Molecular Size Heterogeneity of Human Leukocyte Interferon†

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ABSTRACT: Molecular sieving of human leukocyte interferon revealed an apparent molecular weight of 26 000. However, after denaturation by guanidine hydrochloride in the presence of a reducing agent and reactivation by extensive dialysis, a molecular weight of only 21 000 was observed. The reactivated human leukocyte interferon (mol wt 21 000) gave a single peak of activity when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, confirming that a single molecular weight species was generated by the denaturation and reactivation procedure. A partial unfolding of the molecule was evident when the interferon preparation was heated to 50 °C in the absence or presence of an unfolding agent and then sieved on Sephadex G-100 Superfine. These results suggest that the interferon molecule undergoes a proteolytic cleavage probably by a protease present in extracellular fluid. Thus, a peptide fragment dissociates from the parent molecule when human leukocyte interferon is denatured in the presence of a reducing agent, resulting in a drop of 5000 in molecular weight; interestingly, the resultant 21 000 molecular weight form still retains its antiviral activity.

The heterogeneity of human leukocyte interferon (HL-IF) has now been well established, although the molecular basis for this heterogeneity has remained an enigma. For example, two peaks of leukocyte interferon are eluted from antileukocyte interferon antibody column (Anfinsen et al., 1974). After denaturation, two peaks of activity corresponding to different molecular weights are also observed on $NaDodSO_4$–polyacrylamide gel electrophoresis[1] (Stewart and Desmyter, 1975); two components are also noted during hydroxylapatite chromatography of HL-IF in the presence of $NaDodSO_4$ (Törmä and Paucker, 1976). Chromatography of HL-IF under conditions which do not result in partial (low pH) or extensive ($NaDodSO_4$) denaturation of the molecule also reveal considerable heterogeneity: two chromatographic components are readily obtained on both DEAE-Bio-Gel A and bovine serum albumin–CH-Sepharose 4B (Chen et al., 1976). Similarly, the chromatography of HL-IF on blue dextran–agarose results in separation of two components (Jankowski et al., 1976). Remarkably, molecular sieving of HL-IF fails to unravel molecular size heterogeneity under conditions of low pH (Anfinsen et al., 1974), physiological pH (Chen et al., 1976; Jankowski et al., 1976), and in the presence of $NaDodSO_4$ (Törmä and Paucker, 1976). In all instances the molecular weight of HL-IF, as determined by molecular sieving, is 26 000. However, two molecular forms can be observed when its molecular weight is estimated by $NaDodSO_4$ gel electrophoresis (one component with a mol wt of 15 000 and another of 21 000).

Clearly, it would be of considerable importance to understand the basis for this molecular weight heterogeneity. The heterogeneity of HL-IF on ion-exchange may involve only minor differences in the overall charge of the molecule. The size heterogeneity, by contrast, is of paramount importance as it directly relates to the question of the smallest possible HL-IF polypeptide which preserves biological activity.

In earlier studies, we suggested that the differences in molecular weight estimates might be due to limited proteolysis of the native HL-IF molecule (Chen et al., 1976); namely, a significant drop in molecular weight would be observed on $NaDodSO_4$ gel electrophoresis because a polypeptide fragment was removed under the denaturing conditions of detergent treatment. Our current report presents the first data in direct support of this hypothesis.

Experimental Procedures

Chemicals and Reagents. All molecular weight standards, sodium dodecyl sulfate, and ammonium persulfate were purchased from Sigma Chemical Co. Guanidine hydrochloride (Ultra Pure) was obtained form Schwarz/Mann. Sephadex G-100 Superfine and blue dextran were obtained from Pharmacia Fine Chemicals, Inc. Bovine serum albumin, used for stabilization of interferon activity after column fractionation, was purchased from Pentex. Acrylamide, methylenebisacrylamide, *N,N,N′,N′*-tetramethylethylenediamine, β-mercaptoethanol, and bromophenol blue were from Eastman. Other chemicals were reagent grade.

Interferon Preparation and Assay. HL-IF was prepared according to the procedure of Pidot et al. (1972), by infecting leukocytes, obtained from normal donors, with Newcastle disease virus. Interferon assays were performed by the colorimetric technique of Finter (1969). Human fibroblast cells (CL-604), used for interferon assays, were maintained in Eagle's medium (Eagle, 1959) supplemented with 10% fetal calf serum.

Molecular Sieving. The apparent molecular weight of HL-IF was determined by molecular sieving on Sephadex G-100 Superfine. The column (82 × 1.5 cm) was equilibrated and eluted with 0.15 M NaCl in 0.02 M sodium phosphate buffer, pH 7.4 ($NaCl/PO_4$), at 4 °C. The flow rate was adjusted to 3 mL/h and 0.5-mL fractions were collected. To calibrate the column, the following internal markers were used: bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen (25 000), and bovine pancreatic ribonuclease (13 700). Interferon fractions were collected into 0.5 mL of a 1% solution of bovine serum albumin in $NaCl/PO_4$. After its initial packing, each column used for molecular weight determination was calibrated several times until no drift in the elution pattern of molecular weight standards was seen. Im-

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[1] Abbreviations used: HL-IF, human leukocyte interferon; $NaCl/PO_4$, 0.15 M sodium chloride in 0.02 M phosphate buffer, pH 7.4; $NaDodSO_4$, sodium dodecyl sulfate; $NaDodSO_4$ gel electrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl.

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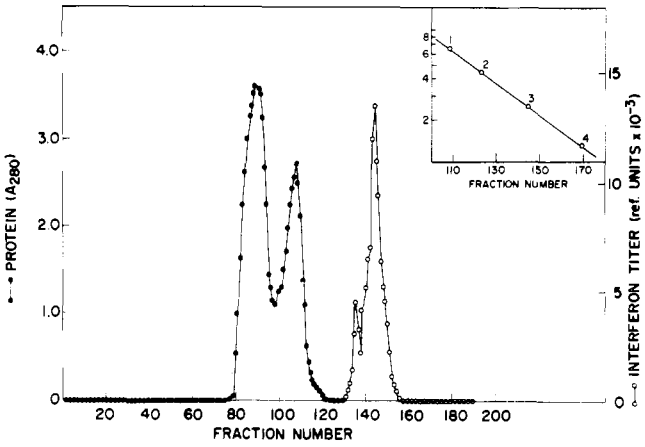

FIGURE 1: Molecular weight determination of HL-IF on Sephadex G-100 Superfine. Two milliliters of an interferon preparation, containing 50 000 units/mL, were dialyzed against NaCl/PO_4 for 18 h at 4 °C. The dialyzed interferon was then centrifuged at 10 000*g* for 15 min and applied to a column (85 cm × 1.25 cm) equilibrated with NaCl/PO_4 at 4 °C. The column was developed with NaCl/PO_4 and 0.5-mL fractions were collected at a flow rate of 3 mL/h. Interferon fractions were collected in 0.5 mL of 1% bovine serum albumin (Pentex) in NaCl/PO_4. Molecular weight standards were: (1) bovine serum albumin (67 000), (2) ovalbumin (45 000), (3) chymotrypsinogen (25 000), and (4) bovine pancreatic ribonuclease A (13 700). Samples were assayed for protein (●—●) and interferon activity (○—○). The overall recovery of interferon activity was 95%.

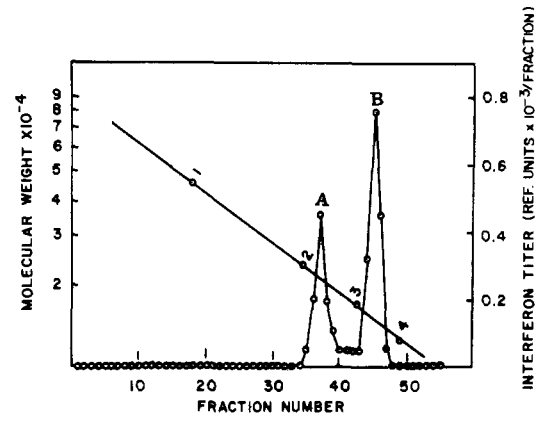

FIGURE 2: $NaDodSO_4$ gel electrophoresis of HL-IF. Interferon was first sieved as described in Figure 1. The peak fractions of interferon (mol wt 26 000) were pooled, concentrated, dialyzed against 0.1 M phosphate buffer (pH 7.5), and then heated to 100 °C for 2 min in a water bath in the presence of 1% $NaDodSO_4$. Aliquots of 100 μL were then applied to gels. One hundred percent of the interferon activity was recovered. Molecular weight standards were: (1) peroxidase II (44 000), (2) α-chymotrypsinogen-A (23 560), (3) myoglobin type III (16 890), and (4) ribonuclease type I-A (13 700). Migration is from left to right, and each fraction represents 0.2 cm of the gel.

portantly, each molecular weight determination of HL-IF was both preceded and followed by a calibration run to confirm the precision of each HL-IF molecular weight determination.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Disc $NaDodSO_4$ gel electrophoresis was performed as described by Summers et al. (1965). The electrophoresis buffer was 0.1 M phosphate, pH 7.5, containing 0.1% $NaDodSO_4$ and separating and stacking gels each contained 0.1 M phosphate buffer (pH 8.0) and 0.1% $NaDodSO_4$. Protein standards were dissolved in electrophoresis buffer (1 mg/mL) and heated to 100 °C for 2 min in a boiling water bath; standards were run both singly and as mixtures. Bromophenol blue was used as tracking dye; electrophoresis was stopped when the dye had migrated to about 140 mm. Gels with protein standards were stained with Coomassie brilliant blue and destained with a glacial acetic acid:methanol mixture until clear; they were stored in a 7.5% acetic acid solution. Gels containing interferon were sliced into 0.2-cm segments and placed in 1 mL of the culture medium with 2% fetal calf serum. After elution at 4 °C overnight on a shaking platform, samples were assayed for interferon.

Results

The sieving of HL-IF on Sephadex G-100 Superfine is shown in Figure 1. The major peak of interferon activity corresponds to a mol wt of 26 000, in good agreement with earlier results obtained by molecular sieving (Anfinsen et al., 1974; Törmä and Paucker, 1976; Chen et al., 1976; Jankowski et al., 1976). It is noteworthy that no interferon activity was ever seen in the region of mol wt 21 000 to 15 000. However, in sieving experiments of five different HL-IF preparations, we did note in a small peak of interferon in the region of mol wt 31 000. This minor component (about 5%) may represent an unprocessed or glycosylated form of HL-IF.

HL-IF fractions from a Sephadex column containing the major interferon peak (mol wt 26 000) were pooled, concentrated, and electrophoresed in 10% $NaDodSO_4$ gels. Earlier, it had been reported that $NaDodSO_4$ gel electrophoresis separates two populations of HL-IF, one of mol wt 15 000 and another of 21 000 (Stewart and Desmyter, 1975). As shown in Figure 2, we also saw two components of interferon corresponding to mol wt 21 000 (A) and 15 000 (B). The relative distribution of interferon activity among these components varied in different preparations, but generally component A contained 40% of the activity and component B 60%.

To reconcile the discrepancy in molecular weight estimates of HL-IF as determined by the two techniques, we proceeded to: (i) denature the molecule by guanidine hydrochloride under reducing conditions; (ii) reactivate it by exposure to molecular oxygen with a concomitant removal of denaturing agents; and (iii) estimate its molecular weight by both molecular sieving and $NaDodSO_4$ gel electrophoresis. The denaturation conditions by guanidine hydrochloride were those described by Mogensen and Cantell (1974); HL-IF preparations were denatured in 5 M guanidine hydrochloride and 0.1 M β-mercaptoethanol at 50 °C for 18 h.

To avoid possible recombination of polypeptide fragment with the remainder of the parent molecule during reactivation, we sieved the denatured HL-IF through a Sephadex G-100 column equilibrated with 5 M guanidine hydrochloride containing 0.1 M β-mercaptoethanol. The fractions corresponding to molecular weight in excess of 10 000 were collected, thus separating the high molecular weight HL-IF from any of the smaller molecular weight peptides which were dislodged during denaturation. The high molecular weight fractions were extensively dialyzed and sieved on a Sephadex G-100 Superfine column: this reactivated HL-IF revealed a molecular weight of 21 000 as illustrated in Figure 3. Therefore, under conditions of denaturation and reactivation, the molecular weight of reactivated HL-IF is reduced by 5000. Even when sieving in the presence of guanidine hydrochloride was omitted, reactivated HL-IF also consistently revealed a molecular weight of 21 000. Thus, the 5000 mol wt fragment must not be able to rejoin efficiently during reactivation; if rejoining with the major portion of the molecule were occurring to a significant extent, a 26 000 mol wt species should be detected when denatured HL-IF is reactivated in the presence of its smaller fragment.

Since reduction of HL-IF was carried out in 5 M guanidine hydrochloride at 50 °C, the temperature itself could have lead

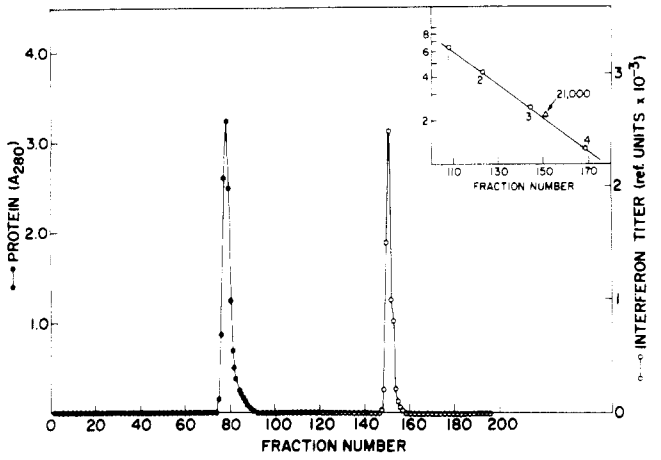

FIGURE 3: Molecular weight determination of HL-IF on Sephadex G-100 Superfine following denaturation and reactivation. Two milliliters of an interferon preparation containing 10 000 units/mL was dialyzed against 0.05 M sodium acetate buffer (pH 5.0) for 18 h at 4 °C. The dialyzed preparation was then centrifuged at 10 000*g* for 15 min and then reconstituted to contain 5 M guanidine hydrochloride and 0.1 M β-mercaptoethanol. The sample was sealed in a glass ampule and heated to 50 °C for 18 h; it was then dialyzed against several changes of $NaCl/PO_4$ (5 × 1 L). The dialyzed sample (2 mL containing 10 000 units of interferon) was then applied to a Sephadex column (Figure 1). Samples were assayed for protein (●—●) and interferon activity (○—○). Eighty-five percent of the activity applied to the column was recovered.

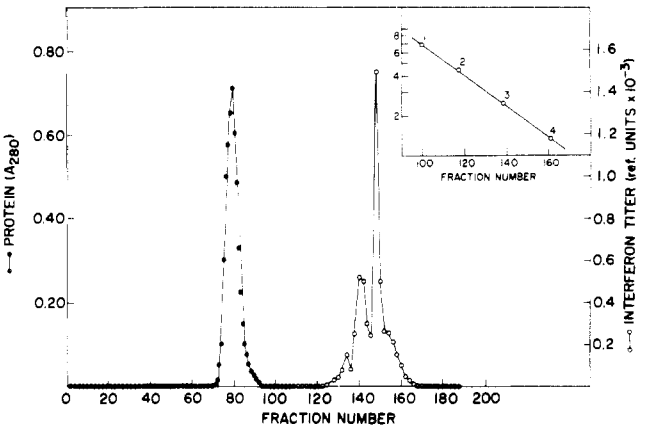

FIGURE 4: Molecular weight determination of HL-IF, after heating to 50 °C, on Sephadex G-100 Superfine. Two milliliters of an interferon preparation containing 10 000 units/mL was dialyzed against $NaCl/PO_4$, centrifuged, and heated to 50 °C for 18 h in a sealed ampule. The heated sample was dialyzed against several changes of $NaCl/PO_4$ and applied to the column (Figure 1). Samples were assayed for protein (●—●) and interferon activity (○—○). Interferon recovery was 25% activity of the original sample and 100% of the activity applied to the column.

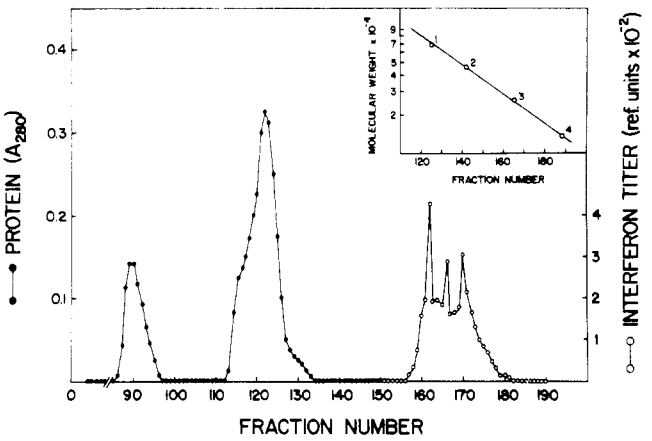

FIGURE 5: Molecular weight determination of HL-IF, after heating in the presence of guanidine hydrochloride, on Sephadex G-100 Superfine. An interferon sample, prepared as described in Figure 4, was reconstituted to contain 5 M guanidine hydrochloride and heated to 50 °C for 18 h. The heated preparation was dialyzed against several changes of $NaCl/PO_4$ (5 × 1 L) and applied to the column (Figure 1). Samples were assayed for protein (●—●) and interferon activity (○—○). The interferon activity fell to 25% of the activity prior to heating, and 75% of the activity applied to the column was recovered.

directly to the observed drop in molecular weight. To test this idea, we heated HL-IF to 50 °C in the absence of both the unfolding and reducing agents. A significant loss of interferon activity, varying between 75 and 85% in different experiments, was observed. The heated interferon preparation was then sieved and, as shown in Figure 4, activity was spread in a wide molecular weight area from 25 000 to 20 000. This downward displacement and widened breadth of elution strongly suggested a partial unfolding and dislodging of a peptide fragment from the molecule simply upon heating.

A further collapse could be induced when interferon was heated to 50 °C in the presence of the unfolding agent, 5 M guanidine hydrochloride, and in the absence of reducing agent. On sieving (Figure 5), the HL-IF displayed both an overall reduction in molecular weight and a suggestion of three species ranging from 25 000 to 18 000.

The data of Figures 3–5 illustrate the three important features describing the overall process of HL-IF denaturation and reactivation: (1) lower molecular weight forms can be generated when it is heated to 50 °C; (2) a reducing agent is required, under denaturing conditions of 5 M guanidine hydrochloride, to displace the 5000 mol wt fragment; and (3) the overall effect of denaturing and reducing agents is to generate a uniform molecular weight form of HL-IF (cf. Figure 3 with Figures 4 and 5).

The reactivated 21 000 mol wt form of interferon first isolated on Sephadex G-100 Superfine was further analyzed on $NaDodSO_4$ electrophoresis. Peak fractions corresponding to 21 000 mol wt (Figure 3) were pooled, concentrated, and applied on $NaDodSO_4$ gel electrophoresis. As shown in Figure 6, this confirmed that only one peak of activity was present; it had a molecular weight of 18 000. The 21 000 mol wt form observed on sieving and the 18 000 mol wt form seen on $NaDodSO_4$ gel electrophoresis are probably identical since these differences in molecular weight can be ascribed to specific technical limitations of the procedures: molecular sieving assumes a globular shape of a protein molecule, and $NaDodSO_4$ gel electrophoresis is particularly subject to error (Fish et al., 1970) when performed without prior use of a reducing agent during detergent treatment. Detergent treatment of HL-IF in the presence of a reducing agent results in irreversible loss of activity (Paucker et al., 1977).

Discussion

The molecular weight of HL-IF, as estimated by molecular sieving, has been consistently reported within a narrow molecular weight range of 26 000, a value which is confirmed here (Figure 1). By contrast, the molecular weights of HL-IF determined by $NaDodSO_4$ gels are different; namely, one form has a mol wt of 21 000 and another 15 000 (Stewart and Desmyter, 1975; Törmä and Paucker, 1976). We now have confirmed these reports (Figure 2).

More importantly, we now find that the apparent molecular weight of HL-IF, as determined by molecular sieving, can be diminished by about 5000 when interferon is subjected to denaturation by guanidine hydrochloride under reducing con-

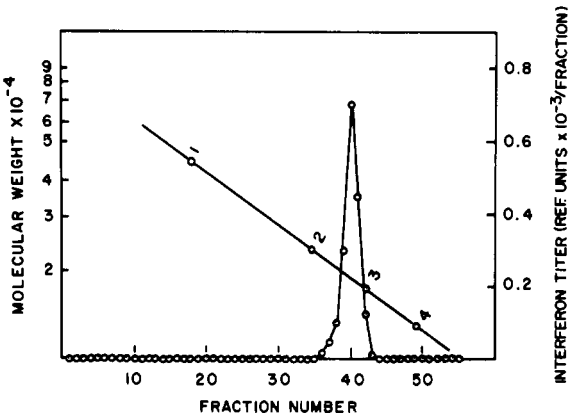

FIGURE 6: $NaDodSO_4$ gel electrophoresis of HL-IF was first isolated as a 21 000 molecular weight form by molecular sieving. Peak fractions of interferon activity from the experiment of Figure 3, corresponding to 21 000 daltons, were pooled and concentrated. Interferon was dialyzed against 0.1 M phosphate buffer (pH 7.5), reconstituted to contain 1% $NaDodSO_4$, and then heated to 100 °C for 2 min in a water bath. Aliquots of 100 μL containing 4×10^4 units/mL were applied to gels; 60% of interferon activity was recovered. Molecular weight standards are the same as those in Figure 2.

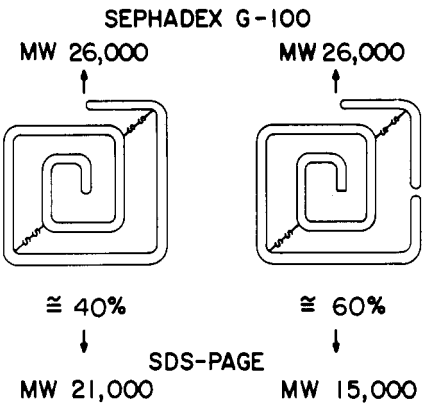

FIGURE 7: Two molecular forms of HL-IF, native and cleaved molecules. The proportions of these two molecular forms might vary as a function of proteolytic activity in different leukocyte interferon preparations.

ditions and then reactivated by dialysis (Figure 3). This reactivated interferon also gives one component on $NaDodSO_4$ gel electrophoresis (Figure 6). The relative ease of inducing a partial collapse of the interferon molecule is illustrated by heating alone (Figure 4) or in the presence of guanidine hydrochloride (Figure 5). A similar drop in molecular weight (mol wt 4000) was also observed by other investigators when HL-IF was treated with glycosidases (Bose et al., 1976); we suggest that this drop could have been due to inherent discontinuity of the HL-IF rather than the action of glycosidases.

Thus, there are actually two subpopulations of HL-IF in each preparation (Figure 7). The major subpopulation (60%) consists of molecules with an inherent discontinuity in their polypeptide chain. The minor subpopulation (40%) consists of interferon molecules which have escaped proteolytic attack, and these seem to represent the truly native form of HL-IF.

Molecular sieving of HL-IF would not distinguish between these two subpopulations if the small fragment (mol wt about 5000) could adhere to the rest of the molecule under nondissociating solvent conditions. Our results suggest that this fragment of HL-IF may be linked by a disulfide bridge to the remainder of the molecule. Even in the absence of a disulfide bridge, complementary forces would be likely to retain a polypeptide fragment with the remainder of the HL-IF molecule, as has been observed for other proteins (Anfinsen and Scheraga, 1975; Li and Bewley, 1976; Richards, 1958). However, under certain conditions—as $NaDodSO_4$ treatment and $NaDodSO_4$ gel electrophoresis (Figure 2)—the 5000 mol wt fragment is displaced, resulting in a major form of 15 000 molecular weight. The minor form, the residual native molecule, yields a molecular weight of 21 000.

The apparent difference in molecular weight on sieving (mol wt 26 000) and on $NaDodSO_4$ gel electrophoresis (mol wt 21 000) is probably simply due to the fact that reliable molecular weights on $NaDodSO_4$ gel electrophoresis can only be obtained after complete denaturation of proteins (Fish et al., 1970). The molecular weight of a $NaDodSO_4$–protein complex is always found lower if it is formed under nonreducing conditions and this is how $NaDodSO_4$ gel electrophoretic analysis of HL-IF is performed.

HL-IF can be reactivated by dialysis after exposure to guanidine hydrochloride under reducing conditions. We isolated this HL-IF as a 21 000 molecular weight entity by molecular sieving and then transferred it to $NaDodSO_4$ gel electrophoresis. On $NaDodSO_4$ gel electrophoresis, it continued to reveal only one component with an apparent molecular weight of 18 000. This was somewhat greater than the value of 15 000 mol wt found for the major component of HL-IF analyzed on $NaDodSO_4$ gels without prior treatment with guanidine hydrochloride under reducing conditions (cf. Figures 2 and 6). The "renaturation" process itself—following the loss of the fragment of 5000 mol wt—must therefore be different during the removal of guanidine hydrochloride and the reducing agent. The minor or native (unnicked) form of HL-IF is also apparently lost during denaturation with guanidine hydrochloride.

Recently, it has been suggested that both size forms of HL-IF are "hydrodynamically identical" and thus indistinguishable on molecular sieving; they are also presumed to have different charge-to-mass ratios of their $NaDodSO_4$ complexes (Törmä and Paucker, 1976). Both of these assumptions seem excessive and can be avoided if our polypeptide cleavage hypothesis is correct.

The existence of a polypeptide fragment of 5000 mol wt may be the result of one of several factors. We considered the possibility that the 5000 mol wt fragment was an extraneous protein adventitiously bound to the HL-IF molecule, but this notion seems rather improbable in view of the requirement for a reducing agent to lower efficiently the molecular weight of HL-IF. A proteolytic cleavage is more likely, and it might occur during the intracellular processing of the nascent interferon polypeptide, resulting in removal of a hydrophobic "signal sequence" (DeVillers-Thiery et al., 1975). Small numbers of the HL-IF molecules which escape the nick would constitute the high molecular weight form of HL-IF.

The most plausible interpretation is that a proteolytic cleavage takes place outside the leukocyte; this notion is supported by the variation seen in the relative proportions of the molecular forms which would be expected if an extracellular protease were present. It will be of interest to probe other properties—such as the cell growth regulatory activity and cross-species antiviral activity—of the remainder of the parent interferon molecule.

Our current work indicating the occurrence of a proteolytic cleavage in HL-IF with preservation of antiviral activity makes plausible additional attempts in further reducing the molecular weight of HL-IF by either enzymatic or chemical means. Ultimately, this kind of approach may facilitate the solid phase synthesis of human interferon.

Note Added in Proof

After this text was submitted, a report appeared (Stewart, W. E., II, et al. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 4200–4204) which claimed a gross heterogeneity of HL-IF as observed on isoelectrofocusing in polyacrylamide gels, suggestive to these authors of an extensive glycosylation of the HL-IF molecule. However, in our hands, a partially purified HL-IF preparation obtained by molecular sieving on Sephadex G-100 did not display any such heterogeneity when electrofocused on Sephadex G-75 Superfine used as a solid support. We believe that the disparity of results is probably due, in part, to a chaotropic salt employed in the purification of the HL-IF preparation used by Stewart et al., which could have resulted in the observed heterogeneity.

Acknowledgment

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