Selective Tryptophan Oxidation in the Antitumor Protein Neocarzinostatin and Effects on Conformation and Biological Activity†

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ABSTRACT: Neocarzinostatin, an antitumor protein of known amino acid sequence, was chemically modified by *N*-bromosuccinimide at its tryptophan residues in a study on correlations of structure, biological activity, and conformation. The protein (mol wt *ca.* 11,500) contains two tryptophan residues in positions 46 and 79 and one buried tyrosine residue at position 32. Completely selective oxidation of the 79-tryptophan residue was observed when neocarzinostatin was titrated with *N*-bromosuccinimide in 70% acetic acid or in 0.1 M sodium phosphate buffer at pH 6.1. Treatment with *N*-bromosuccinimide in 8 M urea–acetic acid buffer (pH 4.0) oxidized both the tryptophan residues. The single tyrosine residue was not affected. The monotryptophan-79 oxidized neocarzinostatin retained full biological activities, *i.e. in vitro* antibacterial potency and antileukemic activity in CCRF-CEM cells, indicating that tryptophan in position 79 does not play a role in the biological effects of neocarzinostatin. The bistryptophan-46,79 oxidized product was totally inactive. The circular dichroic (CD) spectrum of native neocarzinostatin in phosphate buffer at pH 6.1 was characterized by three positive extrema at 195, 222.5, and 295 nm and four negative extrema at 212.5, 255, 320, and 365 nm. The CD spectrum did not resemble those elicited by proteins in which either α-helical or random conformation predominate. An unusual and significant aspect of the spectrum is the presence of a positive peak at 222.5 nm in the far-ultraviolet region. The infrared spectrum for amide I and II regions in D_2O indicated the protein to contain antiparallel β-pleated sheet conformation. The circular dichroic properties of native and both mono- and bis-oxidized neocarzinostatin were compared. Selective oxidation of the tryptophan-79 residue did not result in any conformational change, but oxidation of both the tryptophan residues resulted in a change from β-pleated to random coil conformation. Native neocarzinostatin possesses a tightly folded conformation which is stable at acidic and at alkaline pH below 12, as well as in the presence of 1% sodium dodecyl sulfate, as shown by studies of circular dichroism in the far-ultraviolet region. The tyrosine-32 and tryptophan-46 residues are buried. Tight folding of the protein is in accord with previously observed resistance of the two disulfide bonds to reduction in aqueous medium and of lysyl and arginyl peptide bonds to tryptic digestion. The pK of the tyrosine hydroxyl was determined as 12.5. At pH 12.46 unfolding of neocarzinostatin occurs as indicated by changes in circular dichroism at 210 and 240–260 nm and by the time lag in attaining equilibrium during ultraviolet absorption measurements. Denaturation by 8 M urea (pH 4.0) was completely reversible.

Several proteins with antitumor activity have recently been isolated from the culture filtrates of various microorganisms of the genus *Streptomyces*, including actinoxanthin from *S. globisporus* (Vichrova *et al.*, 1957), carzinostatin from *S. carzinostaticus* (Ishida *et al.*, 1960), macromomycin from *S. macromomyceticus* (Chimura *et al.*, 1968), and mitomalcin from *S. malayensis* (McBride *et al.*, 1969). Ranging in molecular weights between 10,000 and 15,000 daltons these are relatively small proteins. On the basis of their antibacterial activity and biosynthetic origin they are genuine antibiotics, and by far the largest molecules presently known in that category. The most thoroughly studied antitumor protein is neocarzinostatin of known amino acid sequence (Meienhofer *et al.*, 1972b) which is produced by the F-41 variant of *Streptomyces carzinostaticus*. Its isolation, purification, chemical characterization, biological activity, antitumor potency, and studies on its mechanism of action have been described (Ishida *et al.*, 1965; Maeda *et al.*, 1966; Kumagai *et al.*, 1966; Ono *et al.*, 1966, 1968). Strong interest in the potential of neocarzinostatin for clinical cancer chemotherapy is based (i) on the relatively mild toxic manifestations of the protein in humans during limited preliminary clinical trials (Takahashi *et al.*, 1969) and (ii) on observations of considerable enhancement of therapeutic index by deamination or acylation of the NH_2-terminal alanine residue (Kumagai *et al.*, 1967).

Neocarzinostatin exhibited potent cytostatic activity both *in vitro* and *in vivo* (Ishida *et al.*, 1965). Antibiotic activity against various Gram-positive organisms was shown at minimum inhibitory concentrations (MIC) of 2 μg/ml for *Sarcina lutea* and 8–16 μg/ml for *Staphylococcus aureus* (Ishida *et al.*, 1965). Highly purified material inhibited CCRF-CEM leukemic cells in culture to 50% (ID_{50}) at dose levels of 0.02–0.03 μg/ml. Studies on the mechanism of action indicated that neocarzinostatin interferes with DNA synthesis and, independently, causes DNA degradation (Ono *et al.*, 1966, 1968). The protein was shown to be highly effective against several experimental tumors in mice including leukemia L-

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TABLE I: Amino Acid Composition (in residues) of Native, Monotryptophan-79 Oxidized and Bistryptophan-46,79 Oxidized Neocarzinostatin.[a]

Amino Acid	Native Neocarzinostatin	Monotryptophan-79 Oxidized	Bistryptophan-46,79 Oxidized
Lys	1.0	0.9	1.0
Arg	3.1	2.8	3.2
Asp	11.1	10.9	11.2
Thr	12.2	12.1	11.8
Ser	10.1	9.8	10.2
Glu	5.4	5.2	5.0
Pro	4.2	4.3	4.3
Gly	15.0	15.3	15.1
Ala	16.9	17.2	16.8
Cys(O_3H)	3.6	3.8	3.6
Val	12.2	11.8	12.1
Ile	1.0	1.0	1.0
Leu	6.1	5.9	6.2
Phe	5.1	5.0	4.9
Tyr	0.9	1.0	0.9
Trp[b]	2.0	1.0	0.0

[a] Analyses were conducted on a Beckman Model 121 H instrument, after hydrolysis of samples in 6 N HCl for 22 hr at 105° (Spackman *et al.*, 1958). [b] Determined by *N*-bromosuccinimide titration.

1210 (Bradner and Hutchison, 1966), ascitic sarcoma 180, and leukemia SN-36 (Ishida *et al.*, 1965). In phase I clinical studies, promising results have been obtained in the treatment of tumors of the rectum and stomach (Takahashi *et al.*, 1969) and of bladder and penile carcinoma (K. Matsumoto and K. Nakauchi, 1969, personal communication).

We are engaged in chemical modification of neocarzinostatin for two purposes: (i) attempts to arrive at structure–activity correlations by comparing effects of site-specific alterations on conformation and biological activity and (ii) efforts to prepare derivatives or analogs with improved chemotherapeutic properties, *i.e.* enhanced antitumor activity. Although neocarzinostatin has a particularly high content of alanine, glycine, serine, and threonine, it contains a number of residues that may readily be modified by site-specific reagents, *i.e.* one tyrosine, two tryptophan, one lysine, three arginine, and four half-cystine residues (Meienhofer *et al.*, 1972b). In this article we wish to report in detail about selective oxidation of one or both tryptophan residues, respectively, by *N*-bromosuccinimide and the changes caused by these modifications on the biological activity of native neocarzinostatin and on optical rotatory dispersion (ORD), circular dichroism (CD), and infrared (ir) spectra. The conformation of highly purified native neocarzinostatin was also studied in some detail, and the results are discussed in comparison with previous reports (Maeda and Ishida, 1967; Maeda *et al.*, 1973).

Experimental Procedures

Materials. Reagents were obtained commercially and were of analytical grade. *N*-Bromosuccinimide was recrystallized from warm water and had mp 173°. Urea (Fisher) was acidified and recrystallized twice from 50% aqueous ethanol in deionized water.

Preparation of Neocarzinostatin. Crude neocarzinostatin (lot T55-S11) was obtained from Kayaku Antibiotics Research Laboratory, Tokyo, Japan. It was purified by dialysis followed by chromatography on CM-cellulose through elution with 0.1 M sodium acetate buffer applying a pH gradient from 3.0 to 4.5, and by Sephadex G-50 gel filtration in 0.1 M acetic acid (Meienhofer *et al.*, 1972a). An additional purification step, chromatography on DEAE-Sephadex A-25, in 0.05 M Tris-HCl buffer (pH 8.0) with a NaCl gradient from 0 to 0.5 M, resulted in a highly purified preparation, which was homogeneous on acrylamide gel electrophoresis at pH 8.0, 4.5, and 2.9. Amino acid analysis (Spackman *et al.*, 1958) gave data (Table I, column 1) in accord with the proposed structure (Meienhofer *et al.*, 1972b). Carbohydrate analysis by the orcinol-H_2SO_4 reaction (Winzler, 1955) shows the absence of covalently bound carbohydrate. Highly purified neocarzinostatin inhibited the growth of *Sarcina lutea* cells at a concentration of 0.05 μg/ml in the agar diffusion plate test. The ID_{50} value for inhibition of CCRF-CEM tumor cells was 0.025 μg/ml.

Spectral Measurements. Both ORD and CD measurements were made with a Cary 60 spectrophotometer at 20° under a constant nitrogen flush. The CD instrument was calibrated with an aqueous solution of *d*-10-camphorsulfonic acid. The data are expressed in terms of mean residue ellipticity, $[\theta]$, for CD and mean residue rotation, $[m]$, for ORD as deg cm^2 dmol^{-1}. The mean residue weight of neocarzinostatin was calculated at 99.14 from its amino acid composition. The protein concentration was determined spectrophotometrically at 277 nm using a measured value, $\epsilon_{277} = 9500$ mol^{-1} cm^{-1} (corrected for light scattering).

Infrared measurements were carried out in D_2O solutions with a Perkin-Elmer spectrophotometer, Model 521, using cells of 0.05-mm path length with CaF_2 windows. The path length of the reference cell was adjusted to obtain a fairly straight reference line with the solvent, D_2O.

Ultraviolet absorbancy measurements were made at 21° either with a Zeiss PMQII or, when necessary, with a Cary Model 14 spectrophotometer using 1-cm quartz cells. In a typical experiment, 2.5 ml of buffer of appropriate pH was pipetted into a cell and 25 μl of 55 mg/ml of neocarzinostatin stock solution was added and mixed with a polyethylene rod, and the absorbance was read against the appropriate buffer. Equilibrium was generally reached within 1 min and the absorbance did not change over the approximate 5 min of measurement. An exception, however, was observed at pH 12.46, where a delay of 30 min was necessary to obtain a constant absorbancy. Absorbance readings were made at 252 and 290 nm wavelengths, respectively, of the difference spectrum of ionized (pH 13.19) *vs.* nonionized (pH 7.08) tyrosine hydroxyl groups (Figure 1). However, for purposes of determination of p*K*, the absorbance at 252 nm was taken. For evaluation of the difference curve and theoretical curve during p*K* determination according to Linderstrøm-Lang (1924), the absorbance of a protein solution at pH 7.08, the low reference, was subtracted from the absorbance of protein solutions at high pH values.

N-Bromosuccinimide Oxidation. Oxidation by *N*-bromosuccinimide was performed according to the method of Patchornik *et al.* (1958; see also Witkop, 1961). Titrations were conducted by adding carefully measured increments of *N*-bromosuccinimide to neocarzinostatin solutions in 0.1 M phosphate buffer (pH 6.1), in 70% acetic acid, and in 8 M urea–acetic acid buffer (pH 4.1) and monitored spectrophotometrically at 280 nm.

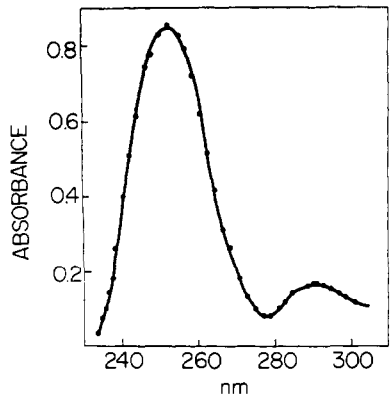

FIGURE 1: Ultraviolet difference spectrum of native neocarzinostatin (3.6×10^{-4} M), at pH 13.19 *vs.* pH 7.08, 21°.

Buffers. Buffer solutions of pH 7.08, 8.26, 9.06, 10.15, 10.96, 11.75, 12.46, and 13.19 were used for spectrophotometric titrations. The buffers were prepared from a stock solution of 0.5 M KH_2PO_4. After appropriate adjustment of pH with 5 M KOH using a Beckman Research pH meter Model 1019 solutions were diluted to 0.2 M with deionized water. Aliquots of 4 M KCl were added wherever necessary before the dilution to maintain a uniform K^+ concentration. The calibration buffers used were those recommended by Bates (1954): pH 4.01, phthalate; pH 6.85, sodium phosphate; and pH 11.69, saturated $Ca(OH)_2$.

NH_2-Terminal amiuo acids were determined using 5-dimethylaminonaphthalene-1-sulfonyl chloride (Woods and Wang, 1967) or 1-fluoro-2,4-dinitrobenzene (Sanger, 1945).

Biological Activities. Native neocarzinostatin and the derivatives were assayed for both antibacterial and antileukemic activities. Bacteriostatic potency against *Sarcina lutea* was assayed on agar gel diffusion plates. Minimal inhibitory concentration was determined by measuring the diameters of inhibition zones and comparing with those obtained by a standard preparation using semilogarithmic graph paper. The log of neocarzinostatin concentrations exhibited linear relationship with diameters (millimeters) up to a concentration of 100 μg/ml. Antileukemic activitity was measured *in vitro* using continuous suspension cultures of human leukemic lymphoblasts (CCRF-CEM cells) and ID_{50} values were determined according to Foley and Lazarus (1967).

Results

ORD, CD, and Ir Spectra of Neocarzinostatin. The ORD and CD spectra of native neocarzinostatin in 0.01 M sodium phosphate buffer (pH 6.1) are given in Figure 2. The ORD spectrum was characterized by negative extrema at 217.5 and 275 nm and a positive extremum at 230 nm. The circular dichroic spectrum was characterized by three positive extrema at 195, 222.5, and 295 nm and four negative extrema at 212.5, 255 (broad), 320, and 365 nm, and did not resemble spectra typical for either α-helical or random coil conformation. Unusual but significant aspects were: (a) the presence of a positive peak at 222.5 nm and (b) relatively low ellipticity values (θ) throughout the far-ultraviolet region, as compared to values reported for other globular proteins.

The infrared absorption spectrum of native neocarzinostatin in D_2O solution, in amide I and amide II regions (Figure 3), showed a strong absorption for amide I around 1635 cm^{-1}, a much weaker one close to 1685 cm^{-1}, and a strong amide II frequency at 1530 cm^{-1}. Polypeptides and proteins possessing

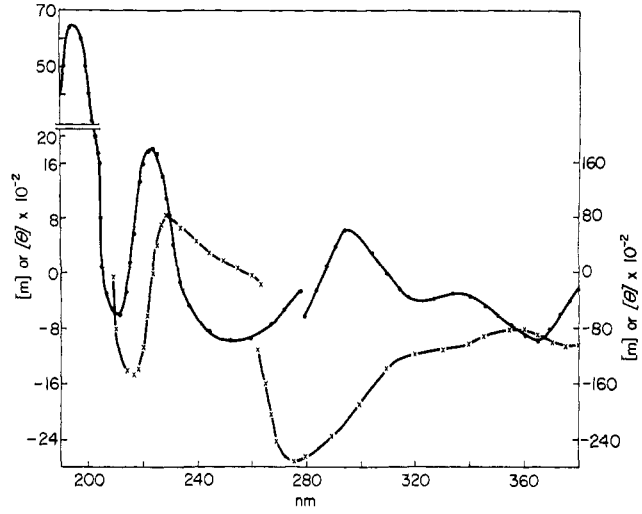

FIGURE 2: Optical rotatory dispersion (×) and circular dichroic (●) spectra of native neocarzinostatin in 0.01 M phosphate buffer (pH 6.1). The spectrum was taken in 10-, 2-, and 0.5-mm quartz cells. The ordinate scale for the long-wavelength region (260–380 nm for ORD and 280–380 nm for CD) is on the right, while the scale for shorter wavelengths is on the left.

antiparallel chain pleated sheet conformation (β structure) exhibited similar amide I and amide II spectral characteristics (Miyazawa, 1962).

The conformation of synthetic polyamino acids has been established by circular dichroic studies in the far-ultraviolet region and successful attempts have been made to correlate CD data with the conformation of native proteins. Conformational transitions caused by pH changes or addition of denaturing agents to the medium are reflected by spectral transitions in this region. The circular dichroic spectra of neocarzinostatin in the 190–240-nm region, taken in buffers at pH 2.2, 6.1, 10.7, and 12.46 and in 1% sodium dodecyl sulfate (pH 8.4), are shown in Figure 4. Changes in pH or the presence of 1% sodium dodecyl sulfate did not affect the spectra to a significant degree, except at pH 12.46, where shifts occurred both in the side-chain region for tyrosine (240–260 nm) and in the region where its peptide bond contributes (around 210 nm). The presence of 8 M urea–acetic acid at pH 4.0 resulted in a complete change of the CD spectrum in the near-ultraviolet region (Figure 5). In the far-ultraviolet region the spec-

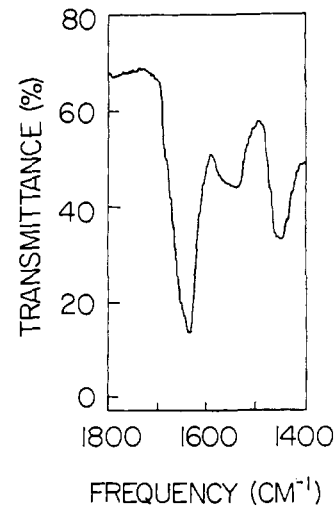

FIGURE 3: Infrared spectrum of native neocarzinostatin in D_2O.

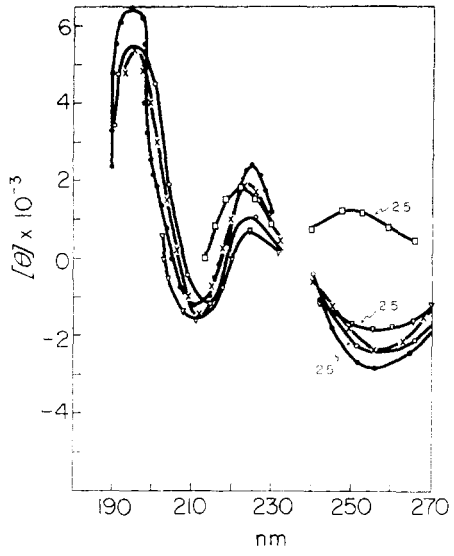

FIGURE 4: Circular dichroic spectra (190–270 nm) of neocarzinostatin (1.2×10^{-5} M) as a function of pH and 1% sodium dodecyl sulfate as indicated: (●) native protein in 0.01 M sodium phosphate buffer (pH 6.1); (○) 0.01 M sodium formate–formic acid buffer (pH 2.2); (×) 0.01 M sodium borate buffer (pH 10.7); (□) 0.01 M potassium phosphate–KOH buffer (pH 12.46); (△) 1% sodium dodecyl sulfate (pH 8.4).

trum could not be recorded because of the high optical density of urea. Subsequent removal of urea either by dialysis or by molecular exclusion chromatography on Sephadex G-10 resulted in the return of the CD spectrum characteristic of native neocarzinostatin. Absence of a conformational change in the peptide region of CD spectrum due to changes in pH (below pH 12) or to the presence of 1% sodium dodecyl sulfate and the complete reversibility of CD after urea denaturation indicated that the protein possesses a tightly folded conformation.

Solvent perturbation effects on the circular dichroism of neocarzinostatin were, however, evident in the near-ultraviolet and visible regions as shown in Figures 4 and 6. No wavelength shift in the negative peak at 255 nm was observed, within the expected range of experimental error, at pH 2.2, 6.1, and 10.7, although there was a change in the degree of ellipticity. At pH 12.46 a broad positive extremum centered around 255 nm was observed instead of a normal negative peak. This change may presumably be due to ionization of the

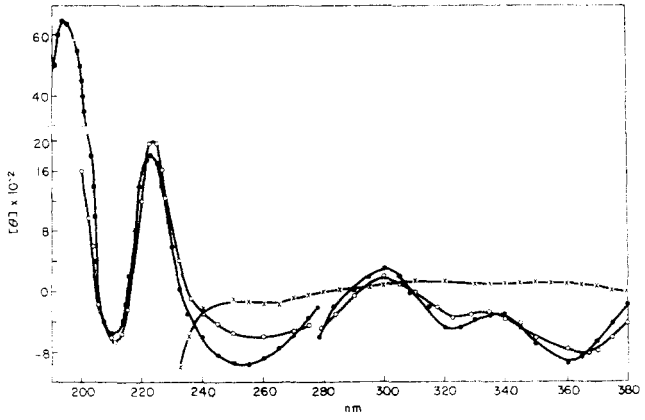

FIGURE 5: Effect of urea on the CD spectrum of neocarzinostatin: (●) native protein in 0.01 M sodium phosphate buffer (pH 6.1); (×) neocarzinostatin in 8 M urea–acetic acid buffer (pH 4.0); (○) protein after removal of urea by chromatography on Sephadex G-10.

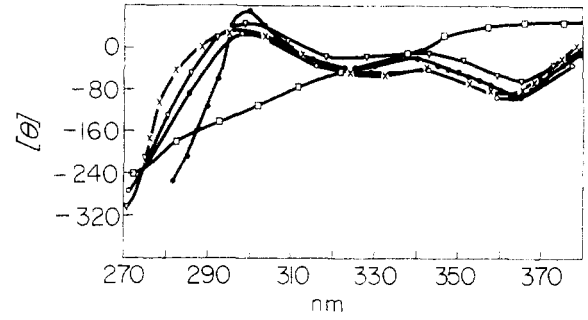

FIGURE 6: Circular dichroic spectra (270–380 nm) of neocarzinostatin (1.2×10^{-5} M) as a function of pH and 1% sodium dodecyl sulfate as indicated: (●) native protein in 0.01 M sodium phosphate buffer (pH 6.1); (○) sodium formate–formic acid (pH 2.2); (×) 0.01 M sodium borate buffer (pH 10.7); (□) 0.01 M potassium phosphate–KOH buffer (pH 12.46); △ 1% sodium dodecyl sulfate (pH 8.4).

single tyrosine. Once again, no appreciable effect was seen in the presence of 1% sodium dodecyl sulfate. In 8 M urea, at pH 4.0, a complete loss of dichroic absorption at 298 (positive) and 364 nm was observed (see Figure 5).

N-Bromosuccinimide Oxidation of Tryptophan Residues. Oxidation of neocarzinostatin with *N*-bromosuccinimide, in 70% acetic acid, in 0.1 M phosphate buffer (pH 6.1) and in 8 M urea–acetic acid buffer (pH 4.0), was monitored spectrophotometrically. The addition of *N*-bromosuccinimide in small increments led to a proportional and instantaneous decrease in tryptophan UV absorption at 280 nm, as shown in Figure 7. When reactions were performed either in 70% acetic acid or in 0.1 M phosphate buffer (pH 6.1) only one tryptophan residue was oxidized with 1.3 mol of *N*-bromosuccinimide. However, in 8 M urea–acetic acid buffer (pH 4.0) both the tryptophan residues were oxidized, and consumption of *N*-bromosuccinimide amounted to 2.8 mol. New NH_2-terminal amino acids were identified by both the dansyl[1] and the N_2ph-F methods, and the results are presented in Table II. *N*-Bromosuccinimide oxidation of one tryptophan residue, in 70% acetic acid or in 0.1 M phosphate buffer (pH 6.1), resulted in the release of glycine as the new NH_2-terminal amino acid. This identified the tryptophan residue which was selectively oxidized, under nondenaturing conditions, as that in position 79 of the primary structure. When *N*-bromosuccinimide oxidation was carried out in the presence of 8 M urea (pH 4.0) aspartic acid and glycine were obtained as new NH_2-terminal amino acids, showing that in the denatured state both the tryptophyl–glycyl bond (positions 79 and 80) and the tryptophyl–aspartyl bond (positions 46 and 47) of neocarzinostatin were cleaved. These findings provided independent confirmation of the sequence positions 46,47 and 79,80 in the proposed amino acid sequence of neocarzinostatin (Meienhofer *et al.*, 1972b). Attempts were made to isolate fragments, after cleavage of the two peptide bonds upon oxidation with *N*-bromosuccinimide. Column chromatography on Sephadex G-50 in 0.01 M ammonia or two-dimensional paper chromatography (*n*-butyl alcohol–acetic acid–water, 4:1:5) and electrophoresis (1, 500 V, 1.5 hr in formic acid buffer, pH 2.8) of *N*-bromosuccinimide-treated neocarzinostatin did not indicate fragmentation. Presumably, the two disulfide bonds of neocarzinostatin might connect half-cystine residues 37 with 84 and 56 with 89, but this assignment still requires further confirmatory work.

The *N*-bromosuccinimide-oxidized preparations were as-

[1] Abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; N_2ph-F, 1-fluoro-2,4-dinitrobenzene.

TABLE II: Analytical Data on Tryptophan Oxidation in Neocarzinostatin.

Reaction Medium	Trp Oxidized	New NH_2-Terminal Amino Acid	Position of Oxidized Trp	Biological Activity: *S. lutea* MIC, (μg/ml)[a]	Biological Activity: CCRF-CEM Cells ID_{50} (μg/ml)[b]
Control[c]	0			0.5	0.022
0.1 M phosphate buffer (pH 6.1)	1	Gly-80	79	0.5	0.025
70% acetic acid	1	Gly-80	79	0.5	0.025
8 M urea–acetic acid buffer (pH 4.0)	2	Asp-47, Gly-80	46 and 79	>200	>2.00

[a] MIC, minimum inhibitory concentration; minimum concentration required for the appearance of zone of inhibition. [b] ID_{50} concentration which caused 50% inhibition of growth. [c] Native neocarzinostatin in deionized water.

sayed for inhibitory activity against *Sarcina lutea* and CCRF-CEM leukemic cells in culture. Selective oxidation of the tryptophan-79 did not affect these activities; see Table II. However, a complete loss of biological activity in these systems was observed when both tryptophan residues in positions 46 and 79 were oxidized.

Amino acid analysis (Table I) showed that the single tyrosine residue in position 32 of neocarzinostatin was not oxidized by *N*-bromosuccinimide under the different reaction conditions described above. This led us to examine whether tyrosine was accessible to hydroxyl ions or not. Spectrophotometric titration of tyrosine residues in proteins has been extensively employed for the determination of "exposed" and "buried" tyrosine residues (Wetlaufer, 1962; Kronman and Robbins, 1970). The ionization constant (pK) of this tyrosine residue, determined according to Linderstrøm-Lang (Figure 8), was ~12.5. This indicated a buried state of the tyrosine residue and is consistent with the CD data shown in Figure 4, where the appearance of a positive CD peak at ~250 nm was noted. This peak, characteristic of ionized tyrosyl side chains, would appear at a pH near 10 for normal tyrosine residues in proteins (Beychok, 1966; Pflumm and Beychok, 1969; Simons *et al.*, 1969), but is displaced in this case to a higher pH because the residue is buried and still inaccessible at a pH below 12.

The effect of tryptophan oxidation on the CD spectrum of neocarzinostatin in the far-ultraviolet region is given in Figure 9. Oxidation of the tryptophan-79 residue did not appreciably change the circular dichroism spectrum of the native protein. Oxidation of both the tryptophan residues at positions 46 and 79 resulted in a complete change of the CD spectrum which became similar to those typical for random coil conformation, with a negative extremum around 198 nm.

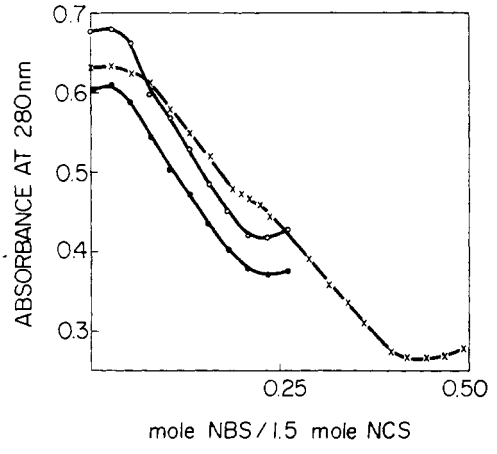

FIGURE 7: Ultraviolet spectrophotometric monitoring (decrease of OD 280) of *N*-bromosuccinimide oxidation of neocarzinostatin: (●) in 70% acetic acid; (○) in 0.1 M phosphate buffer (pH 6.1); (×) and in 8 M urea–acetic acid buffer (pH 4.0). Aliquots (10 μl) of *N*-bromosuccinimide (2.8×10^{-3} M) in deionized water were added to protein solution (5.0×10^{-5} M).

Discussion

Neocarzinostatin is the first antitumor protein for which an amino acid sequence has been proposed (Meienhofer *et al.*, 1972b) and it has considerable promise as a clinically useful cancer chemotherapeutic agent (Takahashi *et al.*, 1969). It appeared to be of particular interest (a) to determine whether the entire intact molecule is required for full biological activity, and (b) to search for neocarzinostatin analogs or derivatives with improved cancer chemotherapeutic properties. A promising lead has been the observation of decreased toxicity and retention of high antitumor activity in mice after deamination or acylation of neocarzinostatin at the NH_2 terminal (Kumagai *et al.*, 1967). The present report on selective oxidation of tryptophan residues represents a first approach toward establishment of correlations between the chemical structure and biological activity of neocarzinostatin.

Another interesting task will be the determination of structure–conformation correlations. In this context we observed during our work on the sequence analysis of neocarzinostatin a quite unusual resistance of the two disulfide bonds to reduction in aqueous solution (Meienhofer *et al.*, 1972a), even in the presence of denaturing agents, after heat denaturation, or at elevated temperature. This led to the use of dithiothreitol in liquid ammonia (Meienhofer, *et al.*, 1971). It was suspected that this chemical inertia and the previously reported resistance of the native protein to digestion by trypsin (Maeda *et al.*, 1966) were due to an extremely tight folding of neocarzinostatin.

Conformation of Neocarzinostatin. The α-helical conformation of proteins in solution is characterized by a positive dichroic maximum at about 190 nm and by well-defined nega-

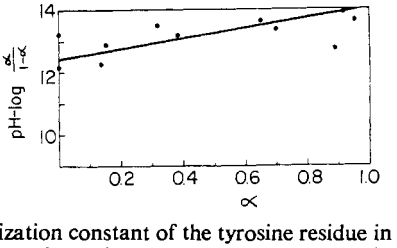

FIGURE 8: Ionization constant of the tyrosine residue in neocarzinostatin. pK_{int} was determined according to the equation of Linderstrøm-Lang (1924): $pH - \log \alpha/(1-\alpha) = pK_{int} - 0.868w\alpha$.

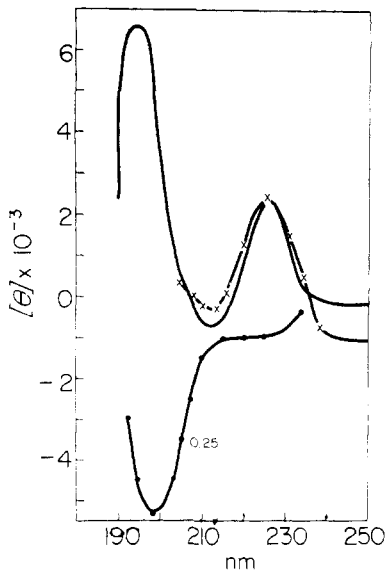

FIGURE 9: Effect of tryptophan oxidation on the circular dichroic spectra of neocarzinostatin in the far-ultraviolet region. The spectrum was taken in 0.01 M phosphate buffer (pH 6.1): (-) native neocarzinostatin; (×) monotryptophan-79 oxidized neocarzinostatin; (●) bistryptophan-46,79 oxidized neocarzinostain.

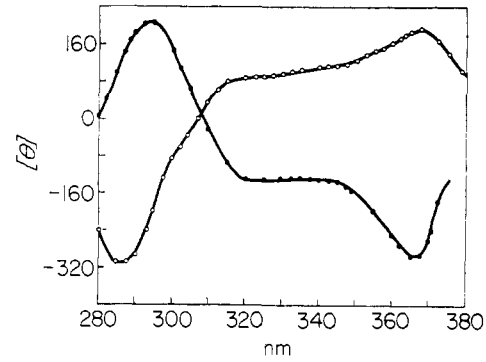

FIGURE 10: Circular dichroic spectra showing individual tryptophan contributions in the region 280–300 nm: (●) monotryptophan-79 oxidized neocarzinostatin exhibiting contribution from tryptophan-46; (○) difference spectrum (obtained by subtracting the above spectrum from that of native neocarzinostatin) containing a contribution from tryptophan-79.

tive extrema at 208–209 and 220–222 nm. The pleated sheet or β conformation has a spectrum with a positive extremum at 195 nm and a negative extremum at about 217 nm. A random coil conformation shows a spectrum with a large minimum around 197 nm, and a weak positive maximum around 218 nm. The Cotton effects and the circular dichroic spectra of neocarzinostatin in the far-ultraviolet region clearly demonstrated that the native protein did not possess helical regions or unordered random coil conformation. Evidence for the presence of a β-pleated segment was provided by infrared spectral studies. Neocarzinostatin exhibited a strong absorption at 1636 cm^{-1} and a weak peak at 1685 cm^{-1} for amide I C=O stretching frequencies. These spectral characteristics are in good agreement with data in the literature on amide I absorption for antiparallel β-pleated structure (Susi *et al.*, 1967; Timasheff *et al.*, 1967). A strong absorption obtained here for amide II frequency at 1530 cm^{-1} also indicated a β-pleated conformation. Since spectral measurements were done in D_2O solutions, the bending mode of H–O–D is likely to overlap the amide II frequency. Maeda *et al.* (1973) have come to similar conclusions in infrared studies using oriented films of neocarzinostatin.

Attempts were made to calculate contributions of α-helical, β-pleated, and randomly coiled segments toward the conformation of neocarzinostatin using, at 3-nm intervals, parameters published by Greenfield and Fasman (1969) and Chen *et al.* (1972). This approach failed because (i) the mole fraction values obtained did not add to unity and (ii) the values of the fractions of helix ($f_h x_h$) were always negative.

The positive CD band exhibited by neocarzinostatin at 222.5 nm is not characteristic of any known polypeptide structure. However, aromatic chromophores and strained disulfides could make strong contributions to this band. In view of the infrared spectral evidence for β structure, the circular dichroism might be interpreted as a superposition of a β-type spectrum from the peptide backbone and the positive 222.5-nm band from side-chain chromophores.

The tertiary structure of neocarzinostatin in aqueous solution is very stable, as shown by the circular dichroic studies in the far-ultraviolet regions; see Figure 4. It remained stable in acidic medium (pH 2.22) and in basic solution below pH 12 as well as in the presence of 1% sodium dodecyl sulfate. Furthermore, the large spectral change observed in 8 M urea solution at pH 4.0 was readily reversed by the removal of urea and the native conformation was fully restored (see Figure 5). The renatured protein possessed the full biological activity of native neocarzinostatin. This stability of the native conformation was probably one reason for the resistance of neocarzinostatin to trypsin hydrolysis and disulfide bond reduction in aqueous media (Meienhofer *et al.*, 1972a).

Contributions of Chromophores to CD Spectra. The chromophores which might contribute to the CD spectrum of neocarzinostatin in the 250–390-nm region are: one tyrosine, two tryptophan, and five phenylalanine residues and the two disulfide bridges. An unusual feature of the CD spectrum of native neocarzinostatin in this region was the presence of a weak but broad band at 365 nm, not previously observed (Maeda *et al.*, 1973). This band was unaffected by the presence of 1% sodium dodecyl sulfate or by pH variations to 2.2 or 10.5. An increase in pH to 12.46 resulted in a change of ellipticity. We do not as yet have evidence for the presence of any chromophoric prosthetic group in the native protein and assume the 365-nm band to arise from the known chromophores of neocarzinostatin [perhaps the disulfides since ultraviolet and visible absorption spectra showed a shoulder at 360 nm with an E_{360} of ~1800 mol^{-1} cm^{-1} after correction for light scattering; see Yamabe *et al.* (1971)].

Another positive circular dichroic band at 297 nm was also unique for neocarzinostatin. *N*-Bromosuccinimide oxidation of the tryptophan-79 residue resulted in a doubling of ellipticity at 365 nm and a large increase at 297 nm. Oxidation of both the tryptophan residues resulted in the disappearance of these circular dichroic bands. This suggested that the tryptophan residues might contribute to the circular dichroism at these wavelengths. Evidence for this was obtained by comparing CD spectra, which exhibited the individual contributions of each of the two tryptophan residues. The spectrum of monotryptophan-79 oxidized neocarzinostatin (Figure 10, closed circles) contains the contribution of the intact tryptophan at position 46. By subtracting this spectrum from that of native neocarzinostatin a difference spectrum was obtained (Figure 10, open circles) containing the tryptophan-79 contribution. This difference spectrum was almost a "mirror image" of the spectrum where residue 46 is the main contributor. This suggested that the location of the tryptophan residues in native neocarzinostatin might favor interactions

with other chromophores that are strong and mutually opposite in direction to each other.

The CD spectrum of neocarzinostatin, in which the disulfide bridges have been reduced and the thiols carboxymethylated, showed no optical activity in the 240–290-nm region suggesting that the main contribution to the 255-nm band of native neocarzinostatin might arise from the disulfide bridges or that the molecule is denatured. Further evidence for such a contribution was seen in the ORD of native neocarzinostatin (Figure 2), where a strong Cotton effect was observed at 270 nm (see Coleman and Blout, 1968).

Although the single tyrosine residue might be buried, a contribution toward the 255-nm CD band is not ruled out, since the increase in pH to 12.46 results in a change in rotation at this wavelength. An overlapping contribution toward this band by tyrosine and phenylalanine residues is also possible. Although the molar residue ellipticity of a phenylalanine residue is small in comparison to tyrosine (Moscowitz *et al.*, 1965), in neocarzinostatin the combined contributions of five phenylalanine residues might still be significant since a majority of them are located in polar or charged environments. These results suggest the possible contributions of disulfides, phenylalanine, and tyrosine residues toward the circular dichroism of neocarzinostatin in the aromatic region, but we are unable to assign unequivocal roles to any amino acid residue on the basis of this study.

N-Bromosuccinimide Oxidation of Tryptophan Residues. In a densely packed protein molecule some amino acid residues are "exposed" and chemically active; others are "buried" and fail to react. Oxidation and solvent perturbation of tryptophan residues and ionization constants of tyrosine residues have been used to differentiate between exposed and buried residues (Spande and Witkop, 1967; Beychok, 1966; Wetlaufer, 1962; Kronman and Robbins, 1970). We analyzed the effects of tryptophan modifications on native conformation by physical methods. Neocarzinostatin is an ideal protein for such studies since it contains only one tyrosine (position 32) and two tryptophan residues (positions 46 and 79) (Meienhofer *et al.*, 1972b). Other highly reactive residues, such as histidine and methionine, are absent. Attempted chemical modifications showed that neocarzinostatin was tightly packed and that several important residues were unavailable to the titrant.

The two tryptophan residues exhibited a differential behavior of striking selectivity toward oxidation by *N*-bromosuccinimide. The residue at position 79 was very reactive and accessible to the reagent in the absence of denaturing conditions and must be situated at or near the surface of the molecule in its native conformation. Under nondenaturing conditions the tryptophan-46 residue remained completely unaffected by *N*-bromosuccinimide. The circular dichroic spectrum of monotryptophan-79 oxidized neocarzinostatin resembled closely that of the native protein indicating that the conformation remained essentially unchanged. This product retained the full biological activities of neocarzinostatin against *Sarcina lutea* and leukemic cells in culture. It was obvious that the intact tryptophan-79 residue is not essential for maintaining the native conformation and does not play a role in the manifestation of cytotoxicity of neocarzinostatin.

The complete resistance to *N*-bromosuccinimide oxidation of the tryptophan-46 residue which must be located in the interior of the native molecule indicated that the native conformation of neocarzinostatin exhibits considerable stability. Denaturation by 8 M urea was required to make this buried tryptophan residue accessible to *N*-bromosuccinimide. The resulting bistryptophan-46,79 oxidized neocarzinostatin exhibited a CD spectrum which was entirely different from that of the native or monotryptophan-76 oxidized protein and was characteristic of random coil conformation. The oxidation of both tryptophan residues, therefore, resulted in a complete loss of ordered structure which was accompanied by a total disappearance of biological activity.

The tyrosine-32 residue of neocarzinostatin was not oxidized by *N*-bromosuccinimide (see amino acid analysis, Table I) and appears to be buried in the native protein and inaccessible to the reagent. This was also indicated by its high pK value of 12.5. In the presence of urea the reactivity of *N*-bromosuccinimide toward tyrosine is known to be greatly reduced (Funatsu *et al.*, 1964).

The data presented in this paper strongly indicate that the antitumor protein neocarzinostatin possesses a very tightly folded conformation, which probably consists to a considerable extent of the β-pleated sheet type. The single tyrosine residue in position 32 and one of the two tryptophan residues, *i.e.* that in position 46, are buried in the interior of this molecule. These findings are in accord with previously observed failures of certain chemical and enzymatic attacks. The two disulfide bonds of neocarzinostatin exhibited an unusually strong resistance toward reduction in aqueous medium by sodium borohydride, mercaptoethanol, or dithiothreitol which could not even be overcome by the addition of urea or guanidine hydrochloride (Meienhofer *et al.*, 1972a), and the one lysyl and three arginyl peptide bonds of the native protein completely resisted digestion by trypsin (Maeda *et al.*, 1966). Presumably, these functional sites are also shielded in the interior of native neocarzinostatin.

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Protein Iodination with Solid State Lactoperoxidase†

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ABSTRACT: Bovine lactoperoxidase covalently coupled to Sepharose 4B is demonstrated to be highly versatile in catalyzing the iodination of proteins. Iodinations can be effectively performed even in the presence of urea or sodium dodecyl sulfate and at temperatures as low as 0°. Lactoperoxidase catalyzed iodination of proteins is optimal when the concentration of hydrogen peroxide is essentially equal to that of the total iodide, while enzymatic activity with respect to protein iodinations is reduced with increasing concentrations of enzyme per unit volume of Sepharose beads. Practical applications of the LP-Sepharose iodination procedure are discussed.

A previous report briefly described an improved method for enzymatic iodination of proteins employing solid-state bovine lactoperoxidase (David, 1972). Unlike other methods of iodination, which utilize relatively strong oxidizing agents such as chloramine-T (Hunter and Greenwood, 1962) or iodine monochloride (McFarlane, 1958), enzymatic iodination with lactoperoxidase (Morrison, 1968, 1970; Marchalonis, 1969; Morrison and Bayse, 1970; Phillips and Morrison, 1970; Morrison *et al.*, 1971; Bauer *et al.*, 1971; Miyachi *et al.*, 1972) has been shown to be quite gentle, resulting in little or no detectable denaturation of the protein substrate. The major disadvantage of the soluble lactoperoxidase procedure has been the necessity of introducing contaminating materials, specifically the enzyme and any impurities in the enzyme preparation, into the iodination reaction mixture. By covalently coupling lactoperoxidase to an insoluble matrix, such as Sepharose 4B, it has been possible to circumvent this problem (David, 1972). The resulting solid-state enzyme not only provides a simple method for the gentle iodination of proteins, but is active over a wide range of conditions of pH and concentrations of protein and iodide.

Previously all iodinations were carried out under conditions of a large excess of H_2O_2 (David, 1972). This report presents data which indicate that the iodination reaction is not greatly

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