Lysozyme: A Major Secretory Product of a Human Colon Carcinoma Cell Line†

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Appendix: Crystallographic and NMR Studies of Human Tumor Derived Lysozyme

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ABSTRACT: One of the major proteins secreted by an established human colon adenocarcinoma cell line has been isolated in 25% yield from the serum-free medium in which the cells were grown and identified as lysozyme. Its purification was achieved by sequential steps of acidification, cation-exchange chromatography, and reversed-phase high-performance liquid chromatography. It was recognized to be a human lysozyme on the basis of its molecular weight (14000), isoelectric point (10.5), amino acid composition, and enzymatic activity. Its identity with previously characterized human lysozymes was established by amino-terminal sequence, peptide composition, immunological properties, NMR, and crystallography. A 4-day, 7-L collection of conditioned medium contained 20.3 mg of secreted protein of which 4.9 mg or ~24% of the total was tumor-derived lysozyme. The intracellular level of lysozyme was ~18 ng per 10^6 carcinoma cells. The possible significance of these findings in regard to the malignant process and tumor maintenance is discussed.

Eukaryotic organisms have evolved complex mechanisms that enable individual cells to influence and control their own environments. This is in large part accomplished by synthesis and secretion of regulatory molecules of which proteins and peptides are particularly important. Some secreted, regulatory proteins such as hormones and growth factors are involved in cell–cell communication while others such as enzymes modify the total milieu by action either in the immediate vicinity of the cell or at some distant site in the organism. Yet others, such as collagens and proteoglycans, serve to maintain extracellular integrity, thus creating conditions conducive to cellular migration, proliferation, and differentiation (Nilsen-Hamilton & Hamilton, 1982).

Investigations of the processes involved in mitogenic stimulation and malignant transformation have shown that such events can be accompanied by both qualitative and quantitative changes in protein secretion which, in some instances, are dramatic (Quigley, 1979). In malignant transformation, these secreted proteins are thought to serve as modulators of the transformed phenotype by effecting loss of cellular growth control, invasiveness and metastasis, and evasion of the host immune response. These changes in secretion may result from overproduction of normal proteins or inappropriate, i.e., ectopic, production of proteins not normally synthesized by the cells from which the tumor originated. Tumor-associated antigens [for reviews, see Herberman (1977) and Raschke (1982)], hormones [for reviews, see Ratcliffe & Podmore (1979) and Furth (1982)], growth factors (DeLarco & Todaro, 1978; Roberts et al., 1983), enzymes [for reviews, see Poole (1973), Schwartz (1978), and Goldberg (1979)], angiogenic factors (Auerback, 1981; Shubik, 1982), so-called major excreted proteins (Gottesman, 1978; Hiwasa et al., 1982), and oncogene products (Doolittle et al., 1983; Waterfield et al., 1983) are among specific examples which have been described. For the most part, definitive roles for these proteins in the malignant process have not been established. However, the chemical and biological characterization of these molecules promises to provide insights into the cellular regulatory processes involved in malignancy which in turn should suggest practical utility in the treatment, detection, or diagnosis of neoplastic diseases.

We have begun to characterize the peptides and proteins secreted from an established human colon adenocarcinoma cell line (HT-29) maintained in a serum-free medium. High-performance liquid chromatography (HPLC)[1] has recently emerged as a method of choice for the isolation of biologically active hormones and growth factors physiologically present in minute amounts (Hearn et al., 1982). It is usually performed under acidic conditions, and we have, therefore, concentrated our initial efforts on isolating secreted proteins and peptides that are acid stable and amenable to this approach. We find that HT-29 cells synthesize and secrete several specific proteins including remarkably large amounts of lysozyme. The lack of diagnostic or prognostic markers as well as the refractory nature of human colon carcinoma to standard chemo-,

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[1] Abbreviations: Pl lysozyme, human placental lysozyme; HEW lysozyme, hen egg white lysozyme; HT lysozyme, human tumor (HT-29) lysozyme; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; SFCM, serum-free conditioned medium; ASCM, acidified serum-free conditioned medium; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CM, carboxymethyl; Tris, tris(hydroxymethyl)aminomethane; Cys(Sp), *S*-(sulfopropyl)cysteine; EDTA, ethylenediaminetetraacetic acid.

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immuno-, and radiotherapy makes this an extremely relevant system to investigate the chemistry and biology of such secreted macromolecules.

Experimental Procedures

Materials. CM-cellulose (grade CM-52) was a product of Whatman Ltd. All dialyses were performed with 6000–8000 molecular weight cutoff tubing (Spectra/Por). Deionized, sterile water was provided by a Milli RO-20 reverse osmosis/Milli Q water purification system (Millipore Corp., Bedford, MA). Human Pl lysozyme was obtained from Alpha Therapeutic Corp. (Los Angeles, CA), and HEW lysozyme was from Sigma Chemical Co. (St. Louis, MO). TPCK-treated trypsin was from Worthington Biochemicals (Freehold, NJ) while pepstatin A was from Sigma Chemical Co.

Cell Line and Culture Conditions. Cells from the human colorectal adenocarcinoma line HT-29 (Fogh & Trempe, 1975) were routinely propagated in T flasks as monolayer cultures utilizing Dulbecco's modified Eagle's medium containing 4.5 mg/mL glucose and supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 50 μg/mL gentamicin, and 0.5 μg/mL fungizone. Cultures were incubated at 37 °C in humidified air maintained at 7% CO_2. Cells were fed every 2–3 days as required and subcultured at confluence by employing standard trypsinization techniques.

Extracellular, tumor-derived products were obtained from large-scale, serum-free cultures of HT-29. Cells (1×10^8) maintained as above were seeded into 6000-cm^2 multilevel cell factories (Vangard International, Inc., Neptune, NJ). Once the cells were confluent, the standard growth medium was replaced with 1.5 L of the supplemented growth medium but without fetal bovine serum. The serum-free medium was replaced every 2 days. The first 2-day collection was discarded while subsequent 4- and 6-day harvests, designated as SFCM, were collected and processed as described below. After 6 days, cultures were discarded.

Processing of Conditioned Medium. Cell debris was removed from the SFCM by sequential passage through Whatman 40 filter paper and Whatman 934-AH glass microfiber filters. Glacial acetic acid was added to the filtrate to a concentration of 5%. The acidified conditioned medium was treated with pepstatin A (5 mg/L), frozen, stored at −20 °C, then thawed, and clarified by filtration through Whatman 934-AH microfiber filters. The filtrate was designated acidified serum-free conditioned medium (ASCM). It was subsequently concentrated 200-fold on a Model DC2 hollow-fiber dialyzer/concentrator unit equipped with HP2 (molecular weight cutoff of 2000) hollow-fiber filters (Amicon Corp., Lexington, MA), dialyzed vs. water, and lyophilized.

CM-cellulose Chromatography and HPLC. CM-52 cation-exchange resin was precycled employing the manufacturer's recommendations, equilibrated with 100 mM sodium phosphate, pH 6.6 (running buffer) at 4 °C, and packed into a 1.5 × 8.0 cm column. Lyophilized ASCM was dissolved in and dialyzed overnight vs. running buffer, clarified by filtration through a 0.45-μm nitrocellulose filter (Millipore Corp.), and applied to the column at 4 °C. The column was washed with the running buffer; the material that did not bind is designated as CM I. Bound material (designated as CM II) was eluted with running buffer containing 1 M NaCl. CM I and CM II were individually pooled, dialyzed extensively vs. water, and lyophilized.

Reversed-phase HPLC was performed by employing a Waters Associates liquid chromatography system consisting of a Model 440 absorbance detector (254 nm), an LKB 2138 206-nm detector, two Model 6000 solvent delivery systems, a WISP 710A automatic sample loader, plus data module and systems controller, and an octadecylsilane Synchropak RP-P column (10-μm particle size, 250 × 4.1 mm) (Synchrom, Inc., Linden, IN). Elution was performed at a flow rate of 1 mL/min at room temperature. Column effluents were monitored simultaneously at 206 and 254 nm. Lyophilized preparations (CM II) were reconstituted in 0.1% (v/v) TFA in water (buffer A) (Mahoney & Hermodson, 1980) and applied to the column through the automatic sample injector. The column was eluted over 80 min with a linear gradient from 12% to 60% acetonitrile/0.1% TFA in water. Collected fractions (1 mL) were lyophilized.

Gel Electrophoresis. Analytical SDS–PAGE utilizing 15% gels was performed according to Laemmli (1970) except that the stacking gel was omitted. Gels were stained with silver by the following modification of established methods (Switzer et al., 1979). After electrophoresis, the gels were washed twice for 5 min per wash in 10% ethanol, fixed overnight in 5% formaldehyde/25% ethanol (Steck et al., 1980), and placed in 10% ethanol, a copper/silver solution, diammine, and a reducing solution as described (Switzer et al., 1979). Staining was stopped with Kodak Rapid-fix followed by a thorough water wash.

Analytical isoelectric focusing was carried out on an LKB 2117 multiphor unit using precast plates (Servalyt Precotes 3-10, Serva Fine Biochemicals, Garden City Park, NY). Gels were stained by conventional procedures employing Coomassie blue and dried.

S-Sulfopropylation of Proteins. Proteins were reduced with 5 μM dithioerythritol in 6 M guanidine hydrochloride, 0.05 M EDTA, and 0.1 M Tris, pH 8.5, and alkylated with 1,3-propane sultone (Sigma Chemical Co.) according to Ruegg & Rudinger (1974). Alkylated samples were desalted by dialysis.

Amino Acid Analysis. Samples of proteins or peptides were hydrolyzed with 6 N HCl and 0.1% phenol in vacuo at 110 °C for 20 h (Sanger & Thompson, 1963), or as stated. The hydrolysates were analyzed on a Durrum D-500 amino acid analyzer equipped with a Hewlett-Packard 3390A integrator, using ninhydrin as reagent. Tryptophan was determined by amino acid analysis of hydrolysates prepared by using 4 N methanesulfonic acid containing 0.2% tryptamine (Pierce Chemical Co., Rockford, IL) (Simpson et al., 1976). The tryptophan content of peptides was estimated from the A_{254}/A_{206} ratio calculated from peptide maps with correction for peptide size.

Proteolytic Cleavage. Reduced and S-sulfopropylated protein was digested with TPCK–trypsin, purified by HPLC according to Titani et al. (1982). Hydrolysis was carried out overnight at 35 °C in 2% ammonium bicarbonate solution at an enzyme to substrate ratio of 0.01 by weight. The peptide mixture was dried and dissolved in 0.1% TFA for HPLC mapping.

Peptide Mapping. Four solvents were utilized for mapping by HPLC: (A) 0.1 M sodium perchlorate and 0.1% orthophosphoric acid buffer at pH 2.2 (Meek, 1980); (B) 75% acetonitrile and 25% solvent A; (C) 0.1% TFA in water (Mahoney & Hermodson, 1980); and (D) 5:5:4 (v/v/v) acetonitrile, 2-propanol, and water containing 0.09% TFA.

Digests were first mapped by using an octadecylsilane reversed-phase column (Altex Ultrapore IP; 5 μm, 250 × 4.5 mm) employing linear gradients between solvents A and B. Initial conditions were 95% solvent A and 5% solvent B. At 35 and 75 min, these were changed to 60% solvent A/40% solvent B and 35% solvent A/65% solvent B, respectively.

Fractions requiring further purification were then rechromatographed on a Synchropak RP-P column (Synchrom, Inc., 250 × 4.1 mm) by using appropriate linear gradients between solvents C and D. The eluate was monitored simultaneously at 206 and 254 nm, and aliquots were taken for amino acid analysis.

Automated Sequence Analysis. A Beckman 890C sequencer (Beckman Instruments, Inc., Palo Alto, CA) equipped with a cold trap attachment was used. The degradation procedure followed Beckman program 121078 for 0.1 M Quadrol as the coupling buffer (Brauer et al., 1975). Phenylisothiocyanate, heptane, and heptafluorobutyric acid were obtained from Beckman. Dithioerythritol was added to the heptafluorobutyric acid (0.01%) (Wittmann-Liebold, 1973) and butyl chloride (0.03%) (Hermodson et al., 1970). The Quadrol buffer (Beckman or diluted from 0.25 M buffer from Pierce Chemical Co.) was stored over aminoethylcellulose (United States Biochemical Corp., Cleveland, OH), and benzene, ethyl acetate, and butyl chloride stocks (Burdick and Jackson, Muskegon, MI) were stored over alumina (neutral, Brockman activity grade 1, Fisher Scientific) as described by Frank (1979). Polybrene was used as carrier (Klapper et al., 1978; Tarr et al., 1978) and was purified in the cup by two degradation cycles prior to loading of sample.

The anilinothiazolinone derivatives were converted to PTH derivatives with 10 μL of 30% TFA (containing 0.1% ethanethiol) under N_2 at 80 °C and dissolved in methanol for analysis by HPLC. PTH-norleucine (50 nmol) as carrier and PTH-norvaline (1 nmol) as internal standard were added to each tube in the fraction collector of the sequencer.

PTH-amino acids were identified by HPLC on a Radialpak C18 column (5-μm particles, Waters Associates). The chromatographic eluate was monitored at 254 nm, and PTH-amino acids were quantitated by using a Waters Associates data module. Two chromatographic systems employing three solvents were used: solvent A, 0.1% triethylamine, 0.3% acetic acid, 1% tetrahydrofuran, and 9% methanol in water; solvent B, 1% triethylamine, 0.25% phosphoric acid, acetic acid to pH 4.45, 10% methanol, and 1% tetrahydrofuran in water; solvent C, 1% tetrahydrofuran, 44% 2-propanol, and 5% methanol in water. System 1 utilized nonlinear gradients from 28% solvent C and 72% solvent A to 65% solvent C and 35% solvent A while system 2 used nonlinear gradients from 10% solvent C and 90% solvent B to 70% solvent C and 30% solvent B. All the commonly occurring PTH-amino acids could be identified and quantitated by the combined use of these two systems.

Protein Assays. Protein concentrations were determined by amino acid analysis.

Ouchterlony Double Diffusion. Goat anti-human lysozyme was from Miles Laboratories (Elkhart, IN), and rabbit anti-human lysozyme was from Accurate Chemical and Scientific Corp. (Westbury, NY). Immunodiffusion gels were prepared on glass microscope slides utilizing 3 mL of 1% Noble agar in sodium borate, pH 7.4, with 0.15 M NaCl.

Enzymatic Assays. Lysozyme activity was determined spectrophotometrically according to the turbidometric method of Locquet et al. (1968). The lysis of *Micrococcus lysodeikticus* cells (Sigma Chemical Co.) in 0.066 M sodium phosphate and 0.09% NaCl, pH 6.2 at 25 °C, was monitored at 650 nm. The concentration of *M. lysodeikticus* cells was varied over a 10-fold range, and kinetic constants were evaluated from least-squares analysis of conventional double-reciprocal plots (Lineweaver & Burk, 1934). The apparent binding constant, K_m(app), is expressed as micrograms of *M. lysodeikticus* cells per milliliter. The maximal rate, V_{max}, is expressed as the absorbance change (ΔA) per microgram of enzyme per minute.

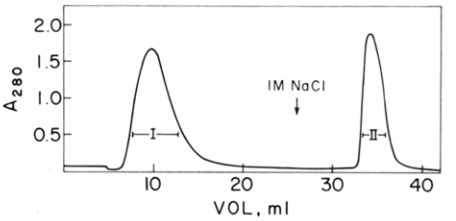

FIGURE 1: Chromatography of ~10 mg of ASCM on a 1.5 × 8.0 cm column of CM-52 cellulose equilibrated with 100 mM sodium phosphate, pH 6.6. The arrow represents the point of addition of the above buffer containing 1 M NaCl. The two fractions (CM I and CM II) were individually pooled, dialyzed against water, and lyophilized.

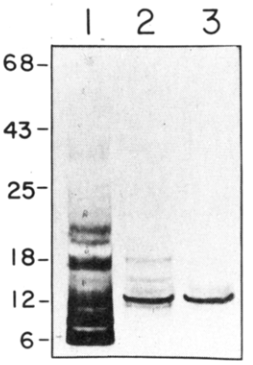

FIGURE 2: SDS–PAGE in 15% polyacrylamide gels of CM I (4.0 μg, lane 1), CM II (4.5 μg, lane 2), and the tumor-derived protein purified by HPLC (6.0 μg, lane 3). Conditions were as described under Experimental Procedures. Elution positions of molecular weight standards are indicated at the left ($\times 10^{-3}$).

Lysozyme concentrations were determined enzymatically from a standard curve based on concentrations of human Pl lysozyme varying from 2.5 mg/mL to 25 ng/mL, the limit of detection. The observed activity varied linearly with enzyme concentration over this range. All assays were performed in triplicate. The concentration of Pl lysozyme was determined by amino acid analysis.

Preparation of Cellular Lysates. HT-29 cells grown as monolayer cultures were harvested with trypsin, pelleted by centrifugation (400g, 10 min, 4 °C), and washed twice in Dulbecco's calcium- and magnesium-free phosphate-buffered saline, pH 7.4. Pelleted cells were then washed once in 20 volumes of a buffer containing 250 mM sucrose, 50 mM Tris, 25 mM KCl, and 5 mM $CaCl_2$, pH 7.5. The washed pellet was resuspended at 10^7 cells per mL in the above buffer containing 0.3% Triton X-100, and the cells were ruptured by homogenization with 10–20 strokes in a Dounce homogenizer with a type B pestle. The homogenate was clarified by centrifugation at 12000*g* for 60 min. The supernatant was dialyzed against water, lyophilized, and resuspended in phosphate-buffered saline for duplicate assays of lysozyme enzymatic activity and total protein.

RESULTS

Isolation Procedures. Acidified serum-free conditioned medium (ASCM) is separated on CM-52 cellulose into two fractions: CM I does not bind to the column, and CM II does and is eluted by salt (Figure 1). These fractions were dialyzed, lyophilized, and analyzed by SDS–PAGE. CM II contains a single, major extracellular protein corresponding to an apparent molecular weight of 12 000–14 000 accompanied by minor contaminants (Figure 2). Further fractionation of CM

Table I: Summary of HT Lysozyme Purification

step	total protein[a] (mg)	HT lysozyme (ΔA/min)	% recovery
SFCM (volume = 7 L)	20.3	1960	100.0
acidification, freeze–thaw, filter (ASCM)	10.9	1440	73.5
CM-cellulose			
CM I	6.2	16	0.8
CM II	2.5	880	44.9
reversed-phase HPLC of CM II	1.13	480	24.5

[a] Determined by amino acid analysis.

Table II: Amino Acid Composition of Tumor-Derived Protein and Human Lysozyme

amino acid	tumor-derived protein[a]	human lysozyme[b]
Cys	8.18 (8)[c]	8
Asp	17.53 (18)	18
Thr	5.05 (5)	5
Ser	6.25 (6)	6
Glu	9.40 (9)	9
Pro	1.89 (2)	2
Gly	11.24 (11)	11
Ala	13.83 (14)	14
Val[d]	9.15 (9)	9
Met	1.90 (2)	2
Ile[d]	4.72 (5)	5
Leu	8.32 (8)	8
Tyr	6.04 (6)	6
Phe	2.08 (2)	2
His	1.08 (1)	1
Lys	5.05 (5)	5
Arg	13.97 (14)	14
Trp	4.66 (5)	5
	130[e]	130[e]

[a] Amino acid ratios are presented along with nearest integer values in parentheses. Average of three determinations. [b] Literature values for milk and leukemic urine lysozymes. [c] Determined as Cys(Sp). [d] Determined on 72-h hydrolysates. [e] Total.

II was achieved by using HPLC with a Synchropak RP-P column and a linear eluting acetonitrile/water/TFA buffer system. The major ultraviolet-absorbing component elutes at 50 min, at an acetonitrile concentration of approximately 42%. Analytical SDS–PAGE showed this component to be free of contaminants and to be identical with the major CM II 12 000–14 000 molecular weight protein (Figure 2). The identification of the protein as a human lysozyme (vide infra) allows calculation of the yield following the individual purification steps based on enzymatic lysis of *M. lysodeikticus* cells (Table I).

Characterization and Amino Acid Composition. Analytical isoelectric focusing indicated that this protein was extremely basic, with an isoelectric point of ~10.5. This, together with its molecular weight of 12 000–14 000, indicated that it belonged to a limited class of known proteins. Amino acid analysis allowed comparison of its composition with those of other members in this class, and it was found to agree completely with that of human milk and leukemic urine lysozymes (Canfield et al., 1971; Jolles & Jolles, 1971, 1972; Thomsen et al., 1972) (Table II).

Enzymatic Characterization. Having identified the tumor-derived protein as human lysozyme (henceforth designated as HT lysozyme), on the basis of composition, molecular weight, and isoelectric point, it was examined for its ability to catalyze the lysis of *M. lysodeikticus* cells (Locquet et al., 1968). Enzymatic activity was indeed demonstrated and compared to activities determined for HEW and Pl lysozymes (Table III). The apparent binding constants for all three enzymes are similar with K_m values of 62, 67, and 70 μg/mL,

Table III: Experimentally Determined Kinetic Parameters[a]

protein	K_m(app) (μg/mL)	V_{max} (ΔA μg^{-1} min^{-1})
HEW lysozyme	67 ± 15	0.11 ± 0.02
Pl lysozyme	70 ± 15	0.40 ± 0.03
HT lysozyme	62 ± 10	0.38 ± 0.03

[a] Determined spectrophotometrically by lysis of *M. lysodeikticus*.

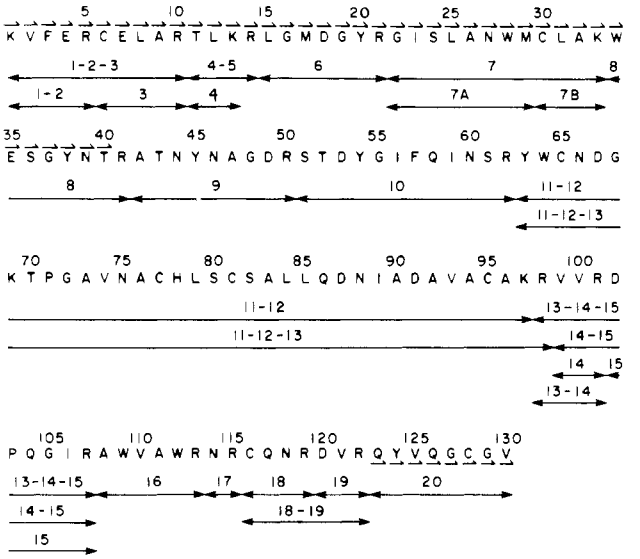

FIGURE 3: Amino acid sequence of human lysozyme (Canfield et al., 1971; Jolles & Jolles, 1971, 1972; Thomsen et al., 1972) showing positions of tryptic peptides isolated in this study from the HT and Pl lysozymes (see Table IV). Nomenclature for peptide designations is described in the legend to Table IV. The half-arrows above the residues show the extent of degradation by the Edman method on the whole molecule, and those below the residues show the extent of degradation for peptide 20 of HT lysozyme.

respectively. These values are in reasonable agreement with published K_m values for HEW and human lysozymes of 115 and 100 μg/mL, respectively (Locquet et al., 1968). The V_{max} values, expressed as the maximal absorbance change at 650 nm per microgram of enzyme per minute, were identical for HT and Pl lysozymes within experimental error. The ratio of activities of previously characterized human and HEW lysozymes expressed as reaction velocity per unit enzyme at a fixed concentration of *M. lysodeikticus* has been reported to be approximately 3.5–4 (Jolles & Jolles, 1967). The ratios of the V_{max} values for both tumor-derived product and Pl lysozyme to HEW lysozyme (Table III) are within this range.

Primary Sequence. Automated sequence analysis of 12.5 nmol of the reduced and S-sulfopropylated tumor-derived protein through 40 cycles of Edman degradation revealed that its sequence was identical with that reported for the two human lysozymes (Figure 3). The sequencing methodology employed resulted in a 67% yield of valine at step 2 with a repetitive yield, based on leucine, of 89%.

Peptide Mapping. Peptide mapping techniques utilizing HPLC were developed to examine the remaining portion of the molecule. The tryptic peptides were prepared from the intact protein and compared with those from human Pl lysozyme which is readily available from commercial sources. Although the complete primary sequence for the placental enzyme has not been determined, it is thought to be identical with that for human milk and leukemic urine lysozymes (Jolles & Jolles, 1967; Mouton & Jolles, 1969). Also, since the conditions for tryptic digestion of human milk lysozyme resulted in the complete excision of Arg-98 (Jolles & Jolles, 1968), lesser amounts of trypsin were employed here in order to isolate larger peptides which would still contain Arg-98. The

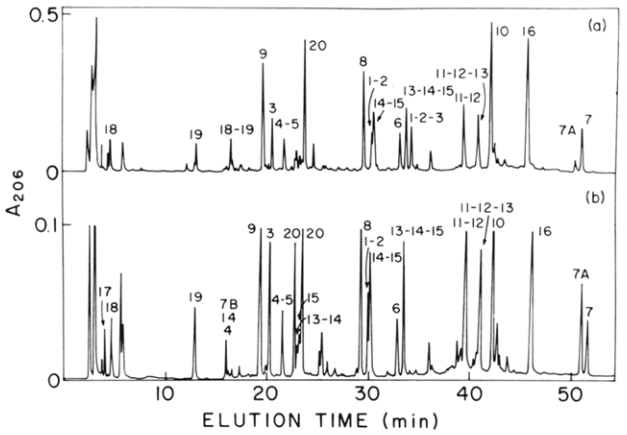

FIGURE 4: Tryptic peptide maps of reduced and sulfopropylated Pl lysozyme (a) and HT lysozyme (b). The HPLC conditions employed are as described under Experimental Procedures. The compositions of individual peptides (1–20) are shown in Table IV and their sequence locations in Figure 3.

tumor-derived protein and Pl lysozyme were both reduced, S-sulfopropylated, and digested with trypsin, and the digests were subjected individually to chromatographic separation on an Altex Ultrasphere IP reversed-phase column (Figure 4). Poorly resolved peptides were purified by rechromatography on a Synchropak RP-P column as described under Experimental Procedures. Peptides derived from the two proteins were subjected to amino acid analysis, and the results were corrected appropriately for background levels of Asp, Ser, Glu, and Gly.

Although the HPLC peptide maps for the two proteins are much the same, some differences are apparent (Figure 4). Preparative mapping allowed the amino acid composition of each fraction to be determined (Table IV). Peptides isolated from HT lysozyme encompassed the entire polypeptide chain (Figure 3) and those from Pl lysozyme all but a dipeptide region (peptide 17, vide infra). Moreover, the compositions of corresponding peptides are the same. The few qualitative and quantitative differences between the tryptic digests of the two proteins (Figures 3 and 4) appear to be the consequence of digestion or chromatography conditions.

One particularly obvious difference occurs with peptide 20 (residues 123–130) from the tumor material which elutes in two positions (Figure 4b). One form elutes at 22.8 min, and the related form, arising by cyclization of its N-terminal glutamine to pyroglutamic acid, elutes at 23.3 min. The sequence of the unblocked peptide, determined by automated Edman degradation, is identical with that of the known human forms. If the digest from either protein is kept for 1 week before HPLC mapping, there is a significant increase in the 23.3-min peak consistent with slow cyclization of the N-terminal glutamine. In the example shown in Figure 4a, peptide 20 from Pl lysozyme elutes almost entirely as the cyclized derivative.

Other differences can be tentatively ascribed to differences in the concentrations of substrate used for each digest. Typically, due to limited availability, 10-fold less tumor-derived material than Pl lysozyme was used during digestion. In addition, partial cleavages at certain lysine and arginine residues as well as one "anomalous" split rendered the peptide maps from both proteins more complex than would be expected based solely on amino acid composition. These will be examined in order starting from the amino terminus of the molecule (Figure 4 and Table IV).

(1) Incomplete cleavage of the Arg_5–$Cys(Sp)_6$ bond in the placental protein generates three peptides (1-2, 1-2-3, and 3)

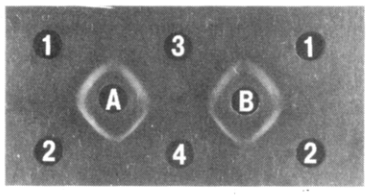

FIGURE 5: Ouchterlony double diffusion gel showing immunological identity between HT and Pl lysozymes. Wells numbered 1 and 2 contained 10 and 5 μg of HT lysozyme, respectively; wells numbered 3 and 4 contained 10 and 5 μg of Pl lysozyme, respectively. The center wells contained goat anti-human lysozyme (A) and rabbit anti-human lysozyme (B).

in contrast to the tumor-derived material where only peptides 1-2 and 3 were obtained. (2) The Lys_{13}–Arg_{14} bond was apparently not cleaved in the placental protein but was split partially in the tumor product. Thus, two peptides, 4 and 4-5, were recovered from the latter digests but not free arginine (Arg_{14}). (3) An anomalous, partial cleavage occurred at the Met_{29}–$Cys(Sp)_{30}$ bond in both proteins which gave rise to peptides 7A and 7B, in addition to the parent peptide 7. Peptide 7B, however, could not be identified in the Pl lysozyme digest. (4) A series of overlapping peptides was generated from both proteins due to the presence of the adjacent basic residues Lys_{97}-Arg_{98}. This produced peptides 11-12, 11-12-13, and 14-15 in both digests. (5) Partial cleavage of the Arg_{101}–Asp_{102} bond in the tumor-derived but not in the placental protein accounted for the recovery of peptides 13-14, 14, and 15 in the former digest. Only peptide 13-14-15 was recovered from the placental digests, suggesting that the Arg_{101}–Asp_{102} bond was resistant to tryptic cleavage under the conditions used for this protein. (6) Peptide 17 was not seen in digests of Pl lysozyme. It should be present, however, since the amino acid compositions of the two proteins are identical and one asparagine and one arginine residue are not accounted for by the tryptic peptides recovered. Additionally, a peptic digest of the Pl lysozyme protein (data not shown) generated a peptide corresponding to residues 112–120 which spans this region. (7) Lastly, partial cleavage of the Arg_{119}–Asp_{120} bond in the placental protein accounted for the isolation of peptide 18-19 from this but not the tumor protein digest.

Immunological Characterization. Ouchterlony double diffusion tests demonstrate the immunological identity of HT and Pl lysozymes. Complete fusion of the immunoprecipitation lines is evident between the two proteins with each of the two anti-human lysozyme antisera (Figure 5).

Intracellular Levels of HT Lysozyme. The enzymatically determined intracellular lysozyme content of HT-29 cellular homogenates was ~18 ng/10^6 cells corresponding to 0.02% of the total intracellular protein by weight. This compares with accumulated, extracellular HT lysozyme levels of ~3.2 μg (10^6 cells)$^{-1}$ (24 h)$^{-1}$ (data not shown). Thus, more than 99% of the lysozyme synthesized during this period is secreted.

X-ray Crystallography and NMR. These studies (see the Appendix) confirm the identity of HT lysozyme to human leukemic and milk lysozymes.

Discussion

Human HT-29 colon adenocarcinoma cells release a relatively large amount of a protein into their growth medium. It has been purified to homogeneity by sequential acidification, CM-cellulose ion-exchange chromatography, and reversed-phase HPLC in an overall recovery of 25% (Table I). The molecular weight, isoelectric point, and amino acid composition of this protein all coincide with those of human lysozyme. In addition, the sequence of its 40 N-terminal amino acid residues

Table IV: Amino Acid Composition of Tryptic Peptides of Human Tumor (HT) Lysozyme and Placental (Pl) Lysozyme

source:	HT	Pl	HT	Pl	HT	HT	Pl	HT	Pl	HT	Pl
peptide:	1-2[a]	1-2	3	1-2-3	4	4-5	4-5	6	6	7	7
Cys(Sp)			0.8 (1)	1.0 (1)		0.2	0.1			(1)[c]	1.0 (1)
Asp	0.2	0.2	0.1			0.2	0.2	1.1 (1)	1.0 (1)	1.3 (1)[c]	0.7 (1)
Thr	0.2	0.1			1.0 (1)	1.0 (1)	0.8 (1)	0.1			
Ser	0.1	0.1	0.2			0.3	0.1	0.1		1.3 (1)	0.9 (1)
Glu	1.1 (1)	1.1 (1)	1.1 (1)	2.0 (2)		0.2	0.2	0.1			
Pro		0.1						0.1			
Gly	0.1	0.4	0.2	0.2		0.4	0.2	2.1 (2)	1.9 (2)	1.4 (1)	1.1 (1)
Ala	0.2	0.1	0.9 (1)	1.0 (1)		0.1	0.2	0.1		2.3 (2)	2.1 (2)
Val	0.9 (1)	0.9 (1)	0.1	1.0 (1)		0.1	0.1				
Met								0.9 (1)	1.0 (1)	(1)[c]	0.9 (1)
Ile	0.1	0.1								1.2 (1)	0.8 (1)
Leu	0.3	0.1	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	2.1 (2)	2.1 (2)
Tyr								0.9 (1)	0.9 (1)	0.2	
Phe	1.0 (1)	0.9 (1)		1.0 (1)						02	
His	0.1										
Lys	1.0 (1)	0.9 (1)		1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)			0.9 (1)	0.9 (1)
Arg	1.0 (1)	1.0 (1)	0.9 (1)	2.0 (2)		1.0 (1)	1.0 (1)	1.1 (1)	1.0 (1)		0.1
Trp[b]										+ (1)	+ (1)
total	5	5	5	10	3	4	4	7	7	12	12
sequence position	1–5	1–5	6–10	1–10	11–13	11–14	11–14	15–21	15–21	22–23	22–33

source:	HT	Pl	HT	HT	Pl	HT	Pl	HT	Pl	HT	Pl
peptide:	7A	7A	7B	8	8	9	9	10	10	11-12	11-12
Cys(Sp)			0.9 (1)		0.1					3.8 (4)	3.7 (4)
Asp	1.5 (1)[c]	0.8 (1)		1.1 (1)	1.0 (1)	3.0 (3)	2.7 (3)	1.8 (2)	2.0 (2)	6.0 (6)	6.3 (6)
Thr				0.9 (1)	1.0 (1)	1.1 (1)	1.4 (1)	0.9 (1)	1.1 (1)	0.9 (1)	0.8 (1)
Ser	1.1 (1)	1.0 (1)		1.1 (1)	1.1 (1)			1.8 (2)	1.9 (2)	2.6 (2)	1.6 (2)
Glu		0.2		1.1 (1)	1.2 (1)			1.1 (1)	1.1 (1)	1.2 (1)	1.2 (1)
Pro		0.1								1.1 (1)	1.4 (1)
Gly	1.2 (1)	1.2 (1)		1.3 (1)	1.2 (1)	1.0 (1)	1.2 (1)	1.0 (1)	1.1 (1)	2.0 (2)	2.3 (2)
Ala	1.1 (1)	1.0 (1)	1.0 (1)		0.1	2.0 (2)	2.1 (2)	0.2		7.1 (7)	6.7 (7)
Val					0.1					2.0 (2)	1.7 (2)
Met	(1)[c]	0.9 (1)									
Ile	0.9 (1)	0.8 (1)						1.6 (2)	1.9 (2)	1.0 (1)	1.0 (1)
Leu	1.0 (1)	1.0 (1)	1.0 (1)					0.3		3.0 (3)	3.0 (3)
Tyr				1.2 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	0.9 (1)	0.9 (1)
Phe								1.0 (1)	0.9 (1)		
His										1.0 (1)	1.0 (1)
Lys			1.0 (1)							2.0 (2)	1.8 (2)
Arg		0.1		1.0 (1)	1.1 (1)	1.3 (1)	1.1 (1)	0.8 (1)	1.0 (1)	0.4	0.3
Trp[b]	+ (1)	+ (1)		+ (1)	+ (1)					+ (1)	+ (1)
total	8	8	4	8	8	9	9	12	12	35	35
sequence position	22–29	22–29	30–33	34–41	34–41	42–50	42–50	51–62	51–62	63–97	63–97

source:	HT	Pl	HT	HT	HT	Pl	Pl	HT	HT	Pl
peptide:	11-12-13	11-12-13	13-14	14	14-15	14-15	13-14-15	15	16	16
Cys(Sp)	1.0 (4)[c]	3.1 (4)[c]								
Asp	5.6 (6)	5.6 (6)			1.2 (1)	1.1 (1)	0.9 (1)	1.0 (1)	0.3	0.3
Thr	1.2 (1)	1.0 (1)			0.2	0.2	0.1		0.1	0.1
Ser	3.4 (2)	2.6 (2)			0.2	0.3	0.2	0.3		0.2
Glu	2.0 (1)	1.8 (1)			1.2 (1)	1.2 (1)	1.0 (1)	1.1 (1)	0.3	0.1
Pro	1.1 (1)	1.2 (1)			1.2 (1)	1.1 (1)	1.0 (1)	0.9 (1)		
Gly	3.6 (2)	3.4 (2)			1.1 (1)	1.5 (1)	1.2 (1)	1.2 (1)		0.2
Ala	6.6 (7)	6.6 (7)			0.2	0.2		0.1	1.9 (2)	2.0 (2)
Val	1.9 (2)	1.9 (2)	1.5 (2)[d]	1.3 (2)[d]	1.1 (2)[d]	1.1 (2)[d]	1.3 (2)[d]		1.0 (1)	1.0 (1)
Met										
Ile	1.3 (1)	1.0 (1)			0.9 (1)	0.9 (1)	0.8 (1)	0.8 (1)	0.3	
Leu	3.4 (3)	3.1 (3)			0.3	0.2			0.2	0.2
Tyr	1.0 (1)	1.0 (1)				0.1			0.2	
Phe		0.2			0.1	0.2			0.2	
His	1.2 (1)	1.0 (1)			0.2	0.1			0.2	0.1
Lys	2.4 (2)	2.0 (2)			0.2	0.2			0.2	0.1
Arg	1.3 (1)	1.1 (1)	2.0 (2)	1.0 (1)	1.7 (2)	1.8 (2)	2.4 (3)	0.9 (1)	1.0 (1)	1.0 (1)
Trp[b]	+ (1)	+ (1)							++ (2)	++ (2)
total	36	36	4	3	9	9	10	6	6	6
sequence position	63–98	63–98	98–101	99–101	99–107	99–107	98–107	102–107	108–113	108–113

source:	HT	HT	Pl	Pl	HT	Pl	HT	Pl
peptide:	17	18	18	18-19	19	19	20	20
Cys(Sp)		0.7 (1)	0.5 (1)	0.9 ((1)			0.6 (1)	0.4 (1)[c]
Asp	0.9 (1)	1.0 (1)	0.9 (1)	2.1 (2)	1.0 (1)	0.9 (1)	0.2	0.5
Thr			0.2				0.1	0.1
Ser			0.3	0.1			0.2	0.2
Glu		1.0 (1)	1.8 (1)	1.0 (1)		0.2	2.0 (2)	2.2 (2)

Table IV (Continued)

source:	HT	HT	Pl	Pl	HT	Pl	HT	Pl
peptide:	17	18	18	18-19	19	19	20	20
Pro								
Gly			0.4	0.4		0.1	2.1 (2)	2.2 (2)
Ala			0.2	0.1			0.2	0.1
Val				1.0 (1)	0.8 (1)	0.8 (1)	1.7 (2)	1.8 (2)
Met								
Ile								0.2
Leu								
Tyr							1.0 (1)	1.0 (1)
Phe								
His								
Lys								0.1
Arg	1.1 (1)	1.0 (1)	1.0 (1)	1.9 (2)	1.1 (1)	1.0 (1)		0.4
Trp[b]								
total	2	4	4	7	3	3	8	8
sequence position	114–115	116–119	116–119	116–122	120–122	120–122	123–130	123–130

[a] The tryptic peptides were numbered (n) according to the nth residue of Lys or Arg present in them, starting with Lys-1 being 1, Arg-5 being 2, etc. If more than one Lys or Arg residue is present in a peptide, the numbers corresponding to positions of the Arg and Lys residues are joined by hyphens, e.g., peptide 1-2-3 contains the first through the third Lys and Arg residues (Lys-1, Arg-5, and Arg-10) (see Figure 3). [b] Tryptophan content of peptides (+, ++) was estimated from the A_{254}/A_{206} ratio calculated from the peptide maps with corrections made for peptide sizes. [c] Peptides HT-7, HT-7A, HT-11-12-13, Pl-11-12-13, and Pl-20 show the effect of hydrolysis in the presence of the perchlorate buffer used for HPLC; i.e., when perchlorate buffer was present, destruction of methionine and high values for aspartic acid, caused by coelution of methionine oxides and partial destruction of Cys(Sp), were observed. [d] Since peptides were hydrolyzed for only 20 h, the values found for valine in peptides 13-14, 14, 14-15, and 13-14-15 are low due to the resistant Val-Val sequence (Thomsen et al., 1972).

is identical with that of human milk and leukemic lysozymes (Figure 3), and its tryptic peptide map is virtually indistinguishable from that of Pl lysozyme (Table IV, Figure 4). Thus, the mapping, sequence, and compositional data strongly indicate that these molecules are indeed the same. In this regard, the enzymatic and immunological characteristics of this protein are similar to those of known human lysozymes. This identity has been independently confirmed by NMR and X-ray crystallography (see Appendix). On the basis of a specific activity of 0.38 $\Delta A\ \mu g^{-1}\ min^{-1}$, HT lysozyme comprises about 24% of the total extracellular protein present in medium conditioned by HT-29 cells (Tables I and III).

Since its discovery and description by Fleming (1922), lysozyme has been the subject of intensive investigations as is apparent from the vast literature dealing with its structural and functional properties and their physiological implications. Indeed, HEW lysozyme was the first enzyme whose structure was established by X-ray crystallography (Phillips, 1966). Lysozymes have been detected in, and in some cases isolated from, several normal human secretions and tissues including milk, tears, saliva, placenta, serum, stomach, and small intestine [see Reitamo et al. (1978) and references cited therein for review]. Lysozyme synthesis occurs in leukocytes, tissue macrophages, or secretory epithelial cells including Paneth cells in the majority of cases cited above. Malignant transformation of these cell types might therefore be expected to increase overall lysozyme production, as has been observed in Hodgkin's disease (Hansen et al., 1981; Ree et al., 1981), various forms of leukemia (Osserman & Lawlor, 1966; Zucker et al., 1970; Seshadri et al., 1981), and, occasionally, solid tumors of the gastrointestinal tract (Tahara et al., 1982; Graffner & Hultberg, 1983). Chemical and biological characterizations performed so far suggest that all human forms of lysozyme, whether normal or pathological, are identical (Jolles & Jolles, 1967; Kraus et al., 1969; Mouton & Jolles, 1969), although only two, the one from milk and the other from leukemic urine, have been sequenced completely (Canfield et al., 1971; Jolles & Jolles, 1971, 1972; Thomsen et al., 1972). The few reports of possible heterogeneity of human lysozymes in serum or urine can be attributed most likely to self-association or heterologous association of the protein or to differences in amide content (Mouton & Jolles, 1969; Virella, 1977; Ward et al., 1978; Wills et al., 1980).

In contrast, normal human colon tissue has not been reported to synthesize or contain appreciable amounts of lysozyme (Klockars & Reitamo, 1975; Mason & Taylor, 1975). Hence, the present finding of the relatively large ectopic production of this enzyme by colon-derived malignant cells is of considerable interest. The question arises as to whether the abnormal synthesis of this protein is due to a nonspecific, seemingly incidental, regulatory defect, perhaps as a consequence of in vitro culture conditions, or to a specific requirement for lysozyme production by the tumor cell. In the latter case, functional roles for lysozyme in addition to those which suggest that it serves as an antibacterial or antiviral agent would have to be postulated. In this regard, a few reports have suggested alternative roles and functions for lysozyme in both avian and mammalian systems. We believe that some of these or yet others might imply potential roles for lysozyme in malignancy.

Lysozymes from bacterial, avian, and human sources influence adhesion and spreading of normal human fibroblasts, and a role for lysozyme in effecting cellular morphology and differentiation has been proposed. Although direct evidence is lacking, this is thought to occur via interaction of lysozyme with cell membrane receptors which can function as substrates for lysozyme enzyme activity (Satta et al., 1980). A direct interaction between lysozyme and tubulin (Levi et al., 1975) with subsequent effects on the cytoskeleton can also be considered. Additionally, it has been demonstrated (Satta et al., 1978; Azzarone et al., 1981) that bacterial lysozyme induces DNA synthesis and cellular proliferation in a number of cell lines in vitro. Similar studies on stimulation of fibroblast proliferation with avian lysozyme support this contention (Takaoka et al., 1972).

However, in contrast, exogenous lysozymes, including the one from human leukemic urine, inhibit DNA synthesis when added to cultures of both normal and transformed mammalian cell lines (Osserman et al., 1973). Therefore, it would seem prudent to reserve judgement on the relevance of these seemingly paradoxical in vitro observations and their relationship to the potential biological effects of lysozyme on the growth,

differentiation, and morphology of normal and transformed cells both in vitro and in vivo. The known possible interactions between lysozyme and nucleic acids (Fasy et al., 1980) and the recent report suggesting a nuclear regulatory function for chromatin-associated lysozyme (Sakagami et al., 1982) are also relevant to the above.

Lysozyme may also have adverse effects on immune function. Under certain conditions, bacterial, avian, and human lysozymes inhibit both in vitro and in vivo responses to mitogenic and antigenic stimulation (Varaldo et al., 1981). Additionally, it has been demonstrated that human leukemic lysozyme, but not HEW lysozyme, decreases the response of human neutrophils to chemotactic stimuli in an in vitro system concomitant with depression of oxidative metabolism and peroxide generation (Gordon et al., 1979). While this was postulated to show that macrophage-derived lysozyme may function in a negative feedback fashion to inhibit neutrophil activity during inflammatory processes, the data could also denote that constitutive lysozyme secretion by malignant cells interferes with host antitumor inflammatory responses. Although undocumented to date, such a process would be of obvious selective advantage to the malignant, lysozyme-secreting cell.

Tumor-secreted lysozyme may participate in the in vivo dissemination and remote proliferation of malignant cells, a potential role hitherto unexplored. The understanding of mechanisms by which malignant cells invade, metastasize, and degrade normal tissue is fundamental to tumor biology. Extensive investigations have focused on this issue, and several lines of evidence implicate tumor-derived enzymes in these processes (Poole, 1973; Poste & Fidler, 1980; Laug et al., 1983). In this regard, lysozyme should be considered a potential degradative agent allowing for tumor dispersion. For such an activity to be important, however, there must be tissue and cellular substrates for lysozyme which, although proposed to be present, have not been defined as yet. Nonetheless, lysozyme may act on glycoprotein, glycolipid, or carbohydrate substrates which have susceptible glycosidic linkages. Importantly, the category of potential tissue substrates may be expanded greatly if human lysozymes, and particularly human tumor-derived lysozymes, are found to exhibit the esterase and weak protease activities recently ascribed to avian forms (Piszkiewicz & Bruice, 1968; Jolles & Jolles, 1983; Oliver & Stadtman, 1983).

A possible role for lysozyme in the metastatic progression of neoplasia can be inferred from several reports of its effect on prostaglandin synthesis and platelet aggregation. The long-standing observations that lysozyme when administered to cancer patients or those infected with herpes zoster reduces pain [for a review, see Bianchi (1981)] have recently been attributed to an ability of lysozyme to interfere with arachidonic acid metabolism and subsequent prostaglandin biosynthesis (Bianchi, 1981, 1982). Such inhibition of prostaglandin synthesis by tumor-derived lysozyme may lead to enhancement of tumor cell–platelet aggregation which has been shown to be an important—if not necessary—step for local tissue invasion and metastasis (Karpatkin & Pearlstein, 1981; Pearlstein et al., 1981). Specifically, this effect would be due to inhibition by lysozyme of prostacyclin and/or prostaglandin D_2 production—potent inhibitors of platelet aggregation (Honn et al., 1982; Tisdale, 1983; Menter et al., 1984). A situation could then potentially exist where tumor-secreted lysozyme would influence the metastatic potential of the malignancy. Thus, enhancement of tumor cell–platelet aggregation as the direct result of the inhibition of synthesis of certain types of prostaglandins by the tumor cell itself or surrounding cell types such as endothelial or smooth muscle could occur. Increased metastatic ability of a malignant melanoma cell line and decreased prostaglandin D_2 synthesis by these cells have been shown to be correlated (Fitzpatrick & Stringfellow, 1979). As tempting as such a speculation may be, it should be viewed with caution.

Lysozyme may also inhibit the synthesis of other classes of prostaglandins, including thromboxane A_2, which enhance platelet aggregation (Moncada & Vane, 1979; Tisdale, 1983). Indeed, Bianchi (1982) has postulated that lysozyme may serve as an antitumor agent by decreasing tumor cell–platelet aggregation via such a mechanism, but the resolution of this issue requires further investigation. The recent clinical finding that the prognosis of patients with advanced gastric tumors containing lysozyme is poorer than that of others with non-lysozyme-containing tumors may be relevant to the above discussion (Tahara et al., 1982).

Clearly, the question arises whether lysozyme could serve as a useful tumor marker for the detection, diagnosis, or monitoring of carcinoma of the colon. Although serum lysozyme activities have been useful in the differential diagnosis of monocytic and myelomonocytic leukemia (Osserman & Lawlor, 1966), there does not seem to be any correlation between serum lysozyme and human malignancies in general (DiLuzio, 1979; Samak et al., 1980). Recent reports, however, have indicated that lysozyme measurement may be useful in the management of gastric carcinomas. In a study by Tahara et al. (1982), lysozyme-containing tumor cells were found in 65 of 171 cases examined. Graffner & Hultberg (1983), who also examined patients with gastric carcinoma, concluded that measurement of gastric juice lysozyme may prove useful for screening patients after gastric resection as well as for screening asymptomatic patients.

Whether or not lysozyme will be of similar usefulness in the case of colon neoplasia has yet to be determined. There is no record of extensive evaluations of the lysozyme content of colon carcinoma tissue or colon carcinoma-derived cell lines. Although the reported incidence of lysozyme-synthesizing colon tumors is thought to be low [e.g., see Kittas et al. (1982)], this may reflect the paucity of investigations in which lysozyme activity was examined specifically. Evaluation of a potential diagnostic role for lysozyme as a colon tumor marker must await such analyses.

In a current study (to be published), several human colon adenocarcinoma cell lines maintained both in vitro and in vivo have been shown to synthesize and secrete lysozyme. This suggests that the frequency of lysozyme-producing colon carcinomas may be higher than generally appreciated. In light of this observation, intensive efforts are under way to examine the role of this enzyme in cell metabolism and tumor maintenance.

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Appendix: Crystallographic and NMR Studies of Human Tumor Derived Lysozyme

Crystallographic Studies (P. J. Artymiuk, S. Collett, and D. C. Phillips). The X-ray crystal structure of human leuk-

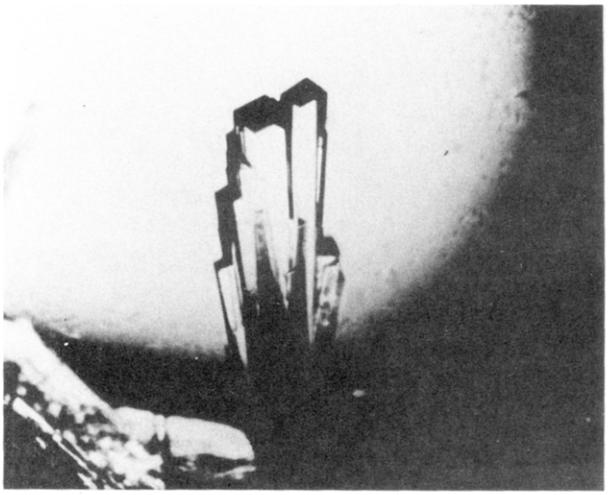

FIGURE 6: Pseudoparallel aggregate of crystals of tumor-derived lysozyme grown in ammonium nitrate: this growth form is characteristic of human lysozyme. The dimensions of the largest individual crystal are about 0.2 mm × 0.2 mm × 0.8 mm.

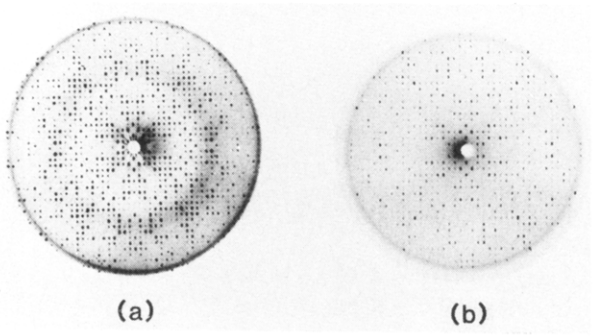

FIGURE 7: (a) 18° precession photograph of the *hk*0 zone of human leukemic lysozyme (Banyard, 1973). (b) 15° precession photograph of the *hk*0 zone of tumor-derived lysozyme: no intensity changes are discernible between the two photographs.

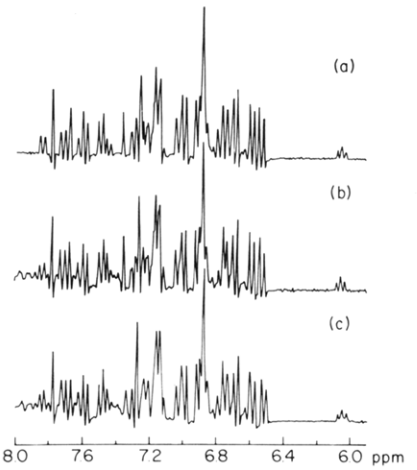

FIGURE 8: Aromatic region of the 300-MHz resolution-enhanced spectrum of human lysozyme at 57 °C and pH 5.3. The human lysozyme was derived from a leukemia patient (a), the HT-29 cell line (b), and normal human milk (c). Small differences in the spectra reflect differences in experimental conditions rather than differences in the sequences of the three proteins. Spectra b and c were obtained immediately after dissolution of lyophilized protein in D_2O. Resonances arising from amide hydrogens can be seen in the region downfield at 7.2 ppm. Spectrum a was obtained by using a sample which had previously been heated to 80 °C to remove all exchangeable hydrogens. Other differences in the spectra are probably the results of small pH differences (<0.2 pH unit).

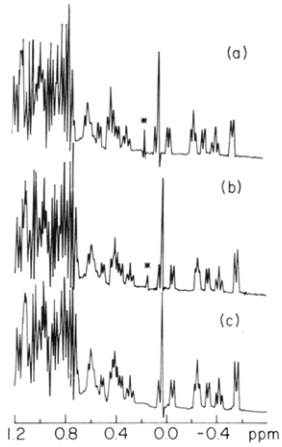

FIGURE 9: Upfield aliphatic region of the 300-MHz resolution-enhanced spectrum of human lysozyme at 57 °C and pH 5.3. The sharp peak labeled with an asterisk arises from an impurity. Small differences in the three spectra reflect small differences in pH. Spectra a–c are as in Figure 8.

emic lysozyme has been solved and refined at 1.5 Å (Artymiuk & Blake, 1981). Crystals of the HT lysozyme have been grown by essentially the same method (Osserman et al., 1969): a drop consisting of 5 μL of 7 M NH_4NO_3 and 5 μL of 20 mg/mL HT lysozyme was allowed to vapor-diffuse against a reservoir of 1 mL of 7 M NH_4NO_3, both buffered at pH 4.7. The characteristic clusters of crystals of up to 0.2 × 0.2 × 0.8 mm^3 in size appeared after several weeks (Figure 6). Fifteen-degree precession photographs (Figure 7) revealed the cell dimensions to be a = 57.2 Å, b = 61.2 Å, and c = 32.9 Å ($\alpha = \beta = \gamma = 90°$), in very good agreement with those of human leukemic lysozyme crystals (a = 57.1 Å, b = 61.0 Å, and c = 32.9 Å). The diffraction intensities appear to be identical.

NMR Studies (C. M. Dobson and C. Redfield). A 300-MHz proton NMR spectrum of HT lysozyme has been obtained: it appears essentially identical with the spectra of human leukemic lysozyme and human milk lysozyme (Figures 8 and 9). Both techniques therefore indicate that HT lysozyme is indistinguishable from the leukemic or the milk enzyme.

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Rotational Diffusion and Self-Association of Band 3 in Reconstituted Lipid Vesicles†

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ABSTRACT: Band 3, the anion transporter of the human erythrocyte membrane, has been purified and reconstituted into phospholipid vesicles of varying composition. Rotational diffusion of band 3 in these vesicles was measured by observing flash-induced transient dichroism of an eosin triplet probe covalently bound to the protein. In egg phosphatidylcholine vesicles of high lipid/protein ratio at temperatures well above the gel to liquid-crystalline phase transition, the absorption anisotropy decays to a constant value of 12 ± 1% (expressed as a percentage of the initial anisotropy). However, higher values of the residual anisotropy and a slower decay are observed upon decreasing the temperature, even though the lipids remain in a liquid-crystalline phase. A similar effect is observed upon decreasing the lipid/protein ratio at constant temperature. It is concluded that self-association of band 3 occurs which is dependent on temperature and protein concentration in the bilayer. It is very probable that similar effects occur in the erythrocyte membrane, where a strong temperature dependence of band 3 rotational mobility is also observed. Reconstitution of band 3 into dioleoylphosphatidylcholine vesicles yields results similar to those obtained with egg phosphatidylcholine. When dimyristoylphosphatidylcholine is used for reconstitution, band 3 is immobilized below the lipid phase transition and remains partially associated above the transition. A precise analysis of the anisotropy decay curves is hampered by the presence of multiple rotating species. Under conditions which favor maximum dissociation of band 3, the data are consistent with the major fraction of band 3 having a rotational relaxation time ($\phi_\parallel$) of ≈40 μs (where $\phi_\parallel$ is defined as the reciprocal of the diffusion coefficient for rotation about the membrane normal). This would be a reasonable value for either the dimer or the tetramer of band 3.

Rotational and lateral diffusion of integral membrane proteins has been extensively investigated in recent years. These studies have yielded much information concerning membrane dynamics and protein–protein interactions [for reviews, see Cherry (1979), Edidin (1981), Hoffmann & Restall (1983), Peters (1983), and Axelrod (1983)]. Some of the most detailed investigations have been performed with band 3, the anion-exchange protein in the erythrocyte membrane. Rotational diffusion of band 3 has been measured by using triplet probes (Cherry et al., 1976; Nigg & Cherry, 1979a; Austin et al., 1979; Johnson & Garland, 1981), while lateral diffusion measurements have involved fluorescence photobleaching recovery (FPR)[1] and fusion techniques (Peters et al., 1974; Golan & Veatch, 1980; Koppel et al., 1981; Schindler et al., 1980; Fowler & Branton, 1977). Lateral and rotational diffusion of band 3 has also been measured in reconstituted vesicles by FPR and saturation-transfer electron-spin resonance, respectively (Chang et al., 1981; Sakaki et al., 1982).

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[1] Abbreviations: eosinyl-MA, eosinyl-5-maleimide; egg PC, egg phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DTE, dithioerythritol; L/P, lipid/protein ratio(s) (w/w); PBS, phosphate-buffered saline; 5PB, 5 mM phosphate buffer; FPR, fluorescence photobleaching recovery; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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