

Fluorescence Studies on Denaturation and Stability of Recombinant Human Interferon-Gamma

Petya Christova^a, Kristina Todorova^b, Ilijana Timtcheva^a, Genoveva Nacheva^b,
Andrey Karshikoff^c, and Peter Nikolov^{a*}

^a Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of Sciences, "Acad. G. Bonchev", Str., bl. 9, 1113 Sofia, Bulgaria, Fax: (+3592) 700225.
E-mail: petnik@orgchm.bas.bg

^b Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

^c Department of Biosciences at Novum, Karolinska Institutet, Huddinge, Sweden

* Author for correspondence and reprint requests

Z. Naturforsch. **58c**, 288–294 (2003); received September 10/November 8, 2002

Unfolding/folding transitions of recombinant human interferon-gamma (hIFN γ) in urea and guanidine chloride (Gn.HCl) solutions were studied by fluorescence spectroscopy. At pH 7.4 Gn.HCl was a much more efficient denaturant (midpoint of unfolding $C^* = 1.1$ M and $\Delta G^0 = 13.4$ kJ/mol) than urea ($C^* = 2.8$ M and $\Delta G^0 = 11.7$ kJ/mol). The close ΔG^0 values indicate that the contribution of electrostatic interactions to the stability of hIFN γ is insignificant. Both the pH dependence of the fluorescence intensity and the unfolding experiments in urea at variable pH showed that hIFN γ remains native in the pH range of 4.8–9.5. Using two quenchers, iodide and acrylamide, and applying the Stern-Volmer equation, a cluster of acidic groups situated in close proximity to the single tryptophan residue was identified. Based on the denaturation experiments at different pH values and on our earlier calculations of the electrostatic interactions in hIFN γ , we assume that the protonation of Asp63 causes conformational changes having a substantial impact on the stability of hIFN γ .

Key words: Human Interferon-Gamma (hIFN γ), Fluorescence Spectroscopy, Denaturation

Introduction

Interferon-gamma (IFN γ) is a cytokine secreted by the T-lymphocytes, which participates in the formation and modulation of immune response. In addition to its immunomodulatory effect, IFN γ induces also antiviral and antiproliferative states in target cells. Due to its numerous biological activities, IFN γ has found application in the treatment of various viral, immunological, cancerous, *etc.*, diseases and has attracted both commercial and scientific interest (Tsanev and Ivanov, 2001).

Mature human interferon-gamma (hIFN γ) consists of 143 amino acids and includes two N-glycosylation sites (Asn25 and Asn97). It is highly enriched in basic amino acid residues (28 lysines and arginines) and does not contain cysteine (Gray and Goeddel, 1982). Human interferon-gamma contains a single tryptophan in a key position (Trp36) in its active form.

The hIFN γ gene has been cloned and expressed in *Escherichia coli* in several laboratories (Gray *et al.*, 1982; Jay *et al.*, 1984; Arakawa *et al.*, 1985; Kung *et al.*, 1986; Perez *et al.*, 1990; Marekov *et al.*,

1991; Zhang *et al.*, 1992). Except for the interferon obtained by Marekov *et al.* (1991), the primary structure of all other recombinant hIFN γ preparations differs from that of the mature natural analogue. The main differences consist in either existence of an additional (initiator) methionine, or presence of residual amino acids belonging to the signal peptide (Cys-Tyr-Cys) or truncation of the C-terminus by three or more amino acids. In all constructs, however, the recombinant protein (unlike the natural one) is not glycosylated. For this reason the recombinant hIFN γ is less stable in solution and tends to aggregate. It aggregates also in the cytoplasm of the cells producing *E. coli* cells forming dense particles called inclusion bodies (Tsanev and Ivanov, 2001).

The procedures for isolation of recombinant hIFN γ from *E. coli* cells include solubilisation of the inclusion bodies in high (denaturing) concentrations of urea or guanidine chloride (Gn.HCl) and purification of the denatured protein by hydrophobic and/or ion-exchange chromatography. Finally, the pure protein is renatured in the presence of appropriate stabilisers such as human al-

bumin, dextran, sucrose, *etc.* In relation to this, the knowledge about the unfolding/folding behaviour of hIFN γ in solutions of urea or Gn.HCl is particularly important for its manufacturing. Unfortunately, such data are scarce in the literature and for this reason the conditions of purification and renaturation (leading to the recovery of biological activity) of hIFN γ are empirically established. The most comprehensive study on denaturation/renaturation of hIFN γ refers to its thermal denaturation in aqueous solutions free of denaturing agents and is carried out with recombinant hIFN γ consisting of 146 (instead of 143) amino acids (Beldarrain *et al.*, 1999).

X-ray analyses of pure hIFN γ (Ealick *et al.*, 1991) and hIFN γ bound to its hIFN γ R α receptor (Walter *et al.*, 1995) have shown that it is organized as a homodimer. Each monomer consists of six tightly associated α -helices (62% of the molecule) running parallel to the axis, which are linked with unstructured areas. In the active form of hIFN γ (Bennett *et al.*, 1995; Sprang and Bazan, 1993), the monomers are organized in an antiparallel fashion so that the N-terminal part of one polypeptide is situated close to the C-terminus of the other. According to (Arakawa *et al.*, 1987; Yphantis and Arakawa, 1987), the active form is extremely stable; some studies (Boteva *et al.*, 1996; Nandi, 1998) have shown that, in solution at room temperature, the equilibrium constant between the monomer and the dimer states of hIFN γ is 10^{-6} M.

The aim of the present study is to investigate, by fluorescent spectroscopy, the denaturation of recombinant hIFN γ consisting of 143 amino acids (with primary structure corresponding exactly to that of the mature natural hIFN γ) as a function of the concentration of two denaturing agents (urea and Gn.HCl) and pH. Since dextran is widely used as an antiaggregating agent and stabiliser of hIFN γ in solution, its effect on the fluorescence properties and stability of hIFN γ is also studied.

Materials and Methods

Recombinant hIFN γ was purified from overproducing *E. coli* cells to a 99.5% purity corresponding to a specific antiviral activity of 10^8 IU/mg, measured on WISH cells (Serially Propagated Heteroploid Cell Line, Amnion, Human) as described earlier (Marekov *et al.*, 1991). To prevent

aggregation during storage, hIFN γ was kept at -20°C in a solution of 20 mM Tris(hydroxymethyl)methylamine/HCl (pH 7.4) and 150 mM NaCl at a concentration of 1 mg/ml. For the spectral studies, the stock protein solution was diluted with the respective solvent to an optical density of $A_{280} = 0.06$, corresponding to $5.4\ \mu\text{M}$ (as calculated on the basis of $\epsilon = 1.1 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$). At this concentration in non-denaturing solutions, hIFN γ exists in the form of active homodimers (see above).

Urea and Gn.HCl denaturation curves were obtained from the fluorescence intensity measurements at 340 nm upon excitation at 295 nm. To avoid ammonium cyanate isomerisation of urea (Stark, 1965), fresh stock solutions containing 0.1% triethylamine were prepared and used for a day. All samples were monitored until constant fluorescence intensity was reached (24 h for samples dissolved in urea and 4 h for Gn.HCl). The fluorescence intensity of the native protein solutions was checked at regular time intervals. No changes were found within 24 h. The pH was adjusted to the respective values by 0.5 M HCl or 0.5 M NaOH and checked before and after the measurements. It remained stable during the experiments and did not fluctuate by more than ± 0.1 pH unit. The reversibility of the unfolding process was established by dilution with buffer of the solution to the desired residual urea/Gn.HCl concentration.

Absorption spectra were recorded on a Specord M40 (Carl Zeiss, Jena) UV-VIS spectrophotometer and a Perkin Elmer MPF44B spectrofluorimeter was used for the fluorescence spectra. The emission spectra were corrected using a standard tungsten lamp, whereas the excitation spectra were corrected with rhodamine B. The fluorescence quantum yield (Q_F) was determined relative to that of the N-Ac-Trp-NH $_2$ (Boteva *et al.*, 1996). The fluorescence decay curves (10000 counts in the maximum, time resolution = 0.1 ns channel) were measured at 25°C on a nanosecond single photon counting spectrofluorimeter system (Photochemical Research Associates Inc., Canada-PRA 2000), using a nitrogen-filled flash lamp with $\lambda_{\text{ex}} = 297\ \text{nm}$ and a detection wavelength corresponding to the maximum of the fluorescence band. The natural lifetime (τ) was estimated by a standard deconvolution procedure. The accuracy of the fit was controlled by the weight residual autocorrelation function of residual and reduced chi-square (χ^2).

Results and Discussion

Steady state and dynamic fluorescence properties

Human interferon-gamma contains a single tryptophan (Trp36) and 4 tyrosines, which allow conformational changes to be investigated by fluorescence spectroscopy.

The corrected excitation spectrum of hIFN γ in a solution of 20 mM Tris-HCl, pH 7.5 and 150 mM NaCl was independent of the emission wavelength in the range of 330–370 nm. Under these conditions, the excitation spectrum consisted of one band only, with a maximum at 280 nm – Fig. 1, curve A, which fairly corresponded to the longest wavelength absorption band. In the shorter wavelength spectral region, where the main absorption band of hIFN γ was observed, the excitation spectrum for the Trp emission with maximum at $\lambda_{em} = 340$ nm did not reproduce the absorption spectrum. Upon excitation at 270 nm the fluorescence spectrum was dominated by a band with a maximum at 300 nm (attributed to the Tyr residues) and a weaker band at about 340 nm corresponding to the Trp emission (Fig. 1, curve B). At $\lambda_{ex} = 295$ nm, only the tryptophan emission was observed (Fig. 1, curve C). The relative fluorescence quantum yield (Q_F) obtained after excitation at 295 nm was 0.038, which was very close to that reported in the literature ($Q_F = 0.042$) (Boteva *et al.*, 1996).

The fluorescence lifetime was measured at 25 °C upon excitation at 297 nm and emission at 340 nm.

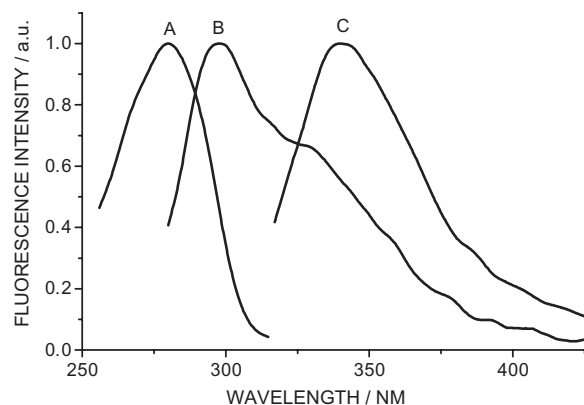


Fig. 1. Corrected excitation (curve A) and emission (curves B and C) spectra of 5.4 μ M hIFN γ in 20 mM Tris-HCl, pH 7.5 at 300 K. Curve A: $\lambda_{em} = 340$ nm; curve B: $\lambda_{ex} = 270$ nm; curve C: $\lambda_{ex} = 297$ nm.

The fluorescence decay curve was fitted with good precision (chi-square = 1.18) to a biexponential linear function with fluorescence lifetimes $\tau_1 = 0.11$ ns and $\tau_2 = 2.87$ ns. These values were close to those reported in (Boteva *et al.*, 1996)- $\tau_1 = 0.11$ ns and $\tau_2 = 3.1$ ns.

Fluorescence quenching effects

Fluorescence quenching experiments were performed with two different types of quenchers, iodide (I^-) and acrylamide. Charged and uncharged quenchers are often used in fluorescent spectroscopy for the assessment of the effect of neighbouring groups on the Trp fluorescence (Lakowicz, 1983). According to Eftink and Ghiron (1981), the two quenchers have the same quenching efficiency ($\gamma = 1.0$) and their molecular volumes are comparable. This means that the difference in the quenching effect of the two substances has to be attributed to their electric charge (determining different accessibility to the Trp residue) only. The fluorescence intensity decreases at increasing concentrations of both quenchers used – iodide and acrylamide, reaching a plateau at values higher than 1 M. The maximum quenching effect achieved with the two quenchers is 30% for the iodide and more than 90% for the acrylamide.

The quenching constants (K_q) were calculated by the Stern-Volmer equation (Stern and Volmer, 1919). In the case of acrylamide, the K_q values were corrected for the acrylamide own fluorescence (Eftink and Ghiron, 1981) using a molar extinction coefficient $\epsilon_{295} = 0.25$ M $^{-1}$ cm $^{-1}$. For both quenchers, the dependence of the normalised fluorescence intensity $I^F(0)/I^F$ on the quencher's concentration was linear, which was an indication of the absence of static quenching contributions (Eftink and Ghiron, 1981). The quenching constants differed by one order of magnitude for the two quenchers: $K_{q(\text{acrylamide})} = 4.68$ M $^{-1}$ and $K_{q(I^-)} = 0.42$ M $^{-1}$. This result is in good agreement with the hIFN γ models predicting strong negative electrostatic potentials (Ealick *et al.*, 1991; Altobelli *et al.*, 2001). Although the hIFN γ molecule is positively charged, a cluster of acidic groups, such as Asp41 together with Glu38 and Glu39, which are located in the vicinity of Trp36, create a region with a strong negative electrostatic potential.

Unfolding/folding transition of hIFN γ in urea and guanidine chloride solutions

The structural stability of recombinant hIFN γ in urea and Gn.HCl at varying pH was studied under isothermal conditions. The unfolding/folding transition was monitored by the changes in the fluorescence intensity and the position of the maximum of the Trp36 band at 25 °C. The free energy of denaturation ΔG was calculated using the equation

$$\Delta G = -RT \ln K = -RT \ln [(I_n - I^f)/(I^f - I_u)] \quad (1)$$

where K is the equilibrium constant, I^f is the observed fluorescence intensity and I_n and I_u are the fluorescence intensities of the native (folded) and denatured (unfolded) conformations, respectively (Ahmad, 1991).

The free energy of denaturation in water (ΔG^0) (*i.e.* ΔG in the absence of denaturant) was determined by the linear extrapolation method (Pace *et al.*, 1989; Monera *et al.*, 1994) plotting ΔG vs. the concentration of the denaturant. The data were fitted to the linear equation:

$$\Delta G = \Delta G^0 - m [\text{denaturant}] \quad (2)$$

where m is a measure of the dependence of ΔG on the denaturant concentration, *i.e.* the extrapolation to zero concentration gives ΔG^0 .

The unfolding curves obtained at pH = 7.4 for urea and Gn.HCl are shown in Fig. 2. Both curves

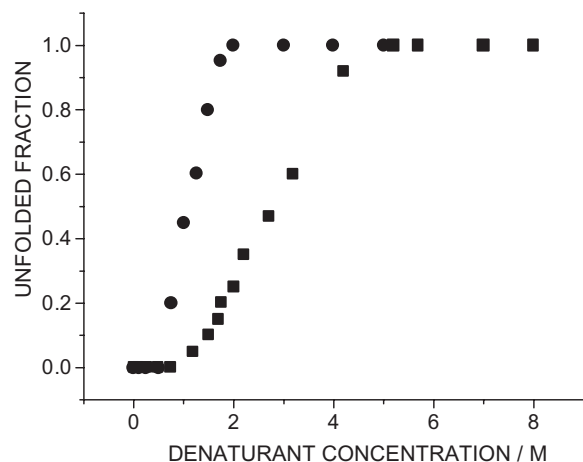


Fig. 2. Fraction of hIFN- γ unfolded as a function of Urea (■) and Gn.HCl (●) concentrations. Experimental conditions: protein concentration 5.4 μM in 20 mM Tris-HCl buffer at pH 7.5, $T = 300\text{ K}$; $\lambda_{\text{ex}} = 297\text{ nm}$, $\lambda_{\text{em}} = 340\text{ nm}$.

are sigmoid, which indicates that the denaturation of hIFN γ in both solutions is a two-state process. Our data clearly show, that the unfolding of hIFN γ is much more effective in Gn.HCl than in urea. As it can be seen in Fig. 2 the midpoint of unfolding (C^*) value is 1.1 M for Gn.HCl and 2.8 M for urea. The free energies of unfolding estimated from these curves by the linear extrapolation method at pH 7.4 are $\Delta G^0 = 11.7\text{ kJ/mol}$ for urea and $\Delta G^0 = 13.4\text{ kJ/mol}$ for Gn.HCl respectively. The two values are very close, indicating that the contribution of electrostatic interactions to the stability of hIFN γ is insignificant (Privalov, 1982). On the other hand the low ΔG^0 value of about 12.6 kJ/mol is an indication of a low stability of hIFN γ under the experimental conditions used (pH = 7.4, 20 mM Tris, 150 mM NaCl, 25 °C). According to the calorimetric studies in (Beldarrain *et al.*, 1999), ΔG^0 of hIFN γ strongly depends on both pH and buffer concentration.

As shown in Fig. 3, in addition to the decrease in fluorescence intensity, the rise of urea and Gn.HCl concentrations leads also to a bathochromic shift of the fluorescence maximum from 335 nm (native form) to 350 nm (denatured form). This is due to the fact that the Trp residue, which is partly buried in the native form is exposed on the surface in the denatured protein.

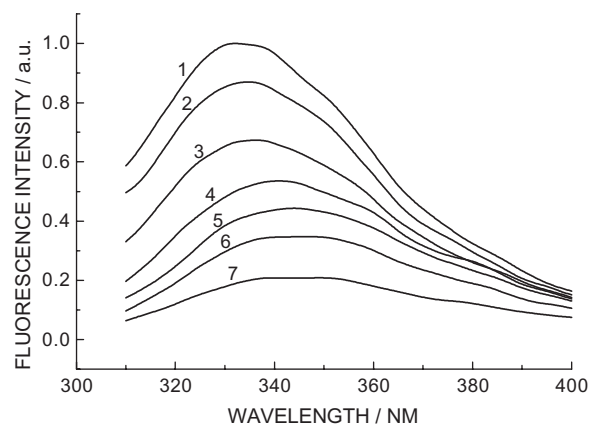


Fig. 3. Changes in the fluorescence spectra of hIFN- γ in 20 mM Tris-HCl buffer at pH 7.5, $T = 300\text{ K}$, protein concentration 5.4 μM , $\lambda_{\text{ex}} = 297\text{ nm}$, at different concentrations of Gn.HCl: (1) – 0 M; (2) – 0.75 M; (3) – 1 M; (4) – 1.2 M; (5) – 1.35 M; (6) – 1.5 M; (7) – 1.75 M.

pH Dependence of the unfolding/folding transition of hIFN γ

To elucidate the effect of electrostatic interactions on the stability of hIFN γ , the pH-dependence of unfolding was studied in urea at 25 °C. Prior to that, the effect of pH on the hIFN γ fluorescence in the absence of urea was investigated in the pH range of 2–12. The experimental data indicate that the fluorescence intensity remained constant in the range of pH 6 to 9 and gradually decreased upon either decrease from 6 to 2 or increase from 9 to 12. When pH was varied in the presence of urea, the fluorescence intensity remained constant at pH 4.8 to 9, which means that the native state of hIFN γ was preserved in this pH interval. This was confirmed also by two bioassays (antiviral and antiproliferative) for quantification of the hIFN γ biological activity (Todorova *et al.*, unpublished). Our results showed that the reversibility of unfolding was higher than 90% when experiments were carried out at pH 4.8–9. Two typical unfolding curves obtained at pH 5.2 and pH 6 are presented in Fig. 4. The same figure illustrates also the respective refolding data.

The free energy of denaturation in water (ΔG^0) of hIFN γ at different pH values is calculated using Eqs. (1) and (2). It was found that the stability of hIFN γ does not depend on pH in the range of

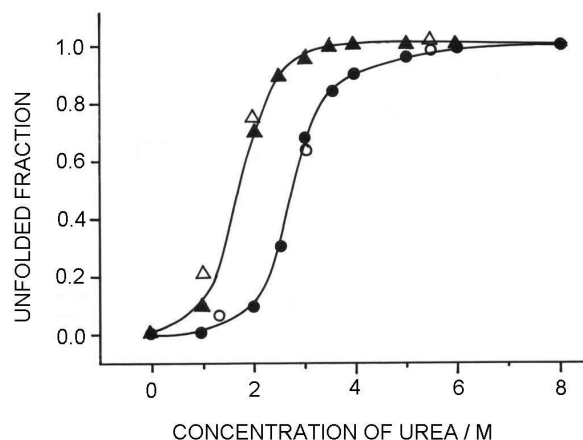


Fig. 4. Fraction of hIFN- γ unfolded as a function of Urea concentration at pH = 5.2 (▲) and pH = 6.0 (●). The refolding data for pH = 5.2 and pH = 6.0 are presented by (△) and (○), respectively. Experimental conditions: protein concentration 5.4 μ M in 20 mM Tris-HCl buffer, $T = 300$ K, $\lambda_{\text{ex}} = 297$ nm, $\lambda_{\text{em}} = 340$ nm.

pH 6.5–9.5, while at pH lower than 6.5 the ΔG^0 value gradually decreases from 11.7 kJ/mol to 8.4 kJ/mol. According to (Beldarrain *et al.*, 1999) the stability of hIFN γ strongly depends on both pH and buffer concentration, which is a reason for ΔG^0 to vary from 9.2 to 32.3 kJ/mol. These values, obtained by thermal unfolding, are of the same order of magnitude as the data presented here, which are based on urea denaturation. They are also close to the ΔG^0 values reported for many other globular proteins (Privalov, 1979; 1982).

The dependence of ΔG^0 on pH was fitted to the titration (sigmoid) curve of a group with $\text{pK} = 5.2$. The fitting parameters for the Boltzmann fit are: $\text{Chi-sqr} = 0.00175\text{E}-4$, $\text{init}(A_1) = 1.4532$, $\text{final}(A_2) = 2.8095$, $\text{XatY50}(x_0) = 5.0587$, $\text{width}(dx) = 0.44818$, $\text{XatY20} = 4.43743$, $\text{XatY80} = 5.68004$. A similar group with $\text{pK} = 5.4$ was registered also by calorimetric measurements (Beldarrain *et al.*, 1999). The authors have concluded that a single group with pK of 5.4 is responsible for the pH effect on the stability of hIFN γ . Based on these results and our earlier calculations of electrostatic interactions in hIFN γ (Altobelli *et al.*, 2001), we conclude that most likely this group is Asp63. The pK value calculated for this residue is 5.1. It is located at the C terminal end of helix C and forms a salt bridge with the N-terminal Tyr14 located in helix A. The salt bridge between these residues connects two large secondary structural elements, namely helix A and the hairpin formed by the helices C and D. The protonation of Asp63 breaks this salt bridge and reduces the attractive interaction between the two secondary structure elements at the C-terminus of helix A and the loop between helices C and D. We assume that the protonation of Asp63 causes conformational changes having substantial impact on the stability of hIFN γ . Other groups involved in interactions with the protein charged multipole or with other groups belonging to the same secondary structural elements seem irrelevant in this respect.

Does the stabiliser (dextran) influence the fluorescence properties and stability of hIFN γ ?

In order to evaluate the effect of dextran (as an antiaggregant and stabiliser of hIFN γ) on the fluorescence properties and stability of hIFN γ in solution, we have compared the fluorescence

parameters and ΔG^0 of fresh non-stabilised and stabilised hIFN γ . Our results indicate that the emission spectra of both preparations are identical. In addition to this, the fluorescence decay measurements show that the presence of dextran does not affect significantly the fluorescence lifetime. For instance, the deconvolution values for dextran-stabilised hIFN γ (at pH = 7.5) are $\tau_1 = 0.13$ ns and $\tau_2 = 2.93$ ns and for non-stabilised preparations they are $\tau_1 = 0.11$ ns and $\tau_2 = 2.87$ ns, respectively. Based on this data we conclude that the presence of dextran does not change significantly the fluorescence properties of hIFN γ .

Two types of experiments were carried out to evaluate the effect of dextran on hIFN γ stability. The pH dependencies of fluorescence intensity of pure and dextran stabilised hIFN γ were compared and it was found that dextran does not interfere

with the fluorescence properties of hIFN γ as a function of pH. The unfolding of pure and dextran-stabilised hIFN γ in urea at two different pH values (5.0 and 7.0) was also studied. The estimated ΔG^0 values are 8.4 kJ/mol and 11.7 kJ/mol for pH 5.0 and pH 7.0, and they remained the same for both stabilised and non-stabilised hIFN γ . Based on these experimental results, we conclude that dextran does not interact with hIFN γ so as to change remarkably its fluorescence behavior and thermodynamic stability.

Acknowledgements

The authors highly appreciate the financial support of FORSKNINGSRADSNAMNDEN (FRN), Sweden (Grants No 981305:4 and D1998-1305:6).

- Ahmad F. (1991), Protein stability from denaturation transition curves. *Indian J. Biochem. Biophys.* **28**, 168–173.
- Altobelli G., Nacheva G., Todorova K., Ivanov I., and Karshikoff A. (2001), Role of the C-terminal chain in human interferon gamma stability: An electrostatic study. *Proteins: Struct., Funct., Genet.* **43**, 125–133.
- Arakawa T., Alton N. K., and Hsu Y. R. J. (1985), Preparation and characterization of recombinant DNA-derived human interferon-gamma. *J. Biol. Chem.* **260**, 14435–14439.
- Arakawa T., Hsu Y. R., and Yphantis D. A. (1987), Acid unfolding and self-association of recombinant *Escherichia coli* derived human interferon- γ . *Biochemistry* **26**, 5428–5433.
- Beldarrain A., Lopez-Lacomba J. L., Furrázola G., Barberia D., and Cortijo M. (1999), Thermal denaturation of human γ -interferon. A calorimetric and spectroscopic study. *Biochemistry* **38**, 7865–7873.
- Bennett M. J., Schlunegger M. P., and Eisenberg D. (1995), 3D domain swapping mechanism for oligomer assembly. *Protein Sci.* **12**, 2455–2468.
- Boteva R., Zlateva Th., Dorovska-Taran V., Visser A. J. W. G., Tsanev R., and Salvato B. (1996), Dissociation equilibrium of human recombinant interferon γ . *Biochemistry* **35**, 14825–14830.
- Ealick S. E., Cook W. J., Vijay-Kumar S., Carson M., Nagabhushan T. L., Trotta P. P., and Bugg C. E. (1991), Three-dimensional structure of recombinant human interferon- γ . *Science* **252**, 698–702.
- Eftink M., and Ghiron C. (1981), Fluorescence quenching studies with proteins. *Anal. Biochem.* **114**, 199–227.
- Gray P. W., and Goeddel D. V. (1982), Structure of the human immune interferon gene. *Nature* **298**, 859–863.
- Gray P. W., Leung D. W., Pennica D., Yelverton E., Najarian R., Simonsen C. C., Derynck R., Sherwood P. J., Wallance D. M., Berger S. L., Levinson A. D., and Doeddel D. V. (1982), Expression of human immune interferon cDNA in *E. coli* and monkey cells. *Nature* **295**, 503–508.
- Jay E., Rommens J., Cloney L., McKnight D., Lutze-Wallace C., Wishart P., Lin W., Asundi V., Dowood M., and Jay F. (1984), High-level of expression of a chemically synthesized gene for human interferon- γ using a prokaryotic expression vector. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 2290–2294.
- Kung H. F., Pan Y. E., Moschera J., Tsai K., Bekesi E., Chang M., Sugino C., and Honda S. (1986), Purification of recombinant human immune interferon. *Methods Enzymol.* **119**, 204–210.
- Lakowicz J. (1983), Chapter 9, Quenching of fluorescence. In: *Principles of Fluorescence Spectroscopy*. Plenum Press, New York p. 258.
- Marekov L. N., Vassileva R. A., Ivanov V. P., Sarafova A. A., Zanev R. G., Ivanov I. G., and Ivanova V. S. (1991), Method for producing human cysteine – gamma-interferon free of methionine at N-terminal. *Eur. Patent App. No. 0446582 A1*.
- Monera O. D., Kay C. M., and Hodges R. S. (1994), Protein denaturation with guanidine hydrochloride or urea provides a different estimate of stability depending on the contributions of electrostatic interactions. *Protein Sci.* **3**, 1984–1991.
- Nandi P. K. (1998), Evidence of molten globule(s) like state of interferon gamma in acidic and sodium perchlorate solutions. *Int. J. Biol. Macromol.* **22**, 23–28.

- Pace N. C., Shirley B. A., and Thomson J. A. (1989). In: Protein structure: a particular approach, (T. E. Creighton, ed.). IRL Press, Oxford, 311–329.
- Perez L., Vega J., Chuai C., Menendes A., Ubieta R., Montero M., Pardon G., Silva A., Santizo C., Besada V., and Herrera L. (1990), Production and characterization of human gamma interferon from *Escherichia coli*. Appl. Microbiol. Biotechnol. **33**, 429–433.
- Privalov P. L. (1979), Stability of proteins: small globular proteins. Adv. Protein Chem. **33**, 167–241.
- Privalov P. L. (1982), Stability of proteins. Proteins which do not present a single cooperative system. Adv. Protein Chem. **35**, 1–104.
- Sprang S. R., and Bazan J. F. (1993), Cytokine structural taxonomy and mechanisms of receptor engagements. Curr. Opin. Struct. Biol. **3**, 815–827.
- Stark G. R. (1965), Reactions of cyanate with functional groups of proteins. III. Reactions with amino and carboxyl groups. Biochemistry **4**, 1030–1036.
- Stern O., and Volmer M. (1919), Über die Abklingungszeit der Fluoreszenz. Phys. Zeitschrift **20**, 183.
- Tsanev R. G., and Ivanov I. G. (2001), In: Immune interferon: Properties and Clinical Applications. CRC Press LLC, Boca Raton, FL, U. S. A.
- Walter M. R., Windsor W. T., Nagabhushan T. L., Lundell D. J., Lunn C. A., Zauondy P. J., and Narula S. K. (1995), Crystal structure of a complex between interferon- γ and its soluble high-affinity receptor. Nature **376**, 230–235.
- Yphantis D. A., and Arakawa T. (1987), Sedimentation equilibrium measurements of recombinant DNA derived human interferon- γ . Biochemistry **26**, 5422–5427.
- Zhang Z., Tong K. T., Belew M., Petterson T., and Janson J. C. (1992), Production, purification and characterization of recombinant human interferon-gamma. J. Chromatogr. **604**, 143–155.