Interferon: Evidence for Subunit Structure*

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Abstract. During the purification of mouse and human interferons, multiple active components have been detected. Mouse interferon was purified over 500-fold by differential precipitation, centrifugation, gel chromatography, and isoelectric focusing. On electrofocusing, two molecular forms (A and B) were noted. Form B (isoelectric point 7.35) had a molecular weight of about 38,000 and Form A (isoelectric point 7.15), which was equally active, a molecular weight of 19,000. Purified Form B was dissociable into Form A, but the reverse reaction occurred to a much lesser extent. Human interferon, purified about 1500-fold, is also composed of multiple molecular forms. Form B (pI 5.60) had a molecular weight of about 24,000 and Form A (pI 5.35), which may contain up to 85% of the total activity, a molecular weight of 12,000. Both forms appear to be equally active. The dissociation of both human and mouse interferons into subunits appears to take place during dialysis versus low salt (0.01 M Tris pH 7.4). The data are consistent with the idea that the native molecule exists as a dimer of similar or identical subunits. Dimer formation, which probably occurs within the cells, does not seem to lead to a measurable cooperative effect between the subunits.

Little is known of the chemical structure of interferon proteins. Chick and mouse interferons have been reported to have molecular weights of about 25,000–35,000 and isoelectric points near neutrality.1,2 Human interferon is reported to have a molecular weight of about 25,000 and an isoelectric point also near neutrality.2 There has been little evidence regarding the possible subunit structure of any of these molecules. Chick interferon is apparently inactivated by the SH-containing compound, β-mercaptoethanol, and the process is enhanced by simultaneous exposure to 8 M urea.1 Sensitivity to −SH reagents has been explained by either a reduction of disulfide bonds, or a dependence on metal ions for function. However, interferon inactivated by β-mercaptoethanol has not been reactivated by either reoxidation or the addition of metal ions.1

As an initial step in the further characterization of interferons, we have extensively purified mouse and human interferons by means of the new technique of electrofocusing in polyacrylamide gels. We observed that both interferons, although initially homogeneous on gel chromatography, display two components on electrophoresis in pH gradients. Molecular weight measurements of the two forms are consistent with the hypothesis that native interferon is a dimer of similar or identical subunits.
Materials and Methods. All of the experiments reported here were carried out with virus-induced interferon from mouse or human cells. Minimal Eagle’s medium, MEM (less amino acids) contained 0.15 M NaCl, 0.01 M sodium phosphate (or Tris hydrochloride, as specified) pH 7.4, and 0.001 M MgCl₂. Mouse L cells and human fetal fibroblasts were grown in monolayers under standard conditions, described more fully elsewhere. The growth and assay of vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) are described elsewhere. Interferon was measured in the centrifuged culture fluid 48 hr after infection with NDV (multiplicity of infection usually 10).

During purification, aliquots of electrophoretic or chromatographic fractions were stored at −70°C to permit repeated assays.

Purification of mouse and human fetal interferons: These steps were used in the purification of interferon proteins from tissue-culture supernatants: acidification to pH 2 for 48 hr followed by centrifugation (8000 × g for 20 min at 4°C), gel chromatography, isoelectric focusing, and a final gel chromatography step. The proteins fulfilled the criteria generally applied to interferon. Mouse interferon after Step 1 was stable at 4°C for over 14 months, while human interferon was stable at 4°C for at least 2 months. Both proteins were relatively unstable after further purification.

Gel chromatography: A column of Sephadex, G-200 superfine, 20 × 1.6 cm, was exhaustively washed with 0.05 M NaCl-0.01 M Tris pH 7.4 at 4°C. The void volume was determined using dextran blue, and the column was calibrated with known molecular weight markers. Protein samples (0.35-0.40 ml) were eluted at a hydrostatic pressure of 11 cm of water. Isoelectric focusing was carried out within 48 hr.

Mouse and human interferons, purified by gel filtration, rechromatographed identically in 0.05 M NaCl-0.01 M Tris pH 7.4. Purified fractions obtained by electrofocusing were mixed with MEM with and without bovine serum albumin (BSA, 100 μg/ml). Different molecular forms chromatographed identically in both solutions. Any form of the protein is relatively stable in the standard chromatographic buffer in respect to activity and molecular size.

Isoelectric focusing: For isoelectric focusing, carrier ampholytes (LKB, Stockholm) were used to create pH gradients in thin layers of acrylamide gel. Gels were prepared by the photopolymerization (riboflavin 0.273 mg/ml) of 5% (w/v) acrylamide containing 2% (w/v) ampholytes. Samples were dialysed versus at least 200 volumes of 0.01 M Tris pH 7.4 for 4-6 hr at 4°C before application. 20-50 μg could be applied without influencing band width or contour. Above 50 μg, the pH gradient deviated slightly from linearity, and the bands became more curvilinear.

After electrophoresis at 400 V for 18 hr, slices from the edges of the gels were placed in 1.0 ml of water and the pH determined. From the inner portion, 7-mm segments were cut for assay and MEM containing BSA (100 μg/ml) was added to each fraction. The hemoglobin standards eluted from the slices into the medium within 4 hr, and 24 hr was allowed for elution of the interferon proteins. Duplicate gels for protein determinations were precipitated and exhaustively washed with 5% TCA.

Chemical determinations: When possible, protein was measured by the method of Lowry et al. using bovine serum albumin as a standard. To estimate protein content in isolated gel slices, in which protein content was as low as 0.2 μg/ml, peptide bond absorbance at 194 nm was determined using a Beckman DBG spectrophotometer with a deuterium lamp. Samples were exhaustively dialyzed against deionized water before spectrophotometry since many inorganic salts absorb in the 190-195 nm range.

Results. Mouse interferon was consistently recovered in yields of 60–100% and human fetal interferon was usually 50–75% recoverable, with a range of 12–80%.

Isoelectric focusing gave the major purification. It is difficult to measure specific activity accurately because of the low protein content, but a lower limit of specific activity could be determined. By these estimates, the cumulative purification of mouse interferon was 593-fold to yield a specific activity (either
form) of 4.5 million units/mg protein. The cumulative purification of human interferon was 1500-fold (Form A) yielding a specific activity of 2.1 million units/mg protein, and 280-fold (Form B) with a specific activity of 386,000 units/mg protein.

**Gel chromatography of unpurified interferons:** Control observations indicated that interferon activity and molecular size were stable in 0.01 M Tris pH 7.4–0.05 M NaCl. Mouse interferon behaved as a single molecular species with a molecular weight of 38,000 (Fig. 3, top panel). Viral-induced human interferon was eluted predominantly in a position corresponding to a molecular weight of 24,000 (Fig. 4, top panel). Both interferons thus exist largely as single molecular species at this stage of purification.

**Isoelectric focusing of mouse and human interferon:** Isoelectric focusing was performed on the interferons obtained from gel chromatography (Fig. 1). Mouse interferon activity was found in two peaks (A and B) containing equal activity, with isoelectric points of 7.15 and 7.35 respectively. Essentially 100% of the input activity was recoverable and the specific activity of the two peaks was the same. In other experiments, the relative proportions of Forms A and B changed. With increasing concentration of protein, peak A contained more activity than peak B (Fig. 5, bottom panel). The observed isoelectric points were constant over the range of protein used.

Human interferon focused with more acidic isoelectric points (Fig. 2). Three peaks were recovered with isoelectric points of 5.35, 5.60, and 5.70. These fractions, unlike those obtained with mouse interferon, contained different percentages of the total activity. The major fractions, Forms A (pI 5.35) and
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**Fig. 2.** Isoelectric focusing of human fetal interferon. Human fetal interferon (sp act 20,000 units/mg protein) was focused on ampholyte gradients as for Fig. 1. 800 units (40 µg) was applied; recovery, 65%. In duplicate gel slices, protein content was estimated by peptide bond absorbance. Human fetal interferon was usually 50–75% recoverable in the focusing step, but yields as low as 12% were obtained. ○, pH gradient; ●, human interferon.

B (pI 5.60), contained 99% of the activity. A minor fraction (Form C) (pI 5.70) with 1% of the total activity was not further characterized. The relative activity existing as Form A or B varied in different electrophoretic runs, but Form A usually contained 50–85% of the total activity.

**Gel chromatography after electrofocusing:** Molecular weight measurements were made on interferon peaks obtained from isoelectric focusing. Mouse Form B yielded 86% of the original 38,000 mol wt species and 14% of a previously undetected 19,000 mol wt species (Fig. 3, bottom panel). Form A yielded 90% of the 19,000 mol wt species and 10% of the original 38,000 mol wt form. Under similar conditions, crude mouse interferon was an exclusively 38,000 mol wt species. Thus, electrofocusing has separated two forms of the molecule.

Similar experiments were performed with human interferon Form A which yielded 90% of a 12,000 mol wt species, previously detected in trace amounts, and 10% of the original 24,000 mol wt form (Fig. 4, bottom panel). It was not possible to study human Form B accurately by the techniques previously applied to the two forms of mouse interferon because of the small amount of activity. We obtained indirect evidence that Form B (pI 5.60) is the undissociated 24,000 species by devising conditions in which the native molecule (mol wt 24,000) could be electrophoresed without dissociation. Under these conditions only one species (pI 5.60) is formed, which has a molecular weight of 24,000.

Both mouse and human interferons are separable into two active forms, one of which is twice the mol wt of the other. The simplest explanation is that the larger mol wt form is a dimer and the lower mol wt form the monomeric unit. While it is possible that the monomeric units are different, it seems more likely that the dimers are made up of identical subunits. Only one monomeric species
is seen, and it has exactly one half the molecular weight of the larger species. The dimer to monomer interconversion may be symbolically written as $I_2 \rightleftharpoons 2I$.

**Interconversions of Forms B and A**: Mouse interferon Form B was isolated by isoelectric focusing, dialyzed versus 0.01 M Tris pH 7.4, and refocused at its isoelectric point. About a quarter of the activity was now present as Form A (Fig. 5). Form B can thus continue to dissociate into A, the presumptive monomeric species. It was not technically possible to carry out similar studies with human interferon Form B because of the much lower activity appearing in this peak.
Fig. 4. **Top panel:** Gel chromatography of human fetal interferon. Unpurified human fetal interferon (sp act 2000 units/mg protein) was chromatographed under conditions described in Fig. 3. About 85% of the 500 units applied was recovered. Interferon was assayed in primary cells of human foreskin.

**Bottom panel:** Gel chromatography of human fetal interferon (Form A) isolated by electofocusing. Form A (pI 5.35), isolated by electrophoresis in the experiment described in Fig. 2, was chromatographed on Sephadex G-200. 400 units of Form A (sp act $2 \times 10^6$ units/mg protein) was applied and all of the activity was recovered. ●, molecular weight standards; ○, purified human interferon Form A.

Something associated with the focusing procedure causes an apparent dissociation of interferon. Could it be the low ionic strength which prevails throughout the electrofocusing procedure? The idea of a salt dependency for stability of the dimeric form was supported by attempts to focus in higher salt concentrations (0.05 M NaCl-0.01 M Tris or phosphate, pH 7.4). When focusing was carried out under these conditions, no evidence for dissociation was obtained. Mouse interferon yielded only Form B (pI 7.35) and human interferon yielded Form B (pI 5.60) as the principal species. In other similar experiments, human Form A has been present from trace amounts to 15% of the recoverable activity.

These observations suggest that low salt concentrations prevailing during
isoelectric focusing allow the detection of subunits. If this were so, dissociation should be demonstrable by gel chromatography. Mouse interferon Form B was dialyzed versus low salt (0.01 Tris pH 7.4) for 16 hr and then chromatographed (Fig. 6, top panel). The dialysis step alone was sufficient to dissociate the molecule as evidenced by the displacement of interferon to a 19,000 mol wt peak. The 38,000 mol wt region contained little activity. There is no concomitant loss of activity, which indicates that the smaller molecular form must be equally active. It provides additional support for subunits of similar structure.

The reverse reaction, the conversion of mouse Form A to Form B was studied by chromatography of Form A which had been dialyzed against MEM (Fig. 6, bottom panel). This buffer was selected since interferon is present as Form B in tissue culture fluid. About 5% of the activity was transformed into Form B, which indicates that the monomeric form is not quantitatively transformed quickly into the oligomer by simply raising the salt concentration. This inefficient transformation of A into B may be due to the low concentration of the monomeric species.13

Mixture experiments performed with the A, B, and C forms of human interferon revealed that the activities were additive. There does not seem to be a measurable cooperative effect between the subunits in the dimer form. Similar studies in progress with polymer-induced human interferon give analogous results.14
Fig. 6. Gel chromatography of mouse interferon Form B dialysed versus low salt concentration. Top panel: Mouse interferon was dialysed versus high (0.05 M NaCl–0.01 M Tris pH 7.4 0.001 M MgCl2) or low (0.01 M Tris pH 7.4) salt for 16 hr before chromatography on Sephadex G-200 (equilibrated with 0.05 M NaCl–0.01 M Tris pH 7.4). In each instance, crude interferon (specific activity 16,000 units/mg protein) was used; over 95% of the 2800 units applied were recovered. No detectable loss of activity occurred on dialysis versus low salt. C, dialyzed versus high salt; ▲, dialyzed versus low salt concentration. Bottom panel: To study the proposed reverse reaction, Form A → B, Form A was isolated in the experiment described in the top panel and dialyzed versus high salt (0.05 M NaCl–0.01 M phosphate pH 7.4–0.001 M MgCl2) with and without fetal calf serum (6% v/v) for 20 hr. The proteins were rechromatographed on Sephadex G-200. The results were unchanged by the presence of the calf serum. ●, molecular weight standards; ○, mouse interferon Form A after dialysis.

Discussion. Mouse and human interferons, viral-induced, initially behaved as single molecular species. At one step during purification (isoelectric focusing), two forms of each protein were detected. These two forms were then characterized as to molecular weight. Form A (mouse) has a molecular weight of 19,000 and Form A (human) a molecular weight of 12,000. Form B (mouse) has a molecular weight of 38,000 and Form B (human) a molecular weight of 24,000. These data strongly suggest that interferon exists as a dimer of two similar, perhaps identical, subunits. In the case of mouse interferon, the monomeric unit mass is 19,000 daltons and with human interferon 12,000 daltons. The different isoelectric points for the dimer and monomer suggest that certain surface charges must rearrange during oligomer formation, but without influencing activity, since there does not seem to be a measurable cooperative effect between the subunits in the dimer form.

The conversion of Form B into A is promoted by the exposure to low salt. In the presence of 0.05 M NaCl, little evidence for two molecular forms exists. The mechanism of the conversion, which is currently under study, could relate to either an environment of low ionic strength or the removal of a necessary metal ion by dialysis. The latter possibility seems more likely since high ionic strength generally promotes dissociation because of the electrostatic forces provided by high salt.

Recently, several highly purified glycolytic enzymes, homogeneous by gel electrophoresis, have been found on isoelectric focusing to contain a number of molecular species.15 The authors suggested that these complex profiles may be related to
combinations of closely related subunits which dissociate and rejoin randomly during electrofocusing. Our current studies with molecular weight measurements indicate that isoelectric focusing does in fact separate different oligomeric forms, and that the prevalence of low salt concentrations during electrofocusing may promote the conversions between different molecular species.

Recent preliminary reports suggest many isoelectric components of interferons from different animal tissues\(^6\) (Stanek, Gressnerova, and Paucker, 1970; personal communication). These studies have been carried out largely by electrophoresing in sucrose gradients. It is not clear how to reconcile these results with the findings reported in this paper since these results appear more complex. Further study should serve to clarify the important relationships between subunit structure and the biological properties of interferon.

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Abbreviations:  BSA, bovine serum albumin; MEM, minimal Eagle's medium; TCA, trichloroacetic acid; pi, isoelectric point.

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7 Determann, H., in Gel Chromatography (New York: Springer-Verlag, 1968).
9 The reaction was carried out between two glass plates held apart by 1 mm thick glass slides. A rotary motion of the glass slide lifted one plate off the gel which was then transferred to an electrophoresis apparatus at 4°C with an atmosphere previously equilibrated with water. Several hours at room temperature may cause the outer boundary of the gel to dry out, leading to a curvilinear appearance of the protein bands.
12 There are several possible explanations for the nearly complete recovery and the apparently lower specific activity of one band as compared to the other. The most likely one is the presence of small amounts of inorganic salts which absorb at 194 nm. There was no measurable loss of protein during TCA precipitation and washing.
13 If Form B consists of two nonidentical subunits, complete reassociation of the two subunits into the dimer form can only occur with equal quantities of the two, whereas if the subunits are identical, any admixture should be convertible to the dimeric form. We cannot test these alternatives at present because of the slow conversion of Form A into B.