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Association and dissociation properties of natural human interferon γ

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Abstract

The properties of natural human interferon γ (IFN-γ) molecules dissolved in protein-denaturing and non-denaturing solvents were examined by high-performance size-exclusion chromatography on a gel permeation column. IFN-γ and tritium-labeled IFN-γ molecules formed either dimers (>90.5%) with the molecular mass of 60 kDa or probably tetramers (<9.5%) with the molecular mass of approximately 100 kDa in non-denaturing solvents, and no monomer was detected. These oligomers were dissociated in protein-denaturing solvents such as 6 *M* guanidine hydrochloride, and IFN-γ existed as monomers. There is no effect on formation of the monomer based on the dissociation of oligomers by acid treatment at pH 4.0. The monomers in protein-denaturing solvents formed dimers by association when applied to a column equilibrated with a non-denaturing solvent of phosphate buffer, pH 7.0. In conclusion, natural human IFN-γ forms oligomers, particularly dimers, in non-denaturing solution, and this oligomer formation is a reversible reaction. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Association; Dissociation; Interferon γ

1. Introduction

Interferon γ (IFN-γ) is a cytokine produced by activated T- and NK-cells [1-4], and is clinically prescribed for the treatment of renal cell carcinoma and mycosis fungoids.

The molecular mass of the native form of human IFN-γ have been reported as approximately 40–60 kDa as determined by non-denaturing gel permeation chromatography [5–8], and as 25, 20 and 17 kDa by separation using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [8,9–11]. These findings indicate that the natural human IFN-γ exists in a oligomeric form in non-denaturing solution. This structural nature of a recombinant human IFN-γ appears to be the same as the native form [12]. Ando et al. reported the large-scale production and purification of natural human IFN-γ derived from human myelomonocytic HBL-38 cells by stimulation with lipopolysaccharide [13]. The IFN-γ produced by the cells consisted of three subspecies with the molecular masses of 25, 20 and 17 kDa, similar to its native form described in the above-mentioned studies. The 25 and 20 kDa species were both glycosylated forms, and the 17 kDa species was a non-glycosylated form [13,14].

The molecular size would appear to be an im-

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portant factor in the fate of macromolecules, e.g., glomerular filtration in the kidneys [15,16] and lymphatic absorption [17,18]. However, little is known about the quaternary structure of natural human IFN-γ, in particular the association and dissociation properties of the molecules. In the present study, the properties of natural human IFN-γ molecules which were derived from the HBL-38 cell line and dissolved in non-denaturing or protein-denaturing solvents were examined using high-performance size-exclusion chromatography (HPSEC) on a gel permeation column.

2. Experimental

2.1. Materials

Natural human IFN-γ derived from HBL-38 cells [13] was obtained from Hayashibara Biochemical Laboratories (Fujisaki, Okayama, Japan). The HBL-38 cells were stimulated with bacterial lipopolysaccharide to produce IFN-γ and the cell-free supernatant of the culture medium was concentrated using an ultra-filtration membrane. IFN-γ in the concentrate was purified with immunoaffinity chromatography using an anti-IFN-γ monoclonal antibody. Two production lots of IFN-γ were used in this study; lot No. 703004 at 2 mg protein per ml with the specific activity of 9.14×10^6 IU/mg protein, and lot No. 706005 at 14 mg protein per ml with the specific activity of 9.00×10^6 IU/mg protein. Analyses on SDS–PAGE showed that the IFN-γ preparations consisted of three subspecies with the molecular masses of 25, 20 and 17 kDa, and the composition of each subspecies was 70%, 20% and 10%, respectively. No other protein contaminants were observed in the preparations. The antiviral activity of IFN-γ was assayed in terms of its cytopathic effect on FL cells and Sindbis virus [19], and the titer of the activity is expressed as an international unit (IU).

A low-molecular-weight gel permeation calibration kit consisting of Blue dextran (V_0), ovalbumin (M_r=43 kDa), chymotrypsinogen A (M_r=25 kDa) and ribonuclease A (M_r=13.7 kDa) was purchased from Pharmacia (Uppsala, Sweden); and insulin chain B (M_r=3.5 kDa) from Sigma (St. Louis, MO, USA). All other reagents and solvents were of high analytical grade.

2.2. Methods

2.2.1. Preparation of tritium-labeled IFN-γ

After transferring the IFN-γ solution (lot No. 706005) to a dialysis tube (8 kDa MWCO, Spectra/Por. 6, Spectrum, Los Angeles, CA, USA), buffer exchange was performed with 100 m*M* borate buffer, pH 8.5, for approximately 18 h at 4°C.

One mCi (37 MBq.) of *N*-succinimidyl-(2,3-^{3}H)-propionate (toluene solution, Amersham, Little Chalfont, UK) was transferred to a reaction vial, and the solvent was evaporated under nitrogen gas. Then, 15 μl (105.5 μg protein) of buffer-exchanged IFN-γ solution at pH 8.5 was added to the vial, and the reaction was conducted on ice for 1 h. The reaction was terminated by the addition of 0.5 ml of 100 m*M* borate buffer, pH 8.5, containing 200 m*M* glycine. The terminated solution was adjusted to 1 ml by addition of phosphate-buffered saline containing 0.25% gelatin (Bio-Rad, Hercules, CA, USA), and tritium-labeled IFN-γ was purified from the solution by Sephadex G-25 (Pharmacia) column chromatography. Sephadex G-25 was placed in a column (150×10 mm I.D.) which was equilibrated with a solution containing 0.25% gelatin.

2.3. Instrumentation

In the study using non-radiolabeled IFN-γ, HPLC was performed using a CCP and 8000 series HPLC instrument (Tosoh, Tokyo, Japan). A CCPM (computer controlled multi-pump) and UV-8,000 (ultraviolet and visible detector) were used as the pump and the detector, respectively. For the detection of IFN-γ, a wavelength of either UV-215 nm or 280 nm was used depending on the concentration of the sample. Chromatopac C-R1B (Shimadzu, Kyoto, Japan) was used as the recorder and integrator, and the measured elution time of protein samples was recorded automatically. In the study using radiolabeled IFN-γ, an HPLC apparatus manufactured by Waters Assoc. (Milford, MA, USA) was used, together with a U6K injector, a Model 6000A pump, and a Model 440 absorbance detector at 280 nm, all from Waters.

The gel permeation column for HPLC was obtained from Tosoh. A TSK gel G 2000 SW-XL (300×7.8 mm I.D.) was attached to the chromatograph. The following were used as the mobile phase;

(1) 0.25 *M* K/Na phosphate buffer, pH 7.0, (2) McIlvaine buffer (citric acid–Na_2HPO_4), pH 4.0 or 5.0, and (3) 10 m*M* K/Na phosphate buffer, pH 7.0, containing 6 *M* guanidine hydrochloride and 1 m*M* Na_2EDTA. The volume of sample injected was 10–30 μl, the flow-rate was set at 0.5 ml/min, and the column temperature was room temperature.

2.4. Measurement of radioactivity and protein

The sample was placed in a counting vial, and then ACSII scintillation fluid (Amersham) was added and the radioactivity was measured using a liquid scintillation counter (Aloka, Tokyo, Japan). The concentration of IFN-γ protein was determined by the method of Lowry et al. [20].

3. Results and discussion

3.1. Oligomeric forms of IFN-γ

The properties of IFN-γ molecules in an aqueous solvent were examined by high-performance size-exclusion chromatography (HPSEC) on a gel filtration column.

Fig. 1 shows a typical chromatogram obtained in the analysis of IFN-γ (2.25×10^5 IU, 20 μg protein) with 0.25 *M* phosphate buffer, pH 7.0 as the mobile phase. The peaks were observed at the elution volumes (V_e, ml) of 6.94 and 7.94, and the molecular weights calculated from the calibration curve were approximately 100 kDa and 60 kDa, respectively. In contrast, when guanidine–HCl buffer was used as the mobile phase in the HPSEC analysis, a single IFN-γ peak was evident at V_e 7.18 and the molecular mass was 24 kDa (Fig. 2).

A similar analysis was conducted using tritium-labeled IFN-γ. In the radio-labeling of IFN-γ with *N*-succinimidyl-(2,3-^{3}H)-propionate, the yield of the conjugation was 74.5% and the specific radioactivity of the radioactive IFN-γ was 4.05 μCi (150 kBq.)/μg protein. The tritium-labeled IFN-γ was applied to HPSEC using phosphate buffer or guanidine–HCl buffer solution as the mobile phase. When the mobile phase was phosphate buffer, two radioactive peaks were noted (Fig. 3A). The molecular masses were approximately 100 kDa and 60 kDa, corresponding to the elution patterns using the UV detector. When guanidine–HCl buffer solution was used as the mobile phase, the radioactive peak was shifted to a position corresponding to a molecular mass of about 25 kDa (Fig. 3B). Regardless of whether UV detection or radioactivity detection was employed,

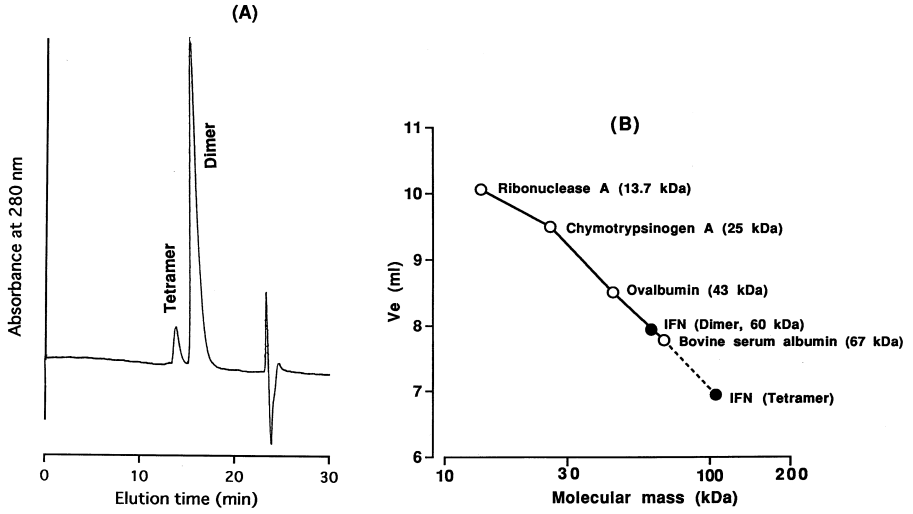

Fig. 1. HPSEC profiles of natural human IFN-γ (A) and molecular mass markers (B) in protein-non-denaturing mobile phase. The mobile phase used for the analysis was 0.25 *M* phosphate buffer, pH 7.0, at a flow-rate of 0.5 ml/min, and the eluent was monitored at 280 nm. The void volume of the column determined with Blue dextran was V_e 5.5.

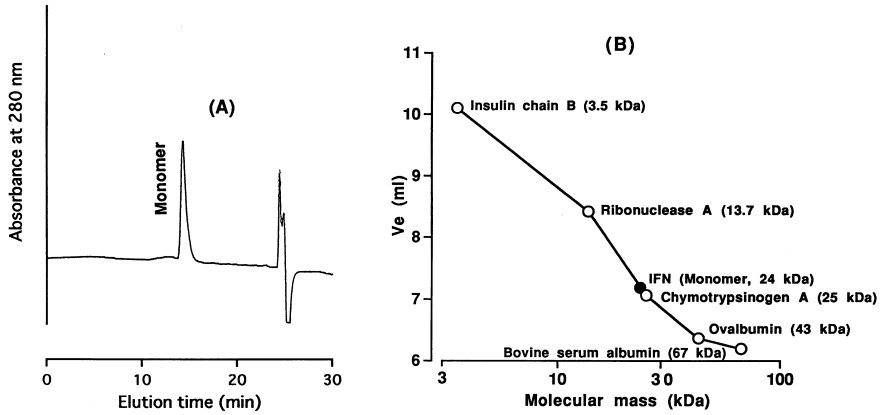

Fig. 2. HPSEC profiles of natural human IFN-γ (A) and molecular mass markers (B) in protein-denaturing mobile phase. The mobile phase used for the analysis was 10 m*M* phosphate buffer, pH 7.0, containing 6 *M* guanidine hydrochloride and 1 m*M* Na_2·EDTA at a flow-rate of 0.5 ml/min, and the eluent was monitored at 280 nm. The void volume of the column determined with Blue dextran was V_e 5.0. 20 μg IFN-γ (10 μl) was analyzed by the HPSEC.

good agreement was obtained in the elution pattern of IFN-γ.

The results of the measurement of the molecular mass of IFN-γ derived from the HBL-38 cell line by SDS–PAGE indicate the presence of subspecies of 25, 20 and 17 kDa; the 25 kDa species is reported to be the major component of IFN-γ [13]. The evidence thus would appear to agree well with that obtained by the HPSEC method using the guanidine–HCl system. Moreover, with the HPSEC method, a difference in the elution patterns of IFN-γ was noted depending on the use of phosphate buffer or guanidine–HCl as the mobile phase. We suspected that when the column for separation was equilibrated with phosphate buffer (pH 7.0), the protein molecules passing through the column were in an intact nature unlikely to be denatured in general. In contrast, in the column equilibrated with a chaotropic agent such as guanidine–HCl, the protein molecules would appear to be denatured and those existing as oligomers or having a quaternary structure were dissociated to produce the monomer or their subunits. Therefore, in the case of IFN-γ, the molecules with a molecular mass of 24 or 25 kDa observed when the protein-denaturing solvent was used as the mobile phase were considered to be monomers, whereas the molecules with a molecular mass of 60 kDa observed when the non-denaturing solvent was used were considered to be dimers, in consideration of the molecular mass of the monomers. We suspected that the molecules with a molecular mass of approximately 100 kDa were tetramers produced by a further association of dimers. Furthermore, with the use of the non-denaturing solvent, no peak was observed in the position corresponding to the molecular mass of the monomer, and its existence under such conditions was not confirmed.

3.2. Influence of IFN-γ concentration on oligomer formation

The influence of IFN-γ concentration on oligomer formation was examined. IFN-γ solutions were prepared diluted with phosphate-buffered saline at the concentration range of 2.25×10^5 to 2.25×10^7 IU/ml, and HPSEC was performed after allowing these solutions to stand for 1 h at room temperature. The results are shown in Table 1. In the case of IFN-γ solution at the concentration of 2.25×10^7 IU/ml, the proportions of dimers and tetramers present were 90.5% and 9.5%, respectively, and most of the IFN-γ molecules existed as dimers. Even at 2.25×10^5 IU/ml, most of the compound (94.2%) were dimers. Furthermore, even at 2.25×10^4 IU/ml in a solution prepared by diluting the IFN-γ solution, no dissociation of dimers or tetramers to monomers was noted.

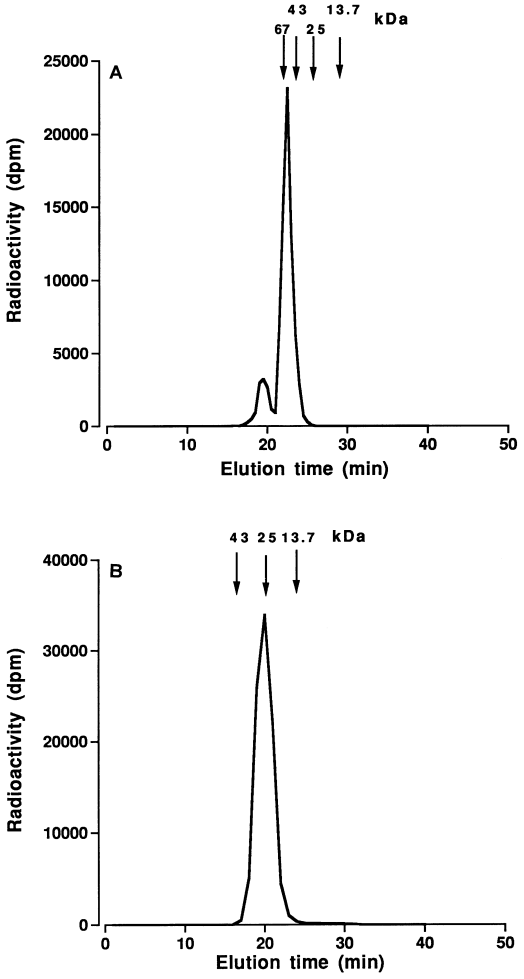

Fig. 3. Radiochromatogram of tritium-labeled natural human IFN-γ in protein-non-denaturing (A) and denaturing (B) HPSEC. The molecular mass markers were ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and bovine serum albumin (67 kDa). 3.75 kBq of the radioactive IFN-γ was added to the unlabeled IFN-γ (20 μg), and the combined sample (10 μl) was injected into the HPSEC column in each occasion.

3.3. Influence of pH on oligomer formation

It is reported that the biological activity of IFN-γ is readily inactivated on exposure to an acidic pH condition [5,7,8]. The influence of pH on oligomer formation by IFN-γ was thus examined. HPSEC was performed by applying 20 μl of IFN-γ solution (2.25×10^7 IU/ml in phosphate-buffered saline at pH 7.4) to a column equilibrated with McIlvaine buffer, pH 4.0 or 5.0. The results revealed that IFN-γ formed dimers and tetramers in this solution, and no dissociation to monomers was noted at both pH 4.0 and 5.0, as in the case of pH 7.0, based on the chromatographic pattern obtained. A tendency of a decrease in the proportion of tetramers present under acidic conditions was observed reflecting the influence of pH on the composition of each oligomer (Table 2). We speculated that some of the tetramers dissociated into dimers. Based on the above evidence, we concluded that the decrease of the biological activity of IFN-γ under acidic conditions is not attributable to a change in the quaternary structure of the IFN-γ molecules.

Table 1
Effect of concentration on oligomer formation of natural human interferon γ in non-denaturing solution[a]

Concentration of IFN-γ (IU/ml)	Composition (%)		
	Monomer	Dimer	Tetramer
2.25×10^5	n.d.[b]	94.2±0.3	5.8±0.3
2.25×10^6	n.d.	92.0±0.1	8.0±0.1
2.25×10^7	n.d.	90.5±0.0	9.5±0.0

[a] The different IFN-γ solutions (20 μl each) were applied to the HPSEC column. The data are expressed as mean±S.E. of three analyses.
[b] n.d.: not detectable.

3.4. Association of monomer IFN-γ

IFN-γ molecules forming oligomers dissociated and existed as monomers in protein-denaturing guanidine–HCl solution. Therefore, the association reaction, the reverse of the dissociation reaction, was examined using HPSEC. In this study, IFN-γ oligomers were dissociated with 6 *M* guanidin–HCl to

Table 2
Effect of pH on oligomer formation of natural human interferon γ[a]

pH	Composition (%)		
	Monomer	Dimer	Tetramer
4.0[b]	n.d.	96.2±0.0	3.8±0.0
5.0[b]	n.d.	95.8±0.2	4.2±0.2
7.0[c]	n.d.	90.5±0.0	9.5±0.0

[a] The data are expressed as mean±S.E. of three analyses.
[b] McIlvaine buffer.
[c] Phosphate buffer; n.d., not detectable.

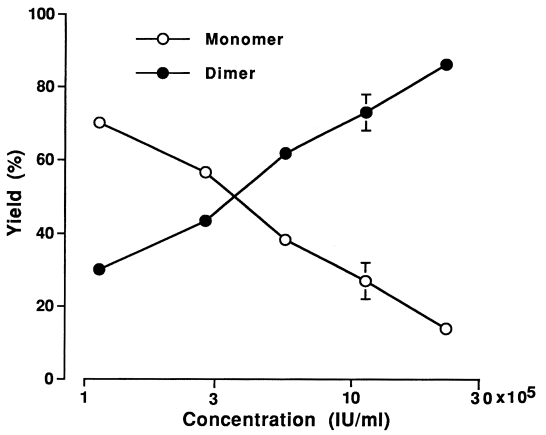

Fig. 4. Effect of concentration on the association of monomer IFN-γ by HPSEC. The data are means±S.D. of triplicate trials.

obtain monomers in advance, and the solution was applied to a column equilibrated with phosphate buffer, pH 7.0 to examine the association reaction in this column. The results are shown in Fig. 4. When the concentration of dissociated IFN-γ was 1.13×10^5 IU equivalent/ml, peaks were observed at V_e 8.15 and 8.89 in the chromatogram, and we surmised that these peaks were the dimer and monomer, respectively, according to the molecular weights as determined from the standard curve. The relative proportions of the two components present, as calculated from the peak area, were 30% dimer and 70% monomer. At the IFN-γ concentration of 2.83×10^5 IU equivalent/ml, the relative proportions of dimer and monomer present were almost equal; 43.4% and 56.6%, respectively. The higher the concentration, the higher the proportion of dimers became, with the relative proportion of dimers being 61.7% at 5.65×10^5 IU equivalent/ml and 73.0% at 1.13×10^6 IU equivalent/ml. The extent of the formation of dimers thus increased in direct proportion to the available concentration of monomers of IFN-γ. No association reaction resulting in the formation of tetramers from monomers or dimers was noted in this experimental system.

4. Conclusion

HPSEC is a useful tool for the study of the oligomeric formation of a protein or glycoprotein. In this study, the association and dissociation properties of natural human IFN-γ were investigated by the analytical method, and the following conclusion was obtained; natural human IFN-γ forms oligomers, particularly dimers, in non-denaturing solution, and this oligomer formation is a reversible reaction.

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