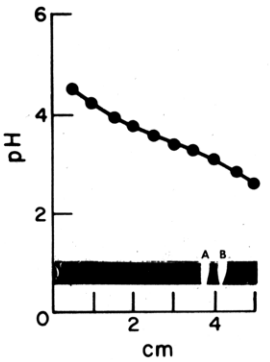

FIGURE 2: Isoelectric focusing of native NCS (A) and bisHPP-NCS (B) in acrylamide gel columns (5 mm × 55 mm). Average concentration of carrier ampholytes in the gel was 1.6% w/v, pH range 2.5–5.0. Protein concentration was 80 μg. Electrofocusing was conducted at 350 V for 2.5 h.

TABLE I: Data on the Ionizable Groups in Native NCS and Its Derivatives Determined by Potentiometric Titration.

Group	pH range	No. of ionizable groups: Native NCS	BisHPP-NCS	Carboxamide-NCS
Carboxyl	4–5	10	10	0
α and ε amino	Ca. 7 and 11	2	<1	2
Guanidyl	>12	3	3	3
Total ionizable groups based on electrometric titration		15	13	5
Phenolic[a]	13	1	1	1

[a] Determined separately by spectrophotometric titration, and the pK was calculated at 12.5 (Samy et al., 1974).

Native NCS (A) was homogeneous, with an isoelectric point (pI) around 3.3. The bisHPP-NCS (B) was also homogeneous and more acidic than native NCS, with an isoelectric point around 3.1. The isoelectric point of the carboxamide-NCS could not, however, be determined by isoelectric focusing experiments. The derivatives could not be located in polyacrylamide gels electrofocused with pH gradients of 2.5–5.0 and 3.5–10.5. The loss of amphoteric character in the carboxamide derivative strongly indicated complete amidation in the protein.

Potentiometric Titration. The electrometric titration of native NCS (Table I and Figure 3) showed that the protein contained a total of about 15 measurable net charges, which included: (a) 10 ionizable carboxyl groups titrated below pH 4.7; (b) 2 ionizable amino groups, 1 α-amino group titrated around pH 7.0, and 1 ε-amino group titrated around pH 11.0; and (c) 3 guanidyl groups which were titrated beyond pH 12.0. In a separate spectrophotometric analysis, a single phenolic group in NCS ionized around pH 13. The pK_a of this group was determined to be 12.5 (Samy et al., 1974). Thus, the total net charges amount to 16 in this protein molecule.

The acylation of the amino groups with HPPoSu ester resulted in a loss of net charge of 1 to 2 units in the protein (Table I). The potentiometric titration of bisHPP-NCS (Figure 3) showed an absence of titratable groups in the pH region 7.0 and 11.0, suggesting that both the amino groups in the protein had been modified by the acylating reagent. The titration of the carboxamide derivative showed the absence of titratable groups in the acidic region (Figure 3) with a concomitant re-

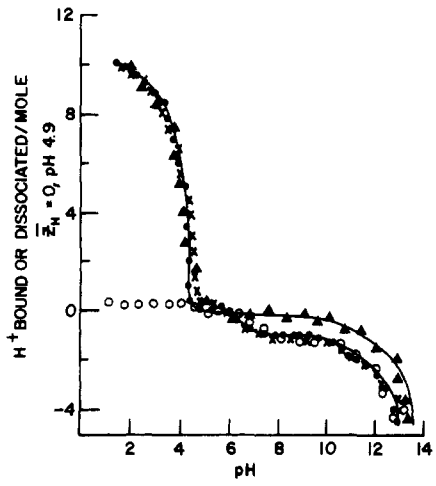

FIGURE 3: Potentiometric titration at 25 °C of native NCS (X), bisHPP-NCS (△), and carboxamide-NCS (○). (●) Represents reverse titration of native NCS. The isoionic point ($\bar{Z}_H = 0$) of native protein was determined after passing an aqueous solution of NCS through a mixed-bed (Dowex 1-Dowex 50) ion-exchange column.

TABLE II: Composition of Amino Acid and Ammonia in Native NCS and Carboxamide-NCS.

Amino acid/ammonia	Native NCS	Carboxamide-NCS
Lysine	1.0[a]	1.0
Ammonia	7.2	17.4
Arginine	2.9	3.1
Aspartic acid	10.9	11.2
Glutamic acid	5.1	5.0

[a] Expressed in residues; the average of four separate analyses. Ratios are obtained with lysine as 1.0 residue. The values for ammonia have been corrected. In the carboxamide-NCS, correction has been made for the introduction of ammonia during reaction with EDC.

duction in the total measurable net charge (ΣN^+) to 5 (Table I). These results suggested that all the carboxyl groups in NCS were amidated during condensation with ammonia in the presence of carbodiimide.

Quantitation of Amino and Carboxyl Groups. The extent of modification of the amino and carboxyl groups in NCS was also determined by qualitative and quantitative chemical analyses. Amino acid and sequence analyses of native NCS showed that it has two reactive amino groups residing in the NH_2-terminal alanine residue and the lysine residue at position 20. Both were modified when the protein was treated with HPPoSu ester, as evident from the failure of bisHPP-NCS to react with (a) 2,4,6-trinitrobenzene-1-sulfonic acid, (b) fluorescamine, (c) 1-fluoro-2,4-dinitrobenzene, and (d) 5-*N*,*N*-dimethylaminonaphthalene-1-sulfonyl chloride. The analytical results with 2,4,6-trinitrobenzene-1-sulfonic acid and fluorescamine indicated that the reaction of NCS with the acylating agent led to modification of 2 amino groups in the protein. These chemical data are in agreement with the electrometric titration results where a loss in net positive charges was observed.

Native NCS contains a total of 16 acidic amino acid residues. The amide content (expressed as ammonia in Table II) amounted to 7 residues. This leaves 10 carboxyl groups in the protein, including the COOH-terminal asparagine residue, free for modification reaction. The chemical data for available carboxyl groups are in agreement with potentiometric titration analysis. Seventeen amide groups were analyzed in carbodiimide treated NCS, indicating a complete amidation of the

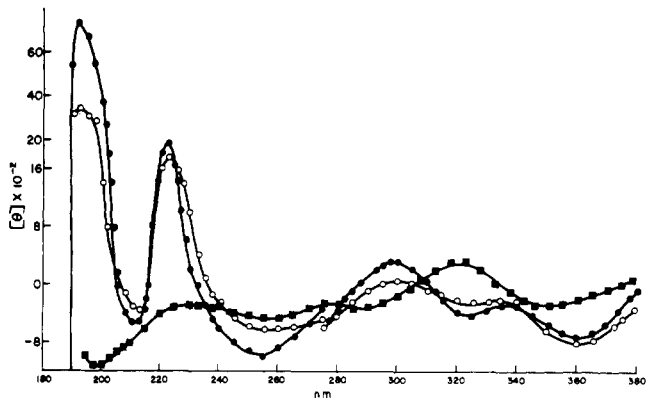

FIGURE 4: Effect of modification of amino and carboxyl groups on the CD spectrum of NCS. (●) Native NCS; (○) bisHPP-NCS; and (■) carboxamide-NCS. The spectrum was taken in 0.01 M sodium phosphate buffer, pH 6.8.

protein. The potentiometric titration (Figure 3) of the carboxamide derivative also showed the absence of any titratable group below pH 6.0 which is in agreement with the chemical data.

CD Spectra. The CD spectrum of native NCS in 0.01 M phosphate buffer, pH 6.8, is characterized by 3 positive extrema at 195, 222.5, and 295 nm and 4 negative extrema at 215, 225 (broad), 320, and 365 nm. It was inferred on the basis of CD and IR spectral properties that native NCS possesses a predominantly β-pleated conformation (Samy et al., 1974). Modification of the amino groups in NCS did not change the CD spectrum of the native protein in the near- and far-UV region (Figure 4); however, amidation of all the carboxyl groups resulted in a complete change of the CD spectrum. The CD spectrum of the carboxamide-NCS was characterized by negative extrema at 195 and 350 nm and positive extrema at 325 nm. The conversion of positive ellipticity at 197 nm to negative might indicate that the protein had lost its native conformation. A similar change in conformation, from β-pleated to unordered or random coil, was observed during denaturation of the protein with 8 M urea (Samy et al., 1974).

Biological Activities. Native NCS and the chemically modified derivatives were assayed for growth inhibitory activities against *Sarcina lutea* and human lymphoblastic leukemic (CCRF-CEM) cells in vitro. Against *Sarcina lutea*, bisHPP-NCS and native NCS were equipotent (MIC, 0.5 μg/mL). BisHPP-NCS inhibited the growth of human leukemic (CCRF-CEM) cells in vitro at an ID_{50} of 0.039 μg/mL (vs. ID_{50} 0.022 μg/mL for NCS). Tritium-labeled NCS also showed similar inhibitory activities in both bacterial and leukemia cell assays. Carboxamide-NCS inhibited the growth of *S. lutea* at an MIC of 2 μg/mL, and against human leukemic cells the ID_{50} concentration was 0.270 μg/mL (vs. 0.022 μg/mL for NCS).

Discussion

NCS is an antibiotic protein with antitumor properties (Ishida et al., 1965). The amino acid sequence and the conformation analyses of the protein were extensively studied in this laboratory (Meienhofer et al., 1972; Maeda et al., 1974a,b; Samy et al., 1974). The aim of our investigation is to establish the optimal structure-activity requirements of NCS through defined chemical modification. The absence of histidine, methionine, and free sulfhydryl groups in the protein simplifies site-specific modification of the two amino groups, the ten carboxyl groups, the single tyrosine, two tryptophan, and three

arginine residues. Disulfide bond modification (Meienhofer et al., 1972; Maeda et al., 1974b), selective tryptophan oxidation by *N*-bromosuccinimide (Samy et al., 1974), unsuccessful attempts to derivatize the buried tyrosine residue (Samy et al., 1974), and acylation of the amino groups (Kumagai et al., 1966; Maeda, 1974) have been reported before.

In the present study, reaction of the amino groups of NCS with HPPoSu ester at pH 8.5 resulted in complete acylation. The bisHPP derivative was purified by chromatography on CM-cellulose in 0.1 M acetic acid and was homogeneous in polyacrylamide gel electrophoresis at pH 8.6 and gel isoelectric focusing between pH 2.5 and 5.0. The isoelectric point of the bisacylated derivative was 3.1 as compared with 3.3 for the native protein. The native conformation of the protein did not change as a result of acylation of the amino groups. The bisacylated derivative was as active as native NCS in the inhibition of growth of *Sarcina lutea* and human leukemic cells (CCRF-CEM) in vitro. The bisacylated NCS was equipotent in antitumor assays in mice with P388 and L1210 leukemias (Samy et al., 1977a). These results clearly suggested that the two amino groups in NCS are not essential for biological activity. Application of the tritiated or ^{125}I-labeled acylating agents, 3-[3,5-^{3}H]-4-hydroxyphenyl)- or 3-([3-^{125}I]-4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester, provided biologically active radiolabeled NCS derivatives.

Preliminary reports on the effects of chemical modification of the amino groups in NCS on some biological properties were reported by Kumagai et al. (1966). Reaction of the amino groups with fluorescein isothiocyanate or conversion into hydroxyl groups by treatment with nitrous acid gave mixtures of mono- and disubstituted derivatives which were separated by chromatography on CM-cellulose. The single modification at the NH_2 terminus produced a NCS derivative which retained antitumor activity and showed decreased toxicity. Treatment of solid S180 tumors in mice with monodeaminated NCS resulted in partial inhibition of tumor growth, while the native protein did not affect solid S180 tumors at maximally tolerated doses. The disubstituted derivatives were inactive (Kumagai et al., 1966). The α-amino group in NCS was also modified by (a) quantitative removal of NH_2-terminal alanine by leucine aminopeptidase or (b) conversion of the amino function into a keto group by treatment with sodium glyoxalate (Samy, 1974). Results obtained from *S. lutea* and CCRF-CEM cell culture assays indicated that both the mono desalanyl and the des-α-amino NCS derivatives were biologically active. From these studies it was then concluded that the α-amino group of alanine was not essential for biological function of NCS whereas the ϵ-amino group of lysine was required. In recent studies (Maeda, 1974), however, a disuccinyl derivative of NCS was reported to retain most of the biological activity as measured by growth inhibition of *Sarcina lutea* and certain mammalian tumor cells in vitro.

Carbodiimide-mediated blocking of carboxyl groups has been used to investigate the functional roles of carboxyl side chains in proteins (Epand and Epand, 1972; Carraway et al., 1969; Eyl and Inagami, 1970; Lewis and Shafer, 1973). This procedure was initially developed by Hoare and Koshland (1967) for carboxyl group analysis in proteins, and Lewis and Shafer (1973) have used ammonium chloride in their EDC-mediated condensation of "exposed" aspartyl and glutamyl residues in enzymes. Amidation of the carboxyl groups of NCS in the presence of EDC at pH 4.75 resulted in quantitative conversion of all 10 carboxyl groups into carboxamides. Potential side reactions with the tyrosine hydroxyl group and to a lesser degree with the serine hydroxyls were anticipated (Abita and Lazdunski, 1969) but could not be detected in reactions with NCS. Spectrophotometric titration of the carboxamide-NCS showed the ionization of tyrosine at pH 13, and the test for the free hydroxyl of tyrosine was qualitatively positive by the dansyl procedure. Carboxamide-NCS differed from native NCS with respect to acrylamide gel electrophoresis at pH 8.6, DEAE-Sephadex chromatography, potentiometric titration, and circular dichroism. The carboxamide derivative, a chemically modified basic protein, retained some ability to inhibit the growth of *Sarcina lutea*, but in human leukemic (CCRF-CEM) cells the growth inhibitory effect was about 10% of the native protein. The loss in biological inhibitory activity may be attributable to the change in conformation of the molecule as evidenced by the change in CD spectral properties.

Ascertaining the nonfunctional role of amino groups led us to prepare a radiolabeled NCS which was biologically active. This resulted in the development of a radioimmunoassay procedure (Samy and Raso, 1976) and in tissue distribution studies of the drug in normal and tumor bearing animals (Lowenthal and Parker, 1977). NCS was covalently coupled to agarose through its nonfunctional free amino side chains and the agarose-immobilized NCS inhibited the growth of human leukemic (CCRF-CEM) cells in vitro, the ID_{50} varying from 6 to 15×10^{-9} M vs. an ID_{50} of 2.4×10^{-9} M for native protein (Lazarus et al., 1977). Since agarose-bound NCS cannot be expected to penetrate into the leukemic cell because of its size, (agarose bead size 75–150 μm as compared with 10–15 μm for the size of the leukemic cell), it is suggested that NCS can induce cytotoxicity by binding to or reacting with receptor(s) on the cell surface. Macromomycin, another antibiotic protein with similar antitumor properties, is also reported to possess similar surface-active properties to induce cytotoxicity (Kunimoto et al., 1972). Generally the cytotoxic agents used in cancer therapy either interact with DNA directly or induce cytotoxicity by intracellular interactions leading to inhibition of nucleic acid or protein synthesis (Maugh, 1974). NCS and macromomycin appear to have novel mode of action, unlike that of most other antitumor agents, and are, therefore, of considerable theoretical as well as practical interest.

In an earlier study (Samy and Raso, 1976) we observed that substitution of the amino groups resulted in a decrease of antigenicity of the protein. This decrease in antigenicity with retention of biological potency may be of clinical significance. If patients undergoing prolonged NCS treatment should show gradually diminished response to the drug because of the production of NCS antibodies, availability of an active, less antigenic derivative would be helpful.

In summary, NCS is a tightly folded acidic protein exhibiting antitumor activity. Modification of all the carboxyl groups in the protein resulted in a change in conformation and loss of biological activity. On the other hand, NCS retained its native conformation after complete acylation of its two amino groups, and biological activity was undiminished indicating that the amino groups are probably not required for biological activity. A radiolabeled NCS was prepared using a radiolabeled acylating ester. The availability of a radiolabeled and biologically active protein has been useful in investigating the mode of action of NCS in leukemic cells and in clinical and experimental pharmacology studies.

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