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

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Unfolding/folding transitions of recombinant human interferon-gamma (hIFNγ) 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 ΔG^0 = 13.4 kJ/mol) than urea (C* = 2.8 M and ΔG^0 = 11.7 kJ/mol). The close ΔG^0 values indicate that the contribution of electrostatic interactions to the stability of hIFNγ is insignificant. Both the pH dependence of the fluorescence intensity and the unfolding experiments in urea at variable pH showed that hIFNγ 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 hIFNγ, we assume that the protonation of Asp63 causes conformational changes having a substantial impact on the stability of hIFNγ.

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

Introduction

Interferon-gamma (IFNγ) is a cytokine secreted by the T-lymphocytes, which participates in the formation and modulation of immune response. In addition to its immunomodulatory effect, IFNγ induces also antiviral and antiproliferative states in target cells. Due to its numerous biological activities, IFNγ 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 (hIFNγ) 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 hIFNγ 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 hIFNγ 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 hIFNγ 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 hIFNγ 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 hIFNγ 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 hIFNγ are empirically established. The most comprehensive study on denaturation/renaturation of hIFNγ refers to its thermal denaturation in aqueous solutions free of denaturing agents and is carried out with recombinant hIFNγ consisting of 146 (instead of 143) amino acids (Beldarrain *et al.*, 1999).

X-ray analyses of pure hIFNγ (Ealick *et al.*, 1991) and hIFNγ bound to its hIFNγRα 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 hIFNγ (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 hIFNγ is 10^{-6} M.

The aim of the present study is to investigate, by fluorescent spectroscopy, the denaturation of recombinant hIFNγ consisting of 143 amino acids (with primary structure corresponding exactly to that of the mature natural hIFNγ) 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 hIFNγ in solution, its effect on the fluorescence properties and stability of hIFNγ is also studied.

Materials and Methods

Recombinant hIFNγ 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, hIFNγ 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 μM (as calculated on the basis of $\varepsilon = 1.1 \times 10^4$ M^{-1} cm^{-1}). At this concentration in non-denaturing solutions, hIFNγ 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 λ_{ex} = 297 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 hIFNγ in a solution of 20 mM Tris-HCl, pH 7.5 and 150 mM NaCl was independed 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 hIFNγ was observed, the excitation spectrum for the Trp emission with maximum at λ_{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 λ_{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.

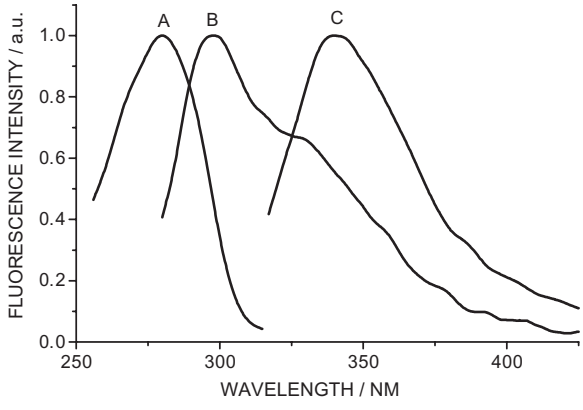

Fig. 1. Corrected excitation (curve A) and emission (curves B and C) spectra of 5.4 μM hIFNγ in 20 mM Tris-HCl, pH 7.5 at 300 K. Curve A: λ_{em} = 340 nm; curve B: λ_{ex} = 270 nm; curve C : λ_{ex} = 297 nm.

The fluorescence decay curve was fitted with good precision (chi-square = 1.18) to a biexponential linear function with fluorescence lifetimes τ_1 = 0.11 ns and τ_2 = 2.87 ns. These values were close to those reported in (Boteva *et al.*, 1996)- τ_1 = 0.11 ns and τ_2 = 3.1 ns.

Fluorescence quenching effects

Fluorescence quenching experiments were performed with two different types of quenchers, iodide (J^-) 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 (γ = 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 ε_{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: $K_{q(acrylamide)}$ = 4.68 M^{-1} and $K_{q(J-)}$ = 0.42 M^{-1}. This result is in good agreement with the hIFNγ models predicting strong negative electrostatic potentials (Ealick *et al.*, 1991; Altobelli *et al.*, 2001). Although the hIFNγ 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 hIFNγ in urea and guanidine chloride solutions

The structural stability of recombinant hIFNγ 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 DG was calculated using the equation

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

where K is the equilibrium constant, I^f is the observed fluorescence intensity and I^f_n and I^f_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:

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

where м 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 are sigmoid, which indicates that the denaturation of hIFNγ in both solutions is a two-state process. Our data clearly show, that the unfolding of hIFNγ 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 м for Gn.HCl and 2.8 м for urea. The free energies of unfolding estimated from these curves by the linear extrapolation method at pH 7.4 are ΔG^0 = 11.7 kJ/mol for urea and ΔG^0 = 13.4 kJ/mol for Gn.HCl respectively. The two values are very close, indicating that the contribution of electrostatic interactions to the stability of hIFNγ 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 hIFNγ under the experimental conditions used (pH = 7.4, 20 мм Tris, 150 мм NaCl, 25 °C). According to the calorimetric studies in (Beldarrain *et al.*, 1999), ΔG^0 of hIFNγ 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.

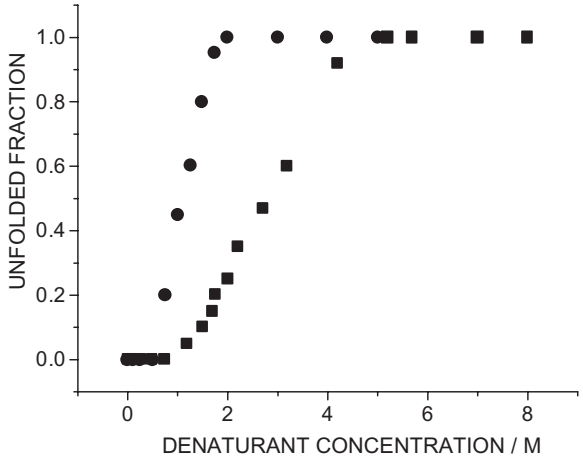

Fig. 2. Fraction of hIFR-γ unfolded as a function of Urea (■) and Gn.HCl (●) concentrations. Experimental conditions: protein concentration 5.4 μм in 20 мм Tris-HCl buffer at pH 7.5, T = 300 K; λ_{ex} = 297 nm , λ_{em} = 340 nm.

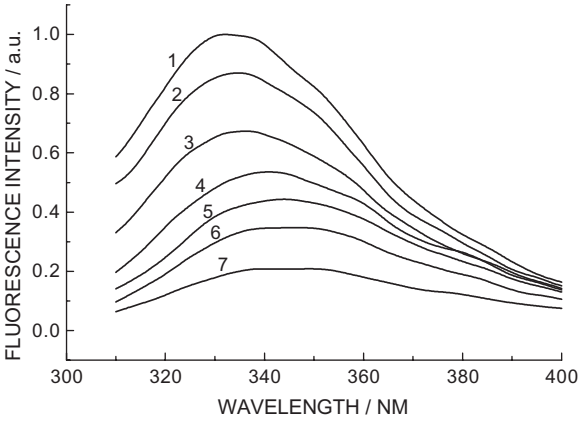

Fig. 3. Changes in the fluorescence spectra of hIFR-γ in 20 мм Tris-HCl buffer at pH 7.5, T = 300 K, protein concentration 5.4 μм, λ_{ex} = 297 nm, at different concentrations of Gn.HCl: (1) – O м; (2) – 0.75 м; (3) – 1 м; (4) – 1.2 м; (5) – 1.35 м; (6) – 1.5 м; (7) – 1.75 м.

pH Dependence of the unfolding/folding transition of hIFNγ

To elucidate the effect of electrostatic interactions on the stability of hIFNγ, the pH-dependence of unfolding was studied in urea at 25 °C. Prior to that, the effect of pH on the hIFNγ 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 hIFNγ was preserved in this pH interval. This was confirmed also by two bioassays (antiviral and antiproliferative) for quantification of the hIFNγ 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 hIFNγ at different pH values is calculated using Equs. (1) and (2). It was found that the stability of hIFNγ does not depend on pH in the range of 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 hIFNγ 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).

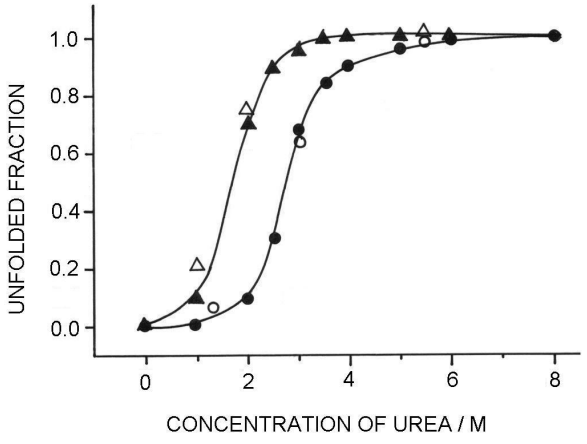

Fig. 4. Fraction of hIFR-γ unfolded as a function of Urea concentration at pH = 5.2 (▲) and pH = 6.0 (●). The refolding data for pH = 5.2 and pH = 6.0 are presented by (△) and (○), respectively. Experimental conditions: protein concentration 5.4 μM in 20 mM Tris-HCl buffer, T = 300 K, λ_{ex} = 297 nm,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

The dependence of ΔG^0 on pH was fitted to the titration (sigmoid) curve of a group with pK = 5.2. The fitting parameters for the Boltzmann fit are: Chi-sqr = 0.00175E–4, init (A_1) = 1.4532, final (A_2) = 2.8095, XatY50(x0) = 5.0587, width(dx) = 0.44818, XatY20 = 4.43743, XatY80 = 5.68004. A similar group with 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 hIFNγ. Based on these results and our earlier calculations of electrostatic interactions in hIFNγ (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 hIFNγ. 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 hIFNγ?

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

parameters and ΔG^0 of fresh non-stabilised and stabilised hIFNγ. 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 hIFNγ (at pH = 7.5) are τ_1 = 0.13 ns and τ_2 = 2.93 ns and for non-stabilised preparations they are τ_1 = 0.11 ns and τ_2 = 2.87 ns, respectively. Based on this data we conclude that the presence of dextran does not change significantly the fluorescence properties of hIFNγ.

Two types of experiments were carried out to evaluate the effect of dextran on hIFNγ stability. The pH dependencies of fluorescence intensity of pure and dextran stabilised hIFNγ were compared and it was found that dextran does not interfere with the fluorescence properties of hIFNγ as a function of pH. The unfolding of pure and dextran-stabilised hIFNγ 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 hIFNγ. Based on these experimental results, we conclude that dextran does not interact with hIFNγ so as to change remarkably its fluorescence behavior and thermodynamic stability.

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