Pressure- and temperature-induced unfolding and aggregation of recombinant human interferon-γ: a Fourier transform infrared spectroscopy study

Koen GOOSSENS*, Joost HAELEWYN†[1], Filip MEERSMAN*, Marc DE LEY† and Karel HEREMANS*[2]

*Laboratorium voor Chemische en Biologische Dynamica, Celestijnenlaan 200 D, B-3001 Leuven, Belgium, and †Laboratorium voor Biochemie, Department of Chemistry, Katholieke Universiteit Leuven, B-3001 Leuven, Belgium

The effect of hydrostatic pressure on the secondary structure of recombinant human interferon-γ (rhIFN-γ) and its biologically inactive truncated form rhIFN-ΔC15 has been studied using Fourier-transform IR (FTIR) spectroscopy. *In situ* observation of the pressure-induced changes using the diamond anvil cell shows that the α-helical structure is mainly transformed into disordered structure at high pressure. Increasing pressure also induces the formation of a gel. Addition of 0.5 M $MgCl_2$ significantly reduces the pressure stability. Releasing the pressure below 300 MPa results in the formation of intermolecular antiparallel β-sheets, which is seldom observed. This suggests that the intermolecular β-sheet of rhIFN-γ is stabilized by electrostatic interactions that are disrupted at high pressure. For comparison we also studied the effect of temperature. Temperature-induced changes reflect extensive transformation of α-helical structure into intermolecular antiparallel β-sheet, as is usually observed for most proteins.

Key words: aggregation, antiparallel β-structure, denaturation, gel formation, human interferon-γ.

INTRODUCTION

The cytokine interferon-γ (IFN-γ), plays a central role in the immune system. It is produced by activated T-cells and natural killer cells. Its antiviral and immunomodulatory activities are mediated through binding to a receptor present on the target cells [1]. Biologically active IFN-γ is a dimeric protein that consists of two identical polypeptide chains of 143 amino acids. It does not contain disulphide bonds, and the pI was found to be 10.4 [2]. The X-ray crystallographic structures of IFN-γ [3] and the IFN-γ/receptor complex [4] revealed a compact and globular dimer with a primarily α-helical (62 %) and no β-sheet structure. Each subunit consists of six α-helices; the extensive intertwining of the helices from the two subunits into one another provides the opportunity for multiple non-covalent interactions that stabilize the dimer. IFN-γ is not stable in acidic medium: below pH 4.5 the dimer dissociates into monomers, with concomitant drastic changes in secondary structure [5]. The C-termini of the molecule do not adopt a rigid conformation [6]; they are exposed to the solvent and accessible to a large number of proteolytic enzymes.

The stability of proteins in solution is dependent on solvent composition, temperature and pressure. One of the aims of molecular biology is to correlate changes in the chemical and physical conditions with changes in the conformation of these bio-macromolecules. Bridgman [7] incidentally discovered that the white of an egg aggregates with increasing hydrostatic pressure. The fact that one can denature proteins with temperature as well as pressure is reflected in their elliptic phase diagram [8,9]. The shape and the position of the phase diagram is determined by the temperature- and pressure-dependence of the enthalpy and volume change of the denaturation process. In the present study we illustrate some of the consequences of the phase diagram for the denaturation of proteins with the specific example of the cytokine recombinant human IFN- γ (rhIFN-γ). The relevance of denaturation studies lies in the fact that such analyses provide insight into the mechanism of protein unfolding and aggregation. The prominent role of aggregation can be observed in molecular diseases, such as Alzheimer's and Parkinson's disease [10], and in inclusion bodies [11].

The differences between the temperature- and pressure-induced changes of the secondary structure during the denaturation process are studied with Fourier-transform IR (FTIR) spectroscopy. Analysis of the amide I′ band allows the determination of the contribution of different secondary structure components of proteins [12,13]. IR spectroscopy is potentially useful in characterizing the difference between temperature- and pressure-induced unfolding and aggregation of proteins [14]. In many proteins, temperature denaturation gives rise to the appearance of IR bands which have been attributed to strong intermolecular β-structures [15,16]. Notable exceptions to this rule are ribonuclease A [17,18] and ribonuclease T1 [19]. No such bands have been observed in pressure-unfolded proteins, although aggregation has been observed in many cases [14,20,21]. In the present study, we show that pressure-unfolded rhIFN-γ develops the typical IR bands that are indicative for intermolecular β-structures after pressure release. This is the first time such an effect has been observed at ambient temperature (25 °C).

EXPERIMENTAL

Sample preparation

Recombinant human IFN-γ was produced by *Escherichia coli* cells and purified as described previously [22]. Briefly, rhIFN-γ inclusion bodies from *E. coli* cells were isolated by means of sucrose-density-gradient ultracentrifugation and solubilized in

Abbreviations used: FTIR, Fourier-transform IR; FWHH, full width at half height; IFN-γ, interferon-γ; rhIFN-γ, recombinant human IFN-γ.

[1] Present address: Innogenetics N.V., Department of Biologicals, Technologiepark 6, B-9052 Zwijnaarde, Belgium

[2] To whom correspondence should be addressed (e-mail Karel.Heremans@fys.kuleuven.ac.be).

6 M guanidine hydrochloride. After subsequent refolding, rhIFN-γ was purified to homogeneity on a Mono S HR 5/5 column, connected to an FPLC system (Pharmacia, Uppsala, Sweden). The resulting electrophoretically pure protein was dialysed twice against 30 mM NH_4HCO_3 for 24 h. Afterwards the solution was filtered using a nitrocellulose filter (0.2 μm pores), and then frozen to -80 °C and freeze-dried for 48 h. This procedure yields the biologically active dimer, as demonstrated by the interaction of the rhIFN-γ with its receptor [23]. Moreover, cross-linking with disuccinimidyl suberate results in a 34 kDa band in SDS/PAGE, indicative of a dimer [22]. No aggregates were present as shown by X-ray scattering analysis (J. Haelewyn and M. De Ley, unpublished work).

In order to remove 15 amino acids from the C-terminal region of the molecule, rhIFN-γ was treated with plasmin (Boehringer Mannheim, Germany), resulting in cleavage of the Lys^{128}–Arg^{129} bond [24]. The rhIFN-γ fragment (which will be referred to as rhIFN-γ ΔC15 herein) was purified by size-exclusion chromatography on a Superdex 75 HR 10/30 column (Pharmacia) and showed an elution peak between the markers myoglobin (17 kDa) and ovalbumin (43 kDa), indicative of the dimeric nature of the protein. After purification, it was similarly dialysed and freeze-dried as described for the native rhIFN-γ. The rhIFN-γ ΔC15 was characterized as described previously [23].

Solutions were prepared at 10 % (w/w) in ^2H_2O at pD 7 in 50 mM Bis-Tris buffer. One temperature titration was carried out by dissolving rhIFN-γ at the same concentration in ^2H_2O at pD 5.3 in 50 mM acetic acid buffer. The samples were stored overnight (16 h) to ensure that all solvent accessible hydrogens would have been exchanged for deuterons. Thus hydrogens buried within the protein will only become exchanged when the protein unfolds. In general, this will not have a significant effect on the analysis of the amide I′ band. However, in the case of ribonuclease A, it has been previously reported that significant changes in the IR spectrum are associated with the H/^{2}H-exchange of the buried amide protons (after unfolding the protein by heating to expose the buried amide hydrogens and subsequent refolding by cooling) [25]. In the present case, the H/^{2}H-exchange associated with a further unfolding of the protein does not seem to have a significant effect on the amide I′ band. Consequently it does not interfere with our analysis of this band. The samples were clarified by centrifugation for 10 min at 12 100 ***g***.

IR spectroscopy at variable temperature and pressure

IR spectra were recorded with a Bruker IFS66 FTIR spectrometer continuously purged with dry air and equipped with a broad band mercury—cadmium—telluride solid state detector. A total of 350 interferograms were accumulated after registration at a resolution of 2 cm^{-1}. Temperature scans were performed with a Graseby Specac low-voltage heating system at a rate of 0.2 °C/min. The time necessary to record one data point was about 3 min. The cell was fitted with CaF_2 windows and had a constant pathlength of 50 μm. For the pressure measurements, the solution was mounted in a stainless steel gasket of a diamond anvil cell (Diacell Products, Leicester, U.K.). The pathlength was 75 μm at ambient pressure. The IR light was focussed on the sample by a NaCl lens [26]. The rate of pressure increase/decrease was approx. 150 MPa/h. The time necessary to record one data point was approx. 10 min because six additional minutes were needed to eliminate absorption due to H_2O vapour. Fine-powdered $BaSO_4$, pressed in the sample hole of the diamond anvil cell, was used as an internal pressure calibrant [27].

Gel formation

Turbidity as a function of pressure was measured in the diamond anvil cell mounted in the Hewlett Packard 8452A diode array spectrophotometer. Pressure was calculated from the ruby fluorescence with a Spex laser Raman spectrometer. The ruby technique has the advantage that it allows easier inspection of the sample under the microscope.

Data analysis

The method to determine the secondary structure of proteins and peptides has been described elsewhere [13]. It is based on a combination of self-deconvolution with band fitting. Fourier self-deconvolution, a mathematical technique of band narrowing [28], was performed with a program developed in our laboratory. The deconvolution and noise reduction factor to be used depended on the quality of the spectral data. The procedure that we developed in order to obtain the maximum amount of significant information from Fourier self-deconvolution is based on a visual inspection of the power spectrum [29]. The data in this paper were analysed with a full width at half height (FWHH) of 23 cm^{-1} with a triangular squared apodization function, with a cutting of 81 % for the noise reduction. This results in a resolution enhancement factor of approx. 1.8. The program also allows the analysis of the frequency shifts as well as the changes in the area of the subcomponents of the amide I′ bands as a function of pressure and temperature. The calculation is based on a Marquardt–Levenberg algorithm.

RESULTS AND DISCUSSION

FTIR spectrum of rhIFN-γ at ambient conditions

A fit of the deconvoluted amide I′ region of the IR spectrum of rhIFN-γ at ambient conditions (25 °C and 0.1 MPa) is shown in Figure 1. The spectrum shows bands at 1611, 1633, 1651 and 1668 cm^{-1}. The band at 1611 cm^{-1} has been assigned to amino acid side chains [30]. The occurrence of bands around 1633 cm^{-1} for highly helical proteins has been assigned to short extended chains connecting the helical cylinders [12]. However, recent model calculations for myoglobin and tropomyosin indicate that

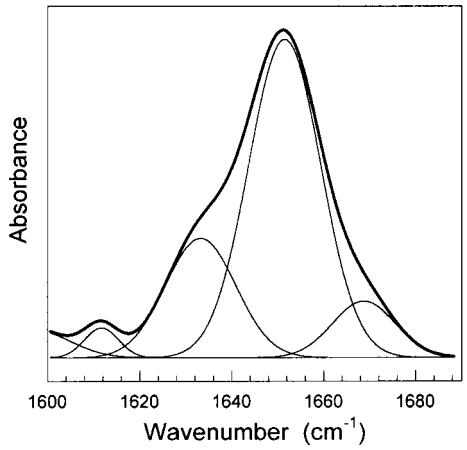

Figure 1 The amide I′ band of native rhIFN-γ

The spectrum was taken at 25 °C and 0.1 MPa. The band was deconvoluted and fitted as described in the Experimental section.

Table 1 Assignments and relative contribution of the different amide I′ components of rhIFN-γ at ambient conditions (25 °C and 0.1 MPa)

Band position (cm^{-1})	Band area (%)	Assignment
1611	3	side chain
1633	23	extended chain
1651	64	α-helix
1668	10	turn

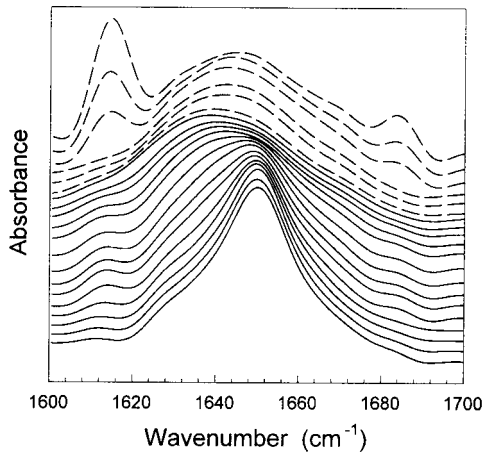

Figure 2 Pressure-dependence of the amide I′ region of native rhIFN-γ at 25 °C

Deconvoluted spectra are shown. Solid lines represent bands at increasing pressures, dashed lines show decreasing pressures. The spectra were taken at (from bottom to top): 0.1, 50, 100, 150, 200, 250, 270, 310, 340, 380, 430, 540, 680, 1040, 890, 560, 490, 230, 170 and 70 MPa.

such an interpretation is not necessarily correct [31,32]. These calculations show that bands below 1640 cm^{-1} may also originate from vibrational motions of solvated α-helices. The band at 1651 cm^{-1} has been assigned to α-helical structure [12]. The relatively high bandwidth (about 19 cm^{-1} FWHH) suggests that the helical sections are non-uniform, and that the broad band consists of other subcomponents which remain unresolved. The band at 1668 cm^{-1} may be assigned to turn structure [12,33]. The assignments and the percentages of the band areas are given in Table 1. The main contribution to the spectrum is the α-helical structure (64 %). The result of our analysis corresponds closely with other methods: X-ray analysis gives 62 % α-helix and no β-sheet [3]; NMR-derived secondary structure [6] essentially confirms the X-ray analysis and circular dichroism measurements confirm that the protein is primarily α-helical (66 % [34]; 67 % [2]; 60 % [35]) and that it still contains α-helical structure (30 %) in the unfolded state at pH = 3.5 [5]; FTIR second derivative spectroscopy [35,36] and the results of secondary structure prediction [37,38] also suggest that rhIFN-γ has a high α-helix content.

Pressure-dependence of the IR spectra

Native rhIFN-γ

The IR spectra reveal no distinct change in the amide I′ band profile with increasing pressure up to 150 MPa, indicating that the secondary structure of rhIFN-γ is unaffected in this pressure range (Figure 2). The small shift in the frequency of the band at

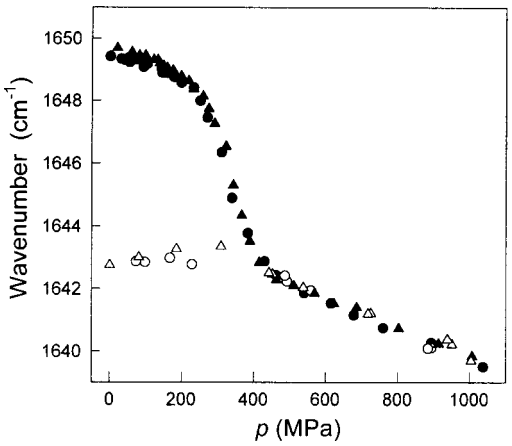

Figure 3 Pressure effect on the frequency maximum of native rhIFN-γ (○) and rhIFN-γ ΔC15 (Δ)

Closed symbols denote pressure increase and open symbols denote pressure decrease.

1651 cm^{-1} ($d\nu/dp \approx -0.2\ cm^{-1}/100$ MPa) is indicative of stronger hydrogen-bonding within the α-helical structure induced by pressure [39].

As the pressure is further increased, the amide I′ band starts to broaden and several changes take place. Bands start to develop at 1614 and 1685 cm^{-1} and their frequencies decrease and increase respectively with increasing pressure. This suggests the formation of intermolecular, hydrogen-stabilized β-structures, as often observed in the temperature denaturation of proteins [15,16,35,40]. However, their low intensity suggests that only part of the molecules are aggregated in such a way. Moreover, it should be emphasized that these bands were not present at 25 °C and 0.1 MPa, suggesting that a possible adsorption of IFN-γ to the powdered barium sulphate is not responsible for aggregation.

Above 200 MPa the intensity of the band at 1650 cm^{-1} decreases, whereas the intensities at 1644 cm^{-1} and 1630 cm^{-1} increase with increasing pressure. The effect of the change in relative intensities with pressure is also reflected in the frequency maximum of the amide I′ band (Figure 3). There is a co-operative transition at approx. 300 MPa. Visual inspection of the movement of a ruby chip shows the formation of a more viscous medium in the cell above 200 MPa. Simultaneously, the development of a gel was measured by observing the turbidity of the sample (results not shown). It is known that rhIFN-γ easily forms a gel upon heating, lowering the pH down to pH 2 or in guanidinium chloride concentrations below 2.0 M [35]. Pressure-induced gelation has been observed before for bovine pancreatic trypsin inhibitor [21] and for many food-related proteins such as β-lactoglobulin and actomyosin [41,42].

Above 500 MPa, no further intensity-redistributions can be observed (Figure 2), and the shift of the amide I′ band maximum reaches a plateau (Figure 3). The resolution-enhanced spectra of the amide I′ band (Figure 2) reveal that the features assigned to α-helix, β-sheet and turns, at ambient conditions, are less pronounced in the spectra above 500 MPa. A broad amide I′ band reflects the presence of many different kinds of hydrogen-bonded carbonyl groups in the protein. In general, one kind of hydrogen-bonding corresponds with one sharp frequency but a mixture corresponds to a broad band. The maximum of the Gaussian band reflects the most probable hydrogen-bonded carbonyl group. A frequency of 1644 cm^{-1} at ambient pressure can be extrapolated from the pressure-dependence of the maxi-

mum of the amide I′ band above 500 MPa (Figure 3). This value results from peptide groups that are hydrogen-bonded to solvent molecules but not to other groups within the protein [12]. At pressures higher than 500 MPa we observe that the contributions of disordered structure and distorted β-sheet are increased at the expense of the decrease in the α-helical structure.

Webb et al. [43] observed the concomitant dissociation and unfolding of the dimeric rhIFN-γ at 140 MPa using second derivative UV spectroscopy. Although we observe the formation of intermolecular β-sheet structure between 150 and 200 MPa, the secondary structure of rhIFN-γ does not unfold up to 200 MPa and the main transition is found at 300 MPa (Figure 3). We suggest that the aggregation results from the pressure-induced dissociation of the dimeric rhIFN-γ. The small increase in pressure stability (200 versus 140 MPa) can be accounted for by the difference in experimental conditions. On the basis of the data on the concentration effect presented in the paper by Webb et al. [43], we have been able to calculate the shift in pressure stability for the protein concentration used in the present study (100 mg/ml) using the following equation:

$$\Delta P = RT \ln(N_{01}/N_{02})/\Delta V_{d}$$

where $\Delta P = P1 - P2$, N_{01} and N_{02} are the initial concentrations for conditions 1 and 2, and ΔV_{d} is the volume change upon dissociation. We can predict a shift of the transition midpoint by ~ 60 MPa to higher pressure. This coincides well with the observed pressure region for the aggregation. Our data therefore seem to suggest that there is a simultaneous dissociation and aggregation of rhIFN-γ. However, because the secondary structure (mainly α-helix) is not significantly affected in this pressure region, it is likely that the α-helix structure does not participate in the intermolecular interactions.

As shown in Figure 2, upon pressure release, the IR spectra reveal no visible change in the amide I′ band profile down to 300 MPa, indicating that the secondary structure of rhIFN-γ remains the same as compared with the secondary structure at 1 GPa. The small shift to higher frequencies with decreasing pressure ($d\nu/dp \approx -0.4$ cm^{-1}/100 MPa) indicates a weakening of the hydrogen-bonding between the C=O groups of the protein backbone and the solvent.

Below 300 MPa, new features appear in the spectrum. The bands with small intensities at 1631, 1649 and 1670 cm^{-1} are due to the amide I′ modes in the intramolecular β-structure, the α-helix and turn structures respectively [12,33]. The most prominent features are the bands with high intensity at 1614 and 1684 cm^{-1}. These bands have been observed in proteins unfolded by heat and have been assigned to intermolecular antiparallel β-structures [15,16]. Although thermally induced aggregation via this mechanism was suggested to be a general phenomenon [44], these bands do not appear upon denaturation by heat in ribonuclease A [17], ribonuclease T1 [19] or horseradish peroxidase [45]. Our results indicate that the pressure-unfolded rhIFN-γ forms aggregates via a similar mechanism as in the temperature denaturation, provided that the pressure is released below 300 MPa. This points to the fact that the intermolecular β-sheets are stabilized by extensive ionic interactions between the carboxylate groups and basic residues of the neighbouring peptide molecules [46,47]. As revealed by our data, the protein is unfolded at high pressure (approx. 500 MPa) but a lining up of the backbones of the peptides upon charge neutralization is not possible because of the positive reaction volume that accompanies electrostatic interactions. Releasing the pressure below 300 MPa allows ionic interactions, resulting in the formation of a network of intermolecular hydrogen bonds [46,48]. To our knowledge, this is the first report of such intermolecular antiparallel β-sheet aggregation after pressure treatment at ambient temperature.

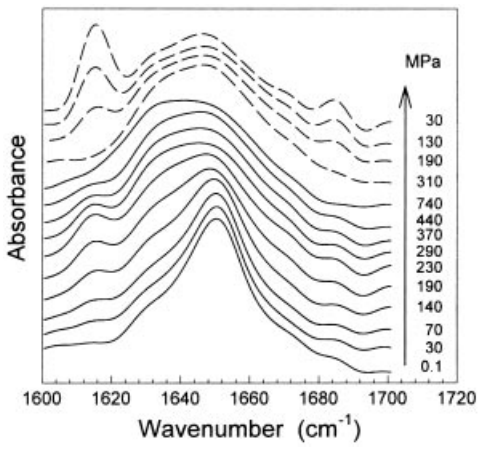

Figure 4 Pressure-dependence of the deconvoluted spectra of native rhIFN-γ in the presence of 0.5 M $MgCl_2$ at 25 °C

Solid lines represent bands at increasing pressures, dashed lines show decreasing pressures.

Addition of 0.5 M $MgCl_2$ results in a strong destabilization of the native rhIFN-γ against high pressure denaturation, as can be seen from Figure 4. At 70 MPa the protein starts to unfold, with the appearance of two bands at 1614 and 1684 cm^{-1}. Increasing the pressure up to 200 MPa results in a further formation of intermolecular antiparallel β-structure, as indicated by the increased intensities of the two bands. Application of higher pressure seems to result in the disruption of the intermolecular hydrogen bonds between the unfolded proteins, as revealed by the disappearance of the bands at 1614 and 1684 cm^{-1}. It is known that pressure can dissociate protein oligomers [49] and protein aggregates [50,51].

When the pressure is released below 300 MPa, the bands appear again, as observed in the absence of salt. This shows that 300 MPa is the upper limit at which formation of intermolecular antiparallel β-sheet is possible for rhIFN-γ. An additional prerequisite for this type of aggregation seems to be a sufficient unfolding of the protein below 300 MPa. The intensities of the bands at 1614 and 1684 cm^{-1} are lower, compared with the situation where no salt is added. This shows the importance of salt bridges in stabilizing strongly hydrogen-bonded structures between unfolded backbone amide groups [52].

Pressure-unfolded rhIFN-γ

After releasing the pressure in the first cycle, a second pressure cycle, in the absence of $MgCl_2$, was applied to study the effect of increasing pressure on the induced antiparallel β-sheet aggregated rhIFN-γ. The result can be seen in Figure 5. The intensities of the bands at 1614 and 1684 cm^{-1} decrease dramatically with increasing pressure relative to the broad band with a maximum at 1644 cm^{-1}. A shoulder develops at 1633 cm^{-1} as a result of a gradual transformation of ordered antiparallel β-sheet structure into a random coil and a highly distorted β-sheet structure. This transformation is accompanied by a small decrease in turbidity of the protein gel and is completely reversible (results not shown). The low and high frequency bands are still present at 1.1 GPa, indicating that it is not possible to completely disrupt the intermolecular antiparallel β-sheets. This has also been found in the case of myoglobin [51]. A possible explanation for this may

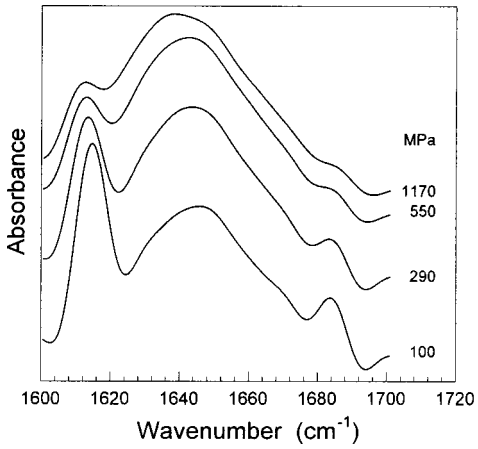

Figure 5 Pressure effect on the deconvoluted spectra of pressure-unfolded rhIFN-γ in the absence of $MgCl_2$

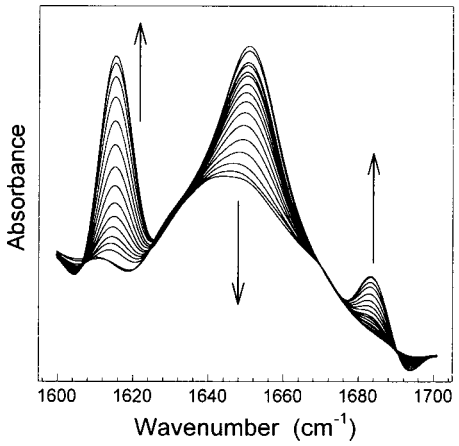

Figure 6 Thermal behaviour of native rhIFN-γ at 0.1 MPa

Deconvoluted spectra at 40, 45, 50 to 63 (in steps of 1 °C), and at 73 °C. Arrows indicate the direction of increasing temperature.

be provided by the fact that the volume decrease, due to the solvation of the disrupted salt-bridges at high pressure [53], is partially compensated by a volume increase, due to the disruption or weakening of hydrogen bonds [54], when transforming anti-parallel β-sheet into disordered structure. The bands observed at 1614 and 1684 cm^{-1} near ambient pressure shift to 1610 and 1686 cm^{-1} with increasing pressure. The increased splitting of these two bands points to a stronger coupling of the backbone carbonyl functions [48].

C-terminal truncated rhIFN-γ

The removal of 15 C-terminal amino acid residues gives rise to rhIFN-γ ΔC15. It was shown by Haelewyn and De Ley [23] that removal of Arg^{129} results in a marked loss of the biological activity. It is therefore interesting to investigate whether this loss of activity is related to a change in secondary structure. The amide I′ band reveals no significant difference in secondary structure between the truncated form and the native intact molecule (results not shown). This is in agreement with the CD-analysis of IFN-γ Δ18 [2], and with the knowledge that the C-terminal region of IFN-γ is of no importance for the structural integrity of the dimer [6]. The pressure-dependence of the deconvoluted amide I′ band of rhIFN-γ ΔC15 is exactly the same as for the native rhIFN-γ (results not shown). The modified protein shows the same pressure stability as compared with the wild type (Figure 3). This result indicates that truncation of the highly positively charged C-terminus does not influence the pressure behaviour of rhIFN-γ. When the pressure is released below 300 MPa, the amide I′ band of rhIFN-γ ΔC15 shows the same shape as the complete rhIFN-γ (results not shown). This shows that the C-terminus does not influence the formation of the intermolecular β-structure of pressure-unfolded rhIFN-γ.

Temperature-dependence of the IR spectra

Figure 6 shows the temperature-dependence of the deconvoluted IR spectra of rhIFN-γ up to 73 °C. It can be seen that the spectrum remains unchanged up to 50 °C, suggesting minor changes in the secondary structure. Above 50 °C, the intensity at 1650 cm^{-1} decreases while new bands appear at 1614 and 1684 cm^{-1}. Since the bands have been assigned to strongly aggregated proteins via intermolecular β-structures [15,16], our results indicate that the temperature-induced unfolded protein strongly aggregates. Furthermore, one can also observe small contributions at 1630, 1650 and 1670 cm^{-1}, indicating the presence of extended chains, α-helix and turn-structure in the aggregated state. The formation of aggregates is an irreversible process, because a decrease in temperature from 73 °C towards room temperature did not alter the features of the amide I′ band. The conformation of the unfolded protein is trapped in the network of aggregates. Our findings are in agreement with the work of Kendrick et al. [35]. They found that the aggregation of rhIFN-γ follows first-order kinetics. Although there is no observable intermediate in the FTIR spectra, these authors were able to describe a pathway in which a transient expansion of the rhIFN-γ native state precedes the aggregation [55]. This pathway was able to explain both the first-order kinetics as well as the inhibitory effect of sucrose on this process.

The ratio of the amide I′ band intensity at 1615 cm^{-1} to that at 1650 cm^{-1} may be used as a convenient parameter to follow the thermal unfolding of rhIFN-γ (Figure 7). The midpoint

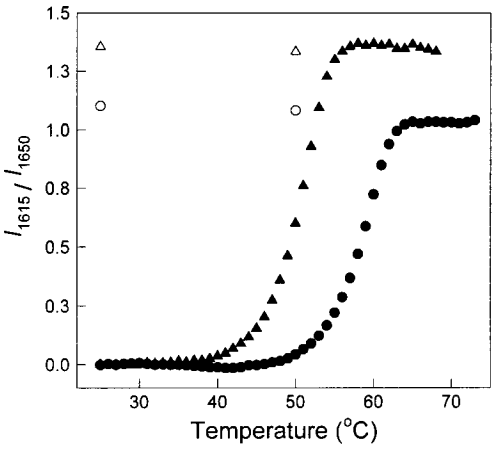

Figure 7 Temperature-dependence of the ratio of the intensity of the amide I′ band at 1615 cm^{-1} to that at 1650 cm^{-1} for native IFN-γ

Results for pD = 7.0 (●) and at pD = 5.3 (▲) are shown. Closed symbols, temperature increase; open symbols, temperature decrease.

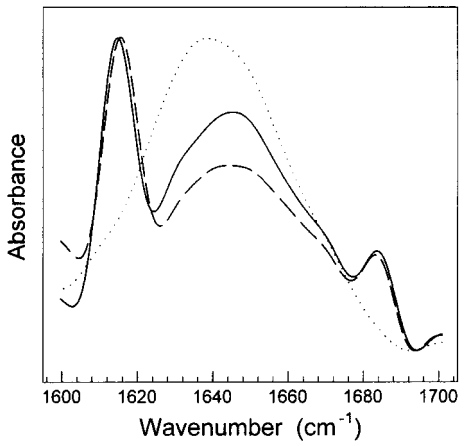

Figure 8 Superposition of the deconvoluted spectrum at 70 °C/0.1 MPa (- - -), at 1 GPa/25 °C (···) and at 70 MPa/25 °C after pressure treatment (—)

denaturation temperature for rhIFN-γ at pD = 7.0, as identified by this parameter, is 58 °C. Decreasing the pD to 5.3 results in a marked decrease in the thermal stability of the protein, with the apparent midpoint temperature of the denaturation at 51 °C. This effect is in agreement with the thermal denaturation profiles of rhIFN-γ at low concentration [56]. The data of Figure 7 also suggest differences in the behaviour of unfolded rhIFN-γ at the two different pD values. The higher I_{1615}/I_{1650} ratio at high temperature for the protein at pD = 5.3 is indicative of an increased aggregation. This result is in contradiction to the results obtained at low concentration, where the aggregation was found to be half-maximal at pH = 5.7 [56]. It was suggested that deprotonated histidine residues may be involved in the aggregation process. When the pH was further decreased to 5.0, rhIFN-γ was found to be stable to irreversible aggregation [56]. Our results indicate that the protonated histidine residues stabilize the observed antiparallel β-structures.

Temperature versus pressure denaturation

Figure 8 shows a superposition of the deconvoluted amide I′ bands of the pressure-unfolded (at 1 GPa as well as after return to 0.1 MPa) and the temperature-unfolded rhIFN-γ. Although the protein is in an aggregated state at 1 GPa, the spectrum is broad and does not show any features. In contrast, the deconvoluted spectrum at 70 °C shows the appearance of two additional bands at low and high frequency of the main band, indicative for the formation of intermolecular antiparallel β-structures [15,16]. This suggests that the interactions in the temperature-induced aggregate are different from the pressure-induced aggregate. This is confirmed by rheology measurements which have shown that temperature-induced gels of β-lactoglobulin are characterized by stronger interactions [57]. However, at 70 MPa the deconvoluted spectrum of the pressure-treated unfolded rhIFN-γ shows the same features as the spectrum at 70 °C. Thus releasing the pressure allows the conversion into a more turbid, temperature-like gel. This has not been observed before. In the case of bovine pancreatic trypsin inhibitor, for instance, the gelation was not accompanied by the formation of these intermolecular bands, not even after pressure release [21]. Compared with the spectrum at 70 °C, the pressure-released rhIFN-γ is less aggregated and consequently has more intramolecular secondary structure left, as indicated by the higher relative ratio between the 1650 and 1614 cm^{-1} bands.

The bands at low and high frequency were only observed at pressures below 300 MPa. This indicates that the observed intermolecular β-sheet structures are stabilized by electrostatic interactions between charged amino acid residues. This hypothesis is supported by the observation that these interactions are weakened in the presence of 0.5 M $MgCl_2$.

Lowering the pD from 7.0 to 5.3 destabilizes the protein but does not affect the aggregation behaviour of rhIFN-γ. This indicates that the histidine residues are not involved in the stabilization of the intermolecular β-sheet structures. In view of the hypothesis of the stabilizing effect of ionic interactions, there are two sequence regions that contain a high number of charged amino acid residues and therefore may play a role in the interaction between the unfolded backbones. The first is the highly positively charged C-terminal part of the sequence. Because the truncation of this part does not influence the pressure-stability and aggregation behaviour of rhIFN-γ, despite the fact that a decrease in biological activity has been observed [23], this region of sequence can be excluded. A second part of sequence is located at the end of helix E, between Phe^{82} and Tyr^{98} [24], and is as follows: Phe^{82}-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp^{90}-Asp-Phe-Glu-Lys-Leu-Thr-Asn-Tyr^{98}. An antiparallel alignment of this sequence shows that the antiparallel β-sheet could be stabilized by the formation of six ion pairs between the two side-chain residues of the polypeptide backbones. It is not excluded that this is the starting point of the aggregation.

In conclusion, the experiments presented in this paper suggest differences in conformation of the temperature- and the pressure-unfolded rhIFN-γ. Temperature-induced denaturation of recombinant human rhIFN-γ readily gives rise to the formation of intermolecular antiparallel β-sheet, whereas pressure induces an aggregate that is maintained by other interactions. Only upon pressure release do the bands typical of temperature-induced aggregation appear. Their appearance implies a strengthening of the gel through the formation of new electrostatic interactions.

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