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Enzymatic Modifications of Human Fibroblast and Leukocyte Interferons

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The sensitivity of highly purified human fibroblast interferon and partially purified human leukocyte interferon to several proteolytic and glycolytic enzymes was determined with respect to antiviral activity, isoelectric point, molecular weight, and thermal stability. Leucine aminopeptidase altered the distribution of isoelectric points for both interferons but produced little change in molecular weights; this enzyme somewhat reduced the activity of only leukocyte interferon. Treatment of fibroblast interferon with carboxypeptidases A and B did not greatly decrease antiviral activity, but it did slightly reduce the molecular weight of the interferon and substantially altered the distribution of isoelectric point values; similar treatment of leukocyte interferon caused some loss in activity, especially of the 17,000-molecular-weight species. Both interferons were inactivated rapidly by treatment with the endoproteases trypsin, pepsin, bromelain, and subtilisin. Chymotrypsin shifted the isoelectric points of both interferons, but only leukocyte interferon was significantly inactivated. Treatment with neuraminidase and β-galactosidase changed the isoelectric point distribution but did not affect the activity or thermal stability of either interferon; such a treatment reduced the molecular weight of fibroblast interferon and the size heterogeneity of leukocyte interferon. Treatment with neuraminidase and then leucine aminopeptidase greatly reduced the activity of both interferons, especially leukocyte interferon. The data indicate that biologically active forms of fibroblast and leukocyte interferons can be distinguished by their relative sensitivity to certain proteases.

Human fibroblast interferon (HFIF) differs from human leukocyte interferon (HLIF) in several physical, antigenic, and biological characteristics (1, 37). Information on the structural and chemical nature of these proteins is sometimes contradictory and has come from several different analytical approaches. That HFIF contains carbohydrate has been deduced from the following findings. (i) Treatment of HFIF with a mixture of glycosidases reduces its molecular weight and charge heterogeneity (3, 23). (ii) HFIF binds to lectins specific for *N*-acetylneuraminic acid, L-fucose, and D-mannose (16) and to immobilized neuraminidase (12). (iii) Purified HFIF oxidized with periodic acid stains with Fuchsin base (18). (iv) Inhibitors of glycosylation, especially 2-deoxy-D-glucose (14), which is known to inhibit addition of mannose, reduce its synthesis. Carter et al. found that HLIF lacks hydrophobic character because it is not retained by a variety of immobilized absorbants, whereas HFIF is retained (16, 17, 35); they also suggested that HLIF probably does not contain carbohydrate (16). Rubinstein et al. were able to purify one component of HLIF on the basis of hydrophobicity (27). Inhibitors of glycosylation also have been reported to inhibit the synthesis of HLIF (34). Other laboratories (3, 28, 33) found that periodate treatment of HLIF resulted in a decrease in molecular weight, as well as elimination of size and charge heterogeneity, indicating that this interferon, like HFIF, contains carbohydrate; however, periodate destroyed more than 70% of the activity of the HLIF. Other information about the molecular structure of these interferons includes observations that each contains sulfur as disulfide (22, 31, 36) and thiol groups (6, 8, 27) and that disulfide reduction in HLIF results in a loss of antiviral activity, whereas HFIF is stable to such reduction. Although sensitivity to trypsin remains a criterion for establishing interferons as proteins (21), their susceptibility to hydrolysis by various other proteases has not been studied systematically.

Recently, preliminary amino acid compositions and sequences of the first 13 or 20 amino-terminal residues have been determined for fibroblast (19) and lymphoblastoid (40) interferons; no apparent homologies were found. We have undertaken an analysis of human interferons, with enzymes that cause known, specific structural modifications. In this study, the sen-

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sitivity of highly purified HFIF and partially purified HLIF to several proteolytic and glycolytic enzymes was determined with respect to changes in isoelectric points (pI), molecular weights, antiviral activities, and thermal stabilities. Our results with highly specific proteases and glycosidases suggest marked structural differences between HFIF and HLIF.

MATERIALS AND METHODS

Interferons. HFIF was purified by immunoaffinity chromatography in the laboratory of C. Anfinsen, National Institutes of Health, from crude fibroblast interferon which was prepared by J. Vilcek by superinduction with polyinosinic acid-polycytidylic acid in the FS-4 strain of human foreskin cells (13). This HFIF preparation had a specific activity of 2.7×10^6 U/mg of protein after 450 μg of horse heart cytochrome *c* per ml was added to help stabilize the interferon and to act as an internal protein marker. HLIF, which was supplied by Kari Cantell, Helsinki, Finland, was induced by Sendai virus in human leukocytes, subjected to treatment at pH 2 for 24 h at 4°C to inactivate the virus, and then purified by differential precipitation at low pH in 0.5 M KSCN (7); this HLIF preparation (PIF) had a specific activity of 10^6 U/mg of protein.

Interferon assay. The antiviral activity of interferon samples was determined from graphic plots of inhibition of yields of encephalomyocarditis viral hemagglutinin in the BUD-8 strain of human skin fibroblasts (15). With this assay, the geometric mean titer of five measurements of National Institutes of Health human fibroblast standard G023-902-527 was 76,400 ± 13,500 U, or about seven times the 10,000 U assigned to this standard; the mean titer of seven determinations of National Institutes of Health HLIF G023-901-527 was 222,000 ± 148,000 U, or about 10 times the 20,000 U assigned to this standard.

Thermal stability. The thermal stability of interferon samples was determined at 68°C. For these tests, 0.1 ml of sample was placed in a sterile, plastic, screw-capped tube (12 by 175 mm; Falcon Plastics, Oxnard, Calif.) and heated for specific intervals. After heating, samples were allowed to cool to room temperature for 10 min. Samples were stored on ice for up to 60 min or at −70°C for longer periods (days) until they were assayed.

SDS-PAGE. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 0.2-ml samples were electrophoresed on cylindrical gels containing 7% acrylamide and 0.1% SDS for 5 to 8 h at 8 mA/gel in 0.1 M sodium phosphate buffer (pH 7.0) by the method of Weber and Osborn (38). Interferon samples in 0.1 M sodium phosphate buffer were prepared for electrophoresis by incubation in 1% SDS at 37°C for 1 h. Ovalbumin (molecular weight, 46,000), soybean trypsin inhibitor (26,000), and cytochrome *c* (12,500) were used as standards. After electrophoresis, the gels were either fixed in 10% trichloroacetic acid and stained with Coomassie brilliant blue G250 (26) or sliced into 2- to 3-mm pieces, from which the interferon was eluted for 1 h at room temperature into 0.25 ml of minimal essential medium in Earle solution (10) supplemented with 4% fetal bovine serum; the base-line value obtained by this method was 100 U/ml. For molecular weight comparisons, corrections for gel shrinkage were made by measuring gels before and after fixing with trichloroacetic acid and by measuring the position in the gel of the cytochrome *c* present in all samples. Untreated HFIF had a molecular weight of 24,000, compared with the value of 20,000 reported by others (3, 14, 18); HLIF was present in two forms, which had molecular weights of 17,000 and 22,000 (9, 20). Samples were stored at −70°C until assayed.

Isoelectric focusing. Gel sheets of 5% acrylamide containing 2.4% (wt/vol) ampholines in the range 3.5 to 9.5 were obtained from LKB, Broma, Sweden. The solution at the anode was 1 N H_3PO_4, and the solution at the cathode was 1 N NaOH. Samples (50 μl) were electrofocused at 50 mA/gel; cytochrome *c* and hemoglobin were used as visible markers. Gels were sliced as described above for SDS-PAGE or fixed in 10% trichloroacetic acid and stained with Coomassie brilliant blue G250.

Enzymes and enzyme assays. L-Leucine aminopeptidase (LAP) (455 U/mg of protein), designated free from other protease activity, was purchased from P-L Biochemicals, Milwaukee, Wis.; proteolytic activity was determined spectrophotometrically by following the cleavage of leucinamide at 238 nm. The activity of three-times-crystallized trypsin (15 U/mg; P-L Biochemicals) was measured by following the hydrolysis of benzoyl-L-tyrosine ethyl ester at 256 nm. Carboxypeptidases A (200 U/mg) and B (170 U/mg), which were purchased from P-L Biochemicals, were from beef pancreas and were treated with diisopropylfluorophosphate, and their activities were measured by monitoring the cleavage of hippuryl-L-phenylalanine and hippuryl-L-arginine, respectively, at 254 nm. All proteolytic enzymes purchased from P-L Biochemicals were designated as suitable for structural studies of proteins. The activity of purified *Vibrio cholerae* neuraminidase (500 U/ml; GIBCO Laboratories, Grand Island, N.Y.), which was reported to be free from proteases, aldolase, and lecithinase and showed one stainable band upon isoelectric focusing, was determined either by measuring the release of sialic acid from fetuin, using the thiobarbituric acid assay of Webster and Pereira (39), or by cleavage of methoxyphenylneuraminic acid (29). β-Galactosidase (300 U/mg; chromatographically purified; P-L Biochemicals) was assayed spectrophotometrically by following the cleavage of *o*-nitrophenyl-β-D-galactoside at 420 nm. Enzyme treatments did not interfere with the interferon assay.

Protein determination. Proteins were assayed by the Coomassie brilliant blue G250 method of Sedmak and Grossberg (30), using bovine serum albumin as a standard.

RESULTS

Exopeptidase treatments of HFIF. Sequential degradation of HFIF was attempted from the amino-terminal end with LAP or from the carboxy-terminal end with carboxypeptidases A and B. LAP treatment did not inactivate

HFIF even after 5 h of incubation at 25°C and pH 7.5. Isoelectric focusing of LAP-treated HFIF revealed a significant change in the pI distribution; whereas untreated HFIF had a major peak with a pI of 5.8 and minor peaks at pI values of 6.3 and 8.5, treated HFIF had a major pI peak at 4.0 and some activity in the pI range of 4.2 to 5.0 (Fig. 1A). These changes in isoelectric nature were accompanied by a very slight but reproducible shift in apparent molecular weight, as detected by SDS-PAGE (Fig. 1B). Untreated HFIF by our measurements had a major peak with a molecular weight of approximately 24,000 and a small shoulder of activity at 30,000. LAP treatment consistently resulted in a single peak of activity at an apparent molecular weight of about 22,000.

Carboxypeptidase A treatment resulted in a negligible loss of original interferon activity, whereas carboxypeptidase B caused a 45% loss (Table 1). Isoelectric focusing of carboxypeptidase A- and B-treated interferon showed a shift in activity to a pI of 3.0 or lower (Fig. 1C). A shift in molecular weight from 24,000 to 20,000 was noted after both carboxypeptidase treatments, representing a loss of approximately 34 to 40 amino acids (Fig. 1D).

Treatment of HLIF with exopeptidases. Unlike the results for HFIF, exposure of HLIF to LAP resulted in losses in antiviral activity of 50% at 15 min (Table 1) and 80% at 180 min; no additional losses were noted after 5 h of exposure. Isoelectric focusing of untreated HLIF showed three bands of activity, a major pI band at 5.3 and two other pI bands at 5.6 and 7.5 (Fig. 2C). The products of LAP treatment of HLIF showed less activity in the species focusing at pH 7.5 and 5.3 and a broader band at pH 5.6.

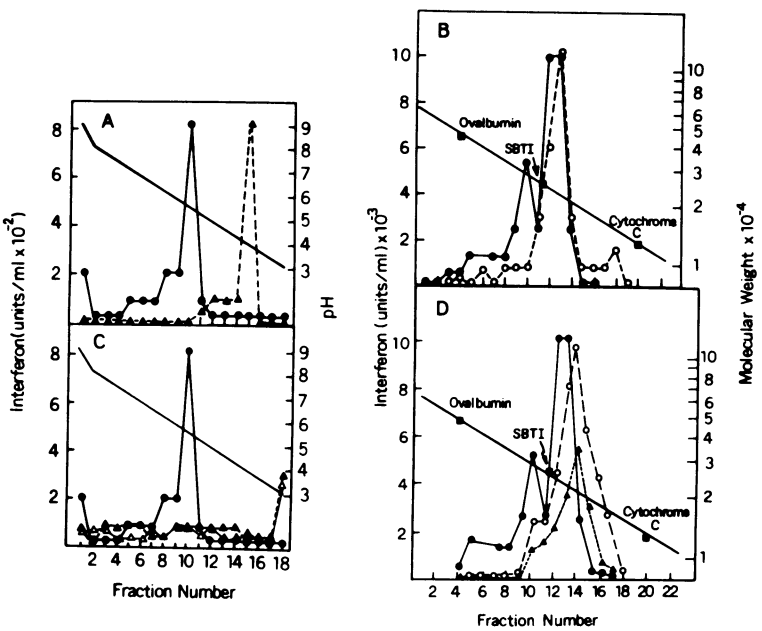

FIG. 1. *Effect of exopeptidase treatment on the molecular weight and isoelectric point of purified HFIF. (A) Isoelectric focusing of interferon treated with LAP. Symbols: ▲, treated (600 U); ●, untreated (600 U). (B) SDS-PAGE of interferon treated with LAP. Symbols: ○, treated (8,000 U); ●, untreated (8,000 U). (C) Isoelectric focusing of interferon treated with carboxypeptidase A (500 U) (▲) and carboxypeptidase B (300 U) (△) and untreated interferon (600 U) (●). (D) SDS-PAGE of interferon treated with carboxypeptidase A (6,400 U) (○) and carboxypeptidase B (4,400 U) (△) and untreated interferon (8,000 U) (●). LAP (0.8 μg of LAP per 4 μg of HFIF) was incubated with interferon for 3 h at 25°C in 0.1 M Tris-hydrochloride, pH 7.5. When the reaction was terminated, the total volume was 1.0 ml. Carboxypeptidase A (0.9 μg of enzyme per 4 μg of HFIF) and carboxypeptidase B (0.85 μg of enzyme per 4 μg of HFIF) were incubated with interferon for 1 h at 37°C in 0.1 M Tris-hydrochloride, pH 7.5. Electrofocusing of samples was on gels containing ampholytes at pH 3 to 10. Gels were sliced into 5-mm fractions, interferon was eluted into cell culture medium, and dilutions were assayed for antiviral activity. Approximately 60 to 80% of the interferon activity was recovered. SDS gels of 10% acrylamide were run at 50 mA/gel for 8 h by the method of Stewart and Desmyter (31). Gels were sliced into 2- to 3-mm fractions, and then interferon was eluted and assayed. Ovalbumin, soybean trypsin inhibitor (SBTI), and cytochrome c were used as markers. Approximately 60 to 80% of the activity applied was recovered.*

SDS-PAGE of LAP-treated HLIF (Fig. 2D) revealed that the majority of the activity was in the 17,000- to 18,000-molecular-weight range and that there was a minor band of activity at about 14,000 molecular weight, whereas the control had two distinct species, at 17,000 and 22,000 molecular weight. Thus, it appears that LAP hydrolyzes the N-terminal ends of the larger-molecular-weight species and that 17,000- and 14,000-molecular-weight species remain.

TABLE 1. *Residual activity and thermal stability of enzyme-treated HFIF and HLIF*

Enzyme treatment[a]	Residual activity			
	After enzyme treatment (% of original)		After heating (% of enzyme-treated control)[b]	
	HFIF	HLIF	HFIF	HLIF
None	100	100	8.4	40
LAP	100	50	8.0	15
Carboxypeptidase A	80	70	6.3	53
Carboxypeptidase B	55	60	14.6	36
Chymotrypsin	70	5	8.0	25
Neuraminidase	100	100	10.0	20
β-Galactosidase	100	100	8.0	25
Neuraminidase and β-galactosidase	100	100	8.0	25
Neuraminidase and LAP	20	2	0.6	80

[a] See figure legends for enzyme concentrations and reaction conditions. LAP, Leucine aminopeptidase.

[b] A total of 10,000 to 40,000 U of interferon and modified interferon per ml in 0.1 M sodium phosphate buffer (pH 7.0) was heated for 30 min at 68°C.

Treatment of HLIF with carboxypeptidase A for 1 h caused a 30% loss of original activity, whereas carboxypeptidase B treatment resulted in a 40% loss (Table 1). The activity lost as a result of carboxypeptidase treatment was predominantly from the HLIF isoelectric moieties banding at pH 5.3 and 5.6, whereas the remaining activity banded at approximately pH 7.5 and 3.0 (Fig. 2A). On SDS-PAGE carboxypeptidase-treated HLIF retained activity in the 21,000- to 23,000-molecular-weight range and showed very little activity in the 17,000-molecular-weight region (Fig. 2B). Thus, the 17,000 dalton form of HLIF appears to correspond to the isoelectric species having pI value(s) of 5.3 to 5.6, since both are relatively sensitive to carboxypeptidase attack, whereas the 22,000-dalton form and the species with a pI value of 7.5 are relatively resistant.

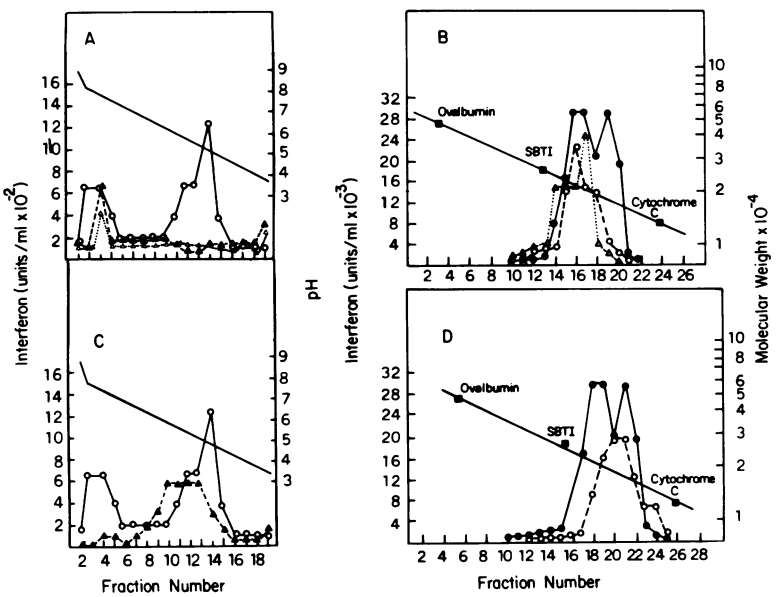

FIG. 2. *Effect of exopeptidase treatment on isoelectric point and molecular weight of HLIF. (A) Isoelectric focusing of interferon treated with carboxypeptidase A (1,000 U) (▲) and carboxypeptidase B (900 U) (△) and untreated interferon (1,500 U) (○). (B) SDS-PAGE of interferon treated with carboxypeptidase A (21,000 U) (○) and carboxypeptidase B (18,000 U) (△) and untreated interferon (30,000 U) (●). (C) Isoelectric focusing of interferon treated with LAP (800 U) (▲) and untreated interferon (1,500 U) (○). (D) SDS-PAGE of interferon treated with LAP (15,000 U) (○) and untreated interferon (30,000 U) (●). Carboxypeptidase A (0.9 μg/5 μg of HLIF) and carboxypeptidase B (0.85 μg/5 μg of HLIF) were incubated with interferon for 1 h at 37°C in 0.1 M Tris-hydrochloride, pH 7.5. LAP (0.8 μg/5 μg of HLIF) was incubated with interferon for 3 h at 25°C in 0.1 M Tris-hydrochloride, pH 7.5. Electrofocusing and SDS gels were run and analyzed as described in the legend to Fig. 1. Approximately 60 to 80% of the activity applied was recovered. SBTI, Soybean trypsin inhibitor.*

Endopeptidase treatments. The sensitivity of interferons to digestion by trypsin (which cleaves bonds at arginine and lysine) has been used extensively as a proof of their protein nature (21). We attempted limited tryptic digestion that might yield smaller, yet active molecules. The extremely rapid inactivation of both HFIF and HLIF at 25°C and pH 7.0 (80% activity lost in 10 min) suggests that only limited attack on peptide bonds involving arginine and lysine is sufficient for inactivation of biological activity.

HFIF was surprisingly resistant to chymotrypsin, which preferentially attacks bonds adjacent to aromatic amino acids (phenylalanine, tyrosine, tryptophan); 64% of the original activity remained after treatment for 30 min at 37°C and pH 7.0. Isoelectric focusing of the treated interferon showed that more than 95% of the activity had a pI of about 3.0 (Fig. 3A), suggesting that at least one bond can be cleaved with little or no loss of activity. SDS-PAGE showed only an insignificant change in the molecular weight (from 24,000 to 22,000) (Fig. 3B). The additional losses of activity which were observed after prolonged chymotrypsin treatment could have been due to the cleavage of other bonds, possibly those involving serine and leucine (11).

In contrast, chymotrypsin rapidly inactivated HLIF, with a loss of 36% of antiviral activity within 30 s and a loss of 95% after 30 min of treatment. The activity remaining after chymotrypsin treatment had a pI of about 3.0 (Fig. 3C). By SDS-PAGE chymotrypsin-treated HLIF migrated in a broad band with an average molecular weight of 21,000 (Fig. 3D), indicating that the 17,000-molecular-weight species was more sensitive to chymotrypsin activity than the larger species.

Subtilisin BPN is an endopeptidase similar to chymotrypsin, but it is less specific and is able to cleave at the carboxy-terminal side of aromatic (phenylalanine, tyrosine, tryptophan) and apolar (leucine, serine, glycine) amino acids. Subtilisin (25 μg/ml) rapidly inactivated both interferons; 98.8% of HFIF activity and 98.4% of HLIF activity were lost in 15 min at 25°C and pH 7.5.

Bromelain, an endopeptidase able to cleave bonds between arginine (position 18) and alanine (position 19) or between alanine (position 19) and glutamine (position 20) in glucagon (24) and exclusively between phenylalanine (position 5) and serine (position 6) in bradykinin (24), at 80 μg/ml destroyed 60% of HFIF activity and 40%

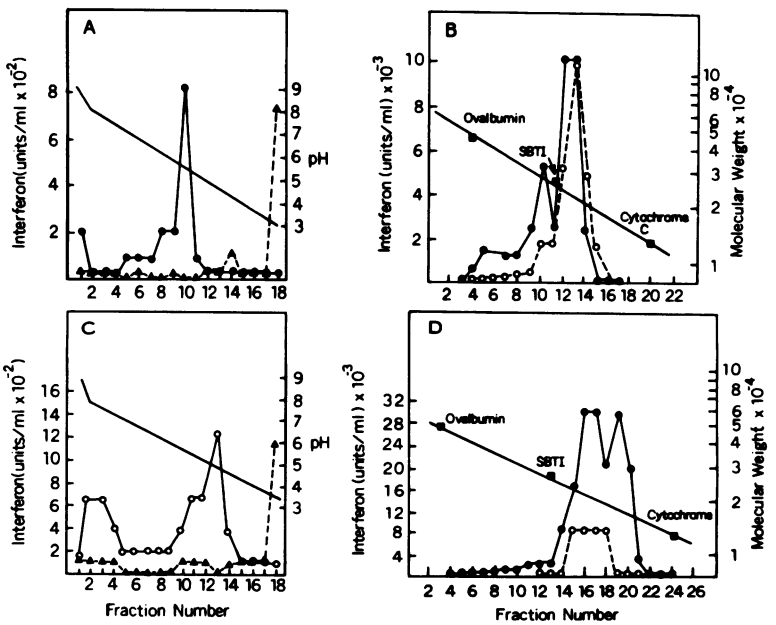

FIG. 3. *Effect of chymotrypsin on the molecular weights and isoelectric points of HFIF (A and B) and HLIF (C and D). (A and C) Isoelectric focusing of treated (▲) and untreated (○ and ●) interferons. (B and D) SDS-PAGE of treated (○) and untreated (●) interferons. Interferon (40,000 U of HFIF or 50,000 U of HLIF) was exposed to chymotrypsin (1 μg/30 μg of HFIF or 50 μg of HLIF) for 30 min at 37°C in 0.1 M sodium phosphate, pH 7.0. The reaction was stopped by adding 50 μg of soybean trypsin inhibitor (SBTI) per ml. The gels were analyzed as described in the legend to Fig. 1. The untreated control profiles from Fig. 1 and 2 are included for comparison.*

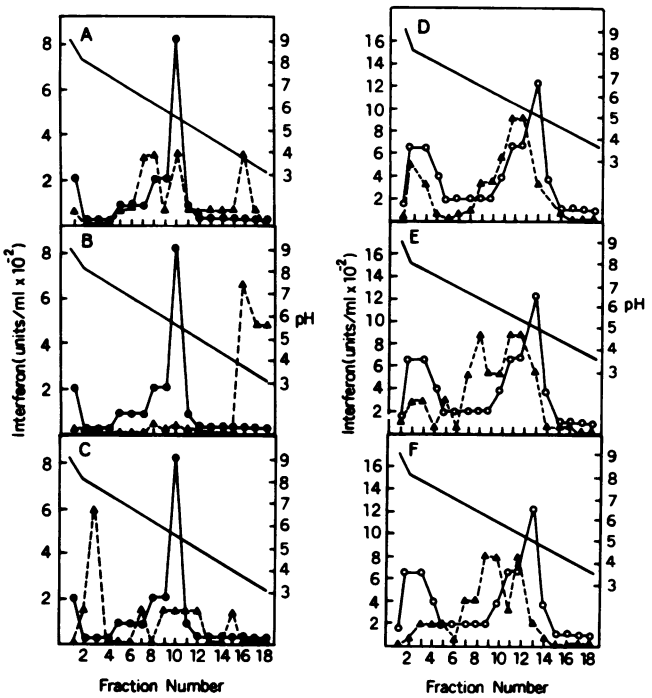

FIG. 4. *Isoelectric distribution of HFIF (A through C) and HLIF (D through F) treated with glycosidases. (A and D) Interferons were exposed to V. cholerae neuraminidase (5 U/125 μg of interferon) for 60 min at 37°C in 0.1 M sodium phosphate (pH 5.9) containing 1 mM $CaCl_2$. Symbols: ▲, treated; ○ and ●, untreated. (B and E) Interferons were exposed to β-galactosidase (1 μg/2 μg of interferon) for 60 min at 37°C in 0.1 M sodium phosphate, pH 6.0. Symbols: ▲, treated; ○ and ●, untreated. (C and F) Interferons were exposed to both neuraminidase and β-galactosidase at the concentrations indicated above. Symbols: ▲, treated; ○ and ●, untreated. Isoelectric focusing was performed as described in the legend to Fig. 1. A total of 600 U of treated or untreated HFIF and 1,500 U of treated or untreated HLIF were applied to the gels.*

of HLIF activity in 5 min and 99.2% of HFIF activity and 98% of HLIF activity in 30 min. Pepsin (another endopeptidase which at low pH cleaves bonds adjacent to dicarboxylic acids and aromatic amino acids) was used because its activity is greatest at low pH, where interferon is most stable. Pepsin (10 μg/ml) destroyed 75% of both interferon activities in less than 1 min and 98% in 5 min.

Glycosidases. Exposure to neuraminidase and/or β-galactosidase for 60 min at 37°C resulted in no loss in biological activity of either HFIF or HLIF. However, the isoelectric values of HFIF treated with neuraminidase were altered (Fig. 4A). Three bands of activity were found; these included two new bands at pH 6.6 and 3.8 and one band coincidental with the major band of untreated interferon at pH 5.8. Further treatment with neuraminidase for up to 3 h resulted in no additional changes in isoelectric points. Analysis of treated HFIF by SDS-PAGE (Fig. 5A) showed one peak of antiviral activity at 23,000 daltons.

β-Galactosidase treatment for 60 min at 37°C did not affect the antiviral activity of HFIF, but all of the activity in the treated sample was in a band extending from about pH 3.1 to 3.8 (Fig. 4B). This shift in isoelectric point was accompanied by a loss in molecular weight from 24,000 to 20,000 (Fig. 5B). Simultaneous treatment for 60 min with neuraminidase and β-galactosidase did not affect the activity of HFIF but altered the electrophoretic and isoelectric mobilities of this molecule (Fig. 4C and 5C). The majority of the activity had a pI of 7.5, and there were minor bands at 3.8, 5.8, and 6.8. The major peak of activity in SDS-PAGE was at 20,000 daltons, although some activity of lower apparent molecular weight was noted. These results suggest that sialic acid and galactose are present in terminal positions on the carbohydrate moieties of HFIF and that the removal of either compound does not affect activity.

Although HLIF activity was not affected by glycosidases, its pI distribution changed. Neuraminidase slightly reduced the activity in the

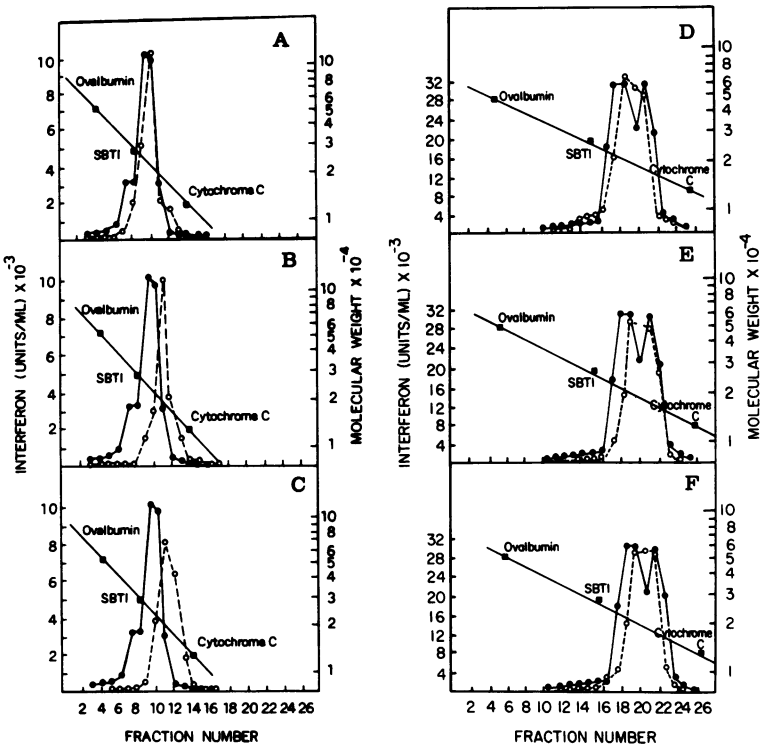

FIG. 5. *SDS-PAGE of HFIF (A through C) and HLIF (D through F) treated with glycosidases. (A and D) Symbols: ○, neuraminidase treated; ●, untreated. (B and E) Symbols: ○, β-galactosidase treated; ●, untreated. (C and F) Symbols: ○, treated with both neuraminidase and β-galactosidase; ●, untreated. Enzyme treatments were performed as described in the legend to Fig. 4, and electrophoresis was performed as described in the legend to Fig. 1, except that the duration of electrophoresis was 6 h. A total of 8,000 U of treated or untreated HFIF and 30,000 U of treated or untreated HLIF were applied to the gels. SBTI, Soybean trypsin inhibitor.*

band at pH 5.6 (Fig. 4D). β-Galactosidase treatment caused shifts in the isoelectric pattern (Fig. 4E); activity was lost in the bands at pH 7.5 and 5.3, activity increased slightly at pH 5.6, and a new band appeared at about pH 6.5. SDS-PAGE (Fig. 5D and 5E) showed a slight increase in HLIF activity in the molecular weight range between 17,000 and 20,000 with either enzyme. HLIF exposed simultaneously to both neuraminidase and β-galactosidase exhibited an isoelectric pattern (Fig. 4F) and a molecular weight distribution (Fig. 5F) essentially the same as those observed with β-galactosidase alone.

Since LAP did not affect activity and only slightly altered the molecular weight of HFIF, it was thought that a carbohydrate moiety might interfere with the action of LAP. To test this hypothesis, HFIF was treated first with neuraminidase for 60 min at 37°C and then with LAP for 120 min. After 30 min of LAP treatment, only 20% of the untreated control activity remained, suggesting that *N*-acetylneuraminic acid may block the action of LAP at or near the amino-terminal end of the molecule. A similar treatment of HLIF was carried out to determine whether the apparent resistance of the 17,000-dalton species was due to sialic acid. As with HFIF, combined treatment resulted in extensive inactivation; only 2% of the activity remained after neuraminidase-treated HLIF was treated with LAP for 30 min.

Thermal stability. Table 1 shows the effects of each enzyme on biological activity and thermal (68°C) stability at pH 7.0. The enzyme treatments had little or no effect on the thermal stability of HFIF, whether or not they reduced original activity. A possible exception was the combined treatment by both neuraminidase and LAP, which reduced both activity and stability. For HLIF, significant changes were observed in only two cases. HLIF treated with LAP was thermolabile, whereas HLIF treated with the combination of neuraminidase and LAP was more resistant to heat inactivation. This apparent increased resistance after the combined treatment must be interpreted in light of the small amount of activity (2% of the original) that remained.

DISCUSSION

Some insight has been gained into structural requirements for activity of HFIF and both molecular weight species of HLIF. Both HFIF and the 22,000-dalton species of HLIF have unblocked N termini sensitive to limited digestion by LAP. However, inactivation by LAP results if the interferons are first exposed to neuraminidase. The mechanism for the neuraminidase-facilitated inactivation of the interferons is not known. The C termini of both HFIF and the 17,000-dalton species of HLIF are unblocked, as evidenced by the action of the carboxypeptidases, whereas the 22,000-dalton HLIF species may have a blocked carboxy terminus which is insensitive to the carboxypeptidases.

The sensitivities of HFIF and HLIF to specific endoproteolytic attack were similar, with one notable exception. Whereas both interferons lost activity after brief exposure to trypsin, pepsin, subtilisin, or bromelain, only HFIF was resistant to inactivation by chymotrypsin; there was little effect on activity or molecular weight after prolonged exposure, although the changes in the isoelectric points of HFIF may indicate limited hydrolysis. Some tentative conclusions about the nature of the accessible amino acids of these interferons can be based on their sensitivity or resistance to the endoproteases employed. Since trypsin, bromelain, and subtilisin attack lysine, arginine, serine, glycine, and alanine residues, these amino acids must be exposed on both HFIF and HLIF. HLIF sensitivity to chymotrypsin indicates the presence of exposed phenylalanine, tyrosine, tryptophan, leucine, methionine, asparagine, or glutamic acid residues, whereas these residues are unavailable on HFIF for attack by chymotrypsin.

The inaccessibility of tyrosine in HFIF to chymotrypsin does not eliminate the possibility of its presence in the molecule, but only the inability of the enzyme to cleave adjacent to it. Berthold et al. (2) have shown by using chemical modification that tyrosine residues are important for the activity of human fibroblastoid interferon. Jankowski et al. (16) have suggested that hydrophobic interaction of HFIF with certain lectins may involve insertion of the lectin into a hydrophobic pocket rather than a binding at the surface. Such a view is consistent with our evidence indicating a lack of amino acids with hydrophobic side chains (such as phenylalanine, tryptophan, methionine, and leucine) at the surface. However, other amino acids with hydrophobic side chains (such as proline, isoleucine, alanine, and valine) could still account for surface hydrophobic interactions; indeed, surface alanine may be present, as indicated by bromelain sensitivity.

It is not surprising that individual glycosidases have no effect on activity or stability since removal of carbohydrates by a mixture of glycosidases was shown previously to have no effect on activity (3); indeed, HFIF could be synthesized in cell-free extracts, presumably without carbohydrates (25). The fact that neuraminidase action provides a more heterogeneous population of HFIF is difficult to explain but may indicate that the terminal sugar residues of the carbohydrate side chain must vary. Our results with highly purified enzyme and interferon differ from those reported by Morser et al. (23), who suggested that sialic acid alone is responsible for the charge heterogeneity; their sialidase preparation reduced the three isoelectric species of crude HFIF to one. Such differences in results may depend on the degree of prior exposure to acid pH, which can hydrolyze glycosidic bonds. However, β-galactosidase, which attacks β-1,6 glycosidic linkages when galactose is the terminal sugar, produces a shift in the whole population of HFIF molecules. Thus, all molecules seem equally sensitive to galactosidase; that is, they may have carbohydrate chains with terminal galactose residues such that the penultimate charge is the same for all molecules. The small apparent shifts in molecular weight which we determined by SDS-PAGE after glycosidase treatment are consistent with the 4,000-dalton changes found by Bridgen et al. (5) for human lymphoblastoid interferon. Glycosidase treatment of HLIF revealed that the 22,000-dalton species is more glycosylated than the 17,000-dalton species; this agrees with the finding of Stewart et al. (33), who showed that extensive periodate treatment, which they assumed removed only carbohydrate, reduced the 21,000-molecular-weight species to the 15,000-molecular-weight size. Furthermore, Rubinstein et al. (27) reported that a homogeneous species of HLIF with a molecular weight of 17,500 had little, if any, carbohydrate. Our results suggest that the species at 22,000 daltons has an isoelectric point of around 7.5 and contains both galactose and sialic acid, a free amino terminus, and a carboxy terminus which is blocked, at least partially. The species at 17,000 daltons has an isoelectric point of 5.6, no exposed sialic acid or galactose, and free carboxy and amino termini. Since interferon becomes more susceptible to hydrolysis by LAP after neuraminidase treatment, it is possible that one function of the carbohydrate on this glycoprotein is to protect it from proteases.

Our studies provide additional evidence that there are important structural differences between HFIF and HLIF and between the two molecular weight forms of HLIF; whereas the

activity of HFIF is insensitive to the action of LAP and largely resistant to chymotrypsin, the two molecular weight forms of HLIF show some differential sensitivities, especially to carboxypeptidases A and B. However, both molecular weight forms of HLIF are very sensitive to the action of chymotrypsin (an observation very recently reported by Braude et al. [4]). Construction of models of the molecular conformations of the biologically active forms of interferon based on amino acid sequence data may be facilitated by knowledge of their sensitivities to proteases and glycosidases. Whether biological activities other than antiviral activity ascribed to human interferons (1), such as cell growth inhibition, differential antigenicity, and degree of heterospecific cell activities, are altered by such enzyme treatments remains to be determined.

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