Susceptibility towards intramolecular disulphide-bond formation affects conformational stability and folding of human basic fibroblast growth factor

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The conformational stability and the folding properties of the all-β-type protein human basic fibroblast growth factor (hFGF-2) were studied by means of fluorescence spectroscopy. The results show that the instability of the biological activity of hFGF-2 is also reflected in a low conformational stability of the molecule. The reversibility of the unfolding and refolding process was established under reducing conditions. Determination of the free-energy of unfolding in the presence of reducing agents revealed that the conformational stability of hFGF-2 ($\Delta G^{H_2O}_{app} \cong$ 21 kJ·mol^{-1}, 25 °C) is low compared with other globular proteins under physiological conditions (20–60 kJ·mol^{-1}). However, the conformational stability of hFGF-2 is particularly low under non-reducing conditions. This instability is attributed to intramolecular disulphide-bond formation, rendering the molecule more susceptible to denaturant-induced unfolding. In addition, denaturant-induced unfolding of hFGF-2 renders the protein more susceptible to irreversible oxidative denaturation. Experimental evidence is provided that the irreversibility of the unfolding and refolding process in the absence of reducing agents is linked to the formation of an intramolecular disulphide bond involving cysteines 96 and 101.

INTRODUCTION

Human basic fibroblast growth factor (hFGF-2) is a single-chain heparin-binding polypeptide synthesized by a variety of different cell types [1,2]. It is a general regulatory mediator of cell growth, stimulating proliferation and inducing or delaying differentiation [1,2]. hFGF-2 is a potent mitogen for endothelial cells [3] and for other cells of mesodermal and neuroectodermal origin [1,2]. The wound-healing activity of hFGF-2 [4,5] renders it a potential therapeutic agent of industrial importance [6,7].

hFGF-2 is known as a notoriously unstable protein. Its mitogenic activity is destroyed by treatment with a variety of solvents, including dilute acid, organic solvents and solutions of guanidinium chloride (GdmCl) [8]. In addition, hFGF-2 displays a marked instability when stored at room temperature, exposed to alkaline pH or incubated in the presence of catalytic amounts of Cu^{2+} ions [9]. Inactivation of hFGF-2 can be reduced upon addition of heparin or heparin-analogue compounds [10–12].

The chemical instability of hFGF-2 is caused by the rapid oxidation of thiol groups. Despite the presence of four cysteines, there is no intramolecular disulphide bond present in native hFGF-2 [9,13–19]. Cysteines 34 and 101 (numbering refers to the 155-amino acid form of hFGF-2) are still present as free cysteines upon treatment of hFGF-2 with alkylating agents [9,17]. Only cysteines 78 and 96 can be modified by thiolation or carboxymethylation [9,17,20]. Determination of the three-dimensional structure of the all-β-type protein revealed that cysteines 78 and 96 are localized on the surface of hFGF-2, whereas cysteine 34 is completely buried and cysteine 101 is partly buried within the folded peptide chain [13,14,18].

Inactivation of hFGF-2 was originally attributed to multimerization caused by oxidation of cysteines 78 and 96, resulting in the formation of intermolecular disulphide bonds [19–22]. Chemical modification of cysteines 78 and 96, or replacement of these cysteines by serines using site-directed mutagenesis techniques, avoided the formation of multimers and increased the stability of hFGF-2 [9,20,22].

In this study we describe the conformational stability and the folding properties of hFGF-2 (155-amino acid form) under reducing and non-reducing conditions using fluorescence emission spectroscopy. In addition, we provide experimental evidence that the irreversibility of the unfolding/refolding process in the absence of reducing agents is linked to the formation of an intramolecular disulphide bond involving cysteines 96 and 101.

MATERIALS AND METHODS

Chemicals

GdmCl, L-arginine, dithiothreitol and 2-mercaptoethanol (2-ME) were from Sigma (München, Germany). Ultrapure urea was from USB (Cleveland, OH, U.S.A.). Urea solutions were prepared fresh before use. All other reagents were analytical grade substances or better from Riedel de-Haën (Seelze, Germany), J. T. Baker (Gross-Gerau, Germany) and Merck (Darmstadt, Germany). MilliQ water was used throughout.

In vitro mutagenesis

The vector pJHLbFGF containing the gene of hFGF-2 (155-amino acid form) was constructed by cloning the *Nde*I–*Sal*I FGF-2 fragment of vector pλFGFB [23] into the vector pThioHis A (InVitrogen, Leek, The Netherlands), which was also digested with *Nde*I and *Sal*I. This procedure removes the thioredoxin gene and generates an FGF-2 gene fused to the strong isopropyl β-D-thiogalactoside (IPTG)-inducible *tac* promoter. For construction of a site-specific mutant, in which the cysteine at position 101 is replaced by a serine, the vector pJHLbFGF was digested with *Xba*I and *Aat*II and the small insert was isolated using Jetsorb (Genomed, Germany). This fragment was amplified using the PCR mutagenesis primer-pair A316 (5′-GAAT-TCAT ATG GCA GCA GGA TCA ATA ACA AC-3′) and

Abbreviations used: hFGF-2, human basic fibroblast growth factor; IPTG, isopropyl β-D-thiogalactoside; 2-ME, 2-mercaptoethanol; GdmCl, guanidinium chloride.

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A317 (5′-GTC ATC TAG ACG TTC AAA GAA GAA GCT TTC GTC AAC GCA-3′). In this way, the sequence encoding cysteine 101 (-GAA **T**GC TTC-) was changed into the sequence (-GAA **A**GC TTC-) coding for serine at position 101. (Bold typeface is used to indicate the mutation.) At the same time a new *Hin*dIII site was created, which was used for selection of positive clones. The mutant DNA fragment was digested with *Xba*I and *Aat*II and ligated into the vector pJHLhbFGF, which was also digested with *Xba*I and *Aat*II. Positive clones (pJHLbFGFC101S) were identified by *Hin*dIII digestion and subsequent sequencing.

Preparation of FGF-2 stock solutions

hFGF-2 was obtained from high-cell-density cultures of recombinant *Escherichia coli* transformed with the temperature-inducible expression vector pλFGFB [23]. The mutant FGF-2(C101S) was obtained from recombinant *E. coli* JM101 transformed with the IPTG-inducible expression vector pJHLbFGFC101S. Cells were grown at 37 °C in shake-flask cultures and production of FGF-2(C101S) was started by the addition of 1 mmol·l^{-1} IPTG as described previously [23]. The yield of mutant FGF-2(C101S) was only 10 % compared with hFGF-2 when the IPTG-inducible expression systems were used for the production of both proteins. The recombinant proteins were purified from the soluble cell fraction as described previously [24]. Fractions containing pure FGF-2 were pooled, incubated for 1 h in 0.1 mol·l^{-1} sodium phosphate buffer (pH 7.0) containing 0.1 mol·l^{-1} dithiothreitol and dialysed extensively against degassed 0.1 mol·l^{-1} sodium phosphate buffer (pH 7.0). The dialysed FGF-2-containing solution was additionally degassed and stored in aliquots at −70 °C until further use. Concentrations of FGF-2 were determined spectrophotometrically by using a specific absorption coefficient of 1.55 cm^2·mg^{-1} at 280 nm for a 0.1 % solution of FGF-2 [24].

Fluorescence spectroscopy

Fluorescence emission spectra were obtained with a Perkin-Elmer LS50B fluorescence spectrophotometer interfaced to a computer and equipped with an integrated magnetic stirrer and a thermostatically controlled cell holder using 4 × 10 mm^2 cells (Perkin-Elmer, Uberlingen, Germany). Spectra of emission fluorescence intensity values were always corrected by subtracting the corresponding solvent spectrum of emission fluorescence values.

GdmCl- and urea-induced unfolding of hFGF-2

Native hFGF-2 was incubated in 0.1 mol·l^{-1} sodium phosphate buffer (pH 7.0) in the presence of various concentrations of denaturant. The concentrations of stock solutions of denaturant were determined by refractive index measurement [25]. The extent of unfolding was determined by measurement of the fluorescence at 355 nm (excitation at 280 nm). The fraction of unfolded protein and the apparent free-energy of unfolding (ΔG_{app}) were fitted as described previously [25–27]. The fraction of unfolded hFGF-2, f_U, was calculated from the fitted values using eqn. (1):

$$f_U = (y_F + m_F[D] - y)\{y_F - y_U + (m_F + m_U)[D]\}^{-1} \quad (1)$$

where y is the observed fluorescence, y_F and y_U are the y-intercepts of the extrapolated fluorescence of the folded and unfolded conformation respectively, m_F and m_U are the slopes of the pre- and post-transition baselines respectively, and [D] is the concentration of denaturant.

The apparent free energy of unfolding in the transition region was calculated from eqn. (2):

$$\Delta G_{app} = -RT\ln[f_U(1-f_U)^{-1}] \quad (2)$$

where R is the gas constant (8.314 J·mol^{-1}·K^{-1}) and T the temperature in K.

The apparent free energy at zero denaturant concentration was determined from eqn. (3):

$$\Delta G_{app} = \Delta G_{app}^{H_2O} - m[D] \quad (3)$$

by extrapolation to zero denaturant concentration.

Refolding of FGF-2

Refolding of hFGF-2 and FGF-2(C101S) was carried out by dilution and monitored by measuring the time-dependent decrease in protein fluorescence at 355 nm (excitation at 280 nm). Details are given in the the Figure legends.

Bioassay

The mitogenic activity of FGF-2 was assayed by stimulation of [^{3}H]thymidine incorporation into the DNA of serum-depleted BALB 3T3 cells as described previously [28]. Commercially available hFGF-2 (Boehringer Mannheim, Mannheim, Germany) was used as standard.

FGF-2 structure visualization

FGF-2 structure data were obtained from the Brookhaven Databank (accession number 4FGF) and visualized using the program RasMol Version 2.6 (RasMol Molecular Renderer; obtained from R. Sayle, Glaxo Research and Development Greenford, Middlesex, U.K.).

RESULTS

Characterization of the folded and unfolded states of hFGF-2 by fluorescence emission spectroscopy

The fluorescence emission of native and unfolded hFGF-2 was studied by simultaneous excitation of the tryptophan and tyrosine

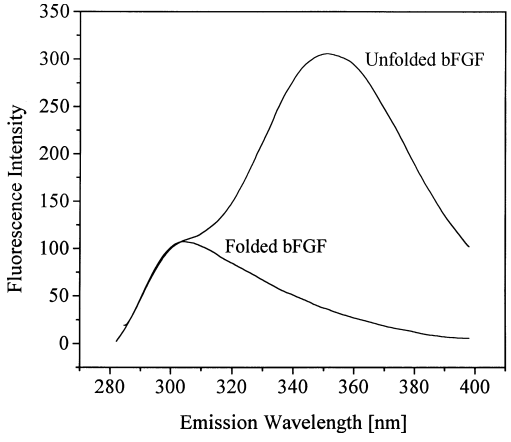

Figure 1 Base-line-corrected fluorescence emission spectra of folded and unfolded hFGF-2

Folded hFGF-2 was in 0.1 mol·l^{-1} sodium phosphate buffer (pH 7.0) at 25 °C. The sample of unfolded hFGF-2 additionally contained 4.0 mol·l^{-1} GdmCl. Fluorescence emission (5 nm bandwidth) was measured after excitation at 280 nm for simultaneous excitation of tryptophan and tyrosine fluorescence (3 nm bandwidth) at a protein concentration of 5 μmol·l^{-1}.

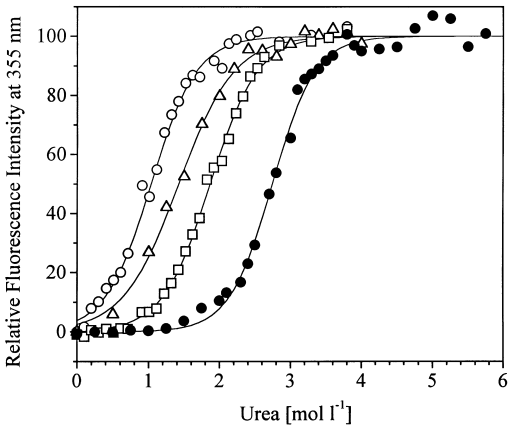

Figure 2 Urea-induced unfolding transitions of hFGF-2 in 0.1 mol·l^{-1} sodium phosphate buffer (pH 7.0) in the absence and presence of reducing agents (0.1 mol·l^{-1} 2-ME)

Unfolding was monitored by measurement of the protein fluorescence emission at 355 nm. Relative fluorescence intensity data are shown to account for temperature dependence of fluorescence and fluorescence quenching by 2-ME. The fluorescence emission of hFGF-2 in 6 mol·l^{-1} urea was set at 100 relative units in each unfolding experiment. Excitation was at 280 nm and the protein concentration was 1 μmol·l^{-1}. The fluorescence emission in the absence of reducing agents was measured after 15 h incubation of hFGF-2 at 10 °C (□), with subsequent incubation for 24 h at 20 °C (△) and with an additional subsequent incubation for 24 h at 25 °C (○). The depicted fluorescence emission data of urea-induced unfolding in the presence of reducing agents were measured after 60 h incubation at 25 °C (●).

fluorescence at 280 nm (Figure 1). Protein fluorescence is usually dominated by tryptophan fluorescence ($\lambda_{max} \approx 320$–330 nm in an apolar environment, $\lambda_{max} \approx 350$ nm in a polar environment) [29], but in native hFGF-2 the fluorescence of the single tryptophan is completely quenched and the emission spectrum is dominated by tyrosine fluorescence ($\lambda_{max} \approx 306$ nm) (see also [30,31]). Unfolding of hFGF-2 is accompanied by a strong increase in fluorescence. This increase is exclusively attributed to an increase in tryptophan fluorescence, with the tyrosine fluorescence still present as a shoulder in the emission spectrum of the unfolded protein. The appearance of tryptophan fluorescence reveals the disruption of the contact between tryptophan and the internal quencher(s), and the emission maximum of 355 nm reflects the complete exposure of tryptophan to the polar environment of the solvent.

Denaturant-induced unfolding of hFGF-2 in the presence and absence of reducing agents

The stability of hFGF-2 against unfolding by urea in the presence and absence of reducing agents was studied by emission fluorescence spectroscopy (Figure 2). The extent of unfolding by increasing concentrations of urea was monitored by the increase in fluorescence emission at 355 nm, where the difference in emission of native and unfolded hFGF-2 is maximum (see also Figure 1). Unfolding of hFGF-2 was additionally monitored by determination of the shift in the maximum of the fluorescence spectrum (results not shown).

The unfolding transition of hFGF-2 in the presence of reducing agents is shifted to notably higher urea concentrations compared with those obtained in the absence of reducing agents. For example, under reducing conditions, stable unfolding transitions are obtained and the mid-point of urea-induced unfolding of hFGF-2 at 25 °C in the presence of 2-ME is observed at a urea concentration of 2.7 mol·l^{-1} (Figure 2). In the absence of reducing agents, the mid-point of urea-induced unfolding of hFGF-2 is

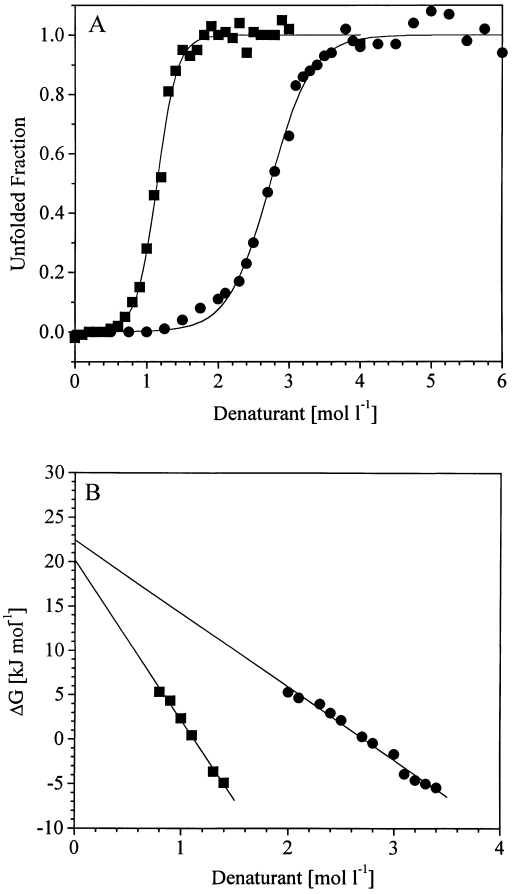

Figure 3 Two-state analysis of urea- (●) and GdmCl-induced (■) unfolding of hFGF-2 (0.1 mol·l^{-1} sodium phosphate buffer, pH 7.0/0.1 mol·l^{-1} 2-ME, 25 °C)

(**A**) The fraction of unfolded hFGF-2 and (**B**) the free-energy of unfolding were determined as a function of denaturant concentration as described in the Materials and methods section. The protein concentration was 1 μmol·l^{-1}.

already observed at a urea concentration of 1.8 mol·l^{-1} after 15 h incubation at 10 °C (Figure 2). Subsequent incubation of these samples for 24 h at 20 °C, followed by further incubation for 24 h at 25 °C, shifted the mid-points of urea-induced unfolding to urea concentrations of 1.4 and 1.0 mol·l^{-1} respectively. The shift of the unfolding transition to lower denaturant concentration in the absence of reducing agents indicates the occurrence of oxidative processes, rendering the protein more susceptible to denaturant-induced unfolding.

Determination of the free-energy of unfolding of hFGF-2

The free-energy of unfolding of hFGF-2 was determined from denaturant-induced unfolding experiments using reducing agents in the incubation buffer to guarantee the reversibility of the unfolding process. By assuming a two-state mechanism of equilibrium-unfolding, the fraction of unfolded hFGF-2 and the apparent free-energy of unfolding (ΔG_{app}) were calculated as a function of denaturant concentration (Figure 3). Determination of the apparent free-energy of unfolding in the absence of denaturant ($\Delta G_{app}^{H_2O}$) by linear extrapolation to zero denaturant concentration revealed that the two denaturants urea and GdmCl result in nearly the same value for $\Delta G_{app}^{H_2O}$, with only 2.5 kJ·mol^{-1} separating the values (Figure 3B and Table 1). The linearity of

Table 1 Parameters characterizing GdmCl and urea denaturation of hFGF-2 in the presence of reducing agents (obtained from Figure 3)

[a] The midpoint ($\Delta G_{app} = 0$) of the denaturation curve. [b] From eqn. (3).

Denaturant	$C_{\frac{1}{2}}$ [a] (mol · l^{-1})	$\Delta G^{H_2O}_{app}$ [b] (kJ · mol^{-1})	m [b] (kJ · mol^{-1})
Urea	2.7	22.6	8.4
GdmCl	1.1	20.1	18.1

the free-energy curve reveals consistency with the assumption of a two-state model for denaturant-induced unfolding of hFGF-2 as studied by fluorescence spectroscopy. The slight difference for $\Delta G^{H_2O}_{app}$, determined by the linear extrapolation method using urea and GdmCl for denaturation, is of the same order of magnitude observed for other globular proteins and proves that $\Delta G^{H_2O}_{app}$ is a property of the protein system and is, essentially, independent of the denaturant [26,32,33]. The value of the ratio of m (GdmCl) to m (urea) (2.15; Table 1) indicates that GdmCl is 2.15 times more effective than urea in unfolding hFGF-2 and that the unfolding units are mainly non-polar [26,34].

Reducing conditions are essential for refolding of hFGF-2

Many proteins are difficult to refold *in vitro*. Refolding of hFGF-2 after denaturation by chaotropic agents is irreversible in the absence of reducing agents. One common obstacle to correct folding is the formation of protein aggregates. However, the irreversibility of refolding of hFGF-2 in the absence of reducing agents is not caused by aggregation. At the low concentrations of hFGF-2 used in the refolding experiments (0.5 μmol · l^{-1}) there was no evidence of protein aggregation detectable by an increase in light scattering (results not shown). Conditions allowing refolding of hFGF-2 in the absence of reducing agents were not found by variation of pH, buffer composition, addition of stabilizing (e.g. ammonium sulphate) or destabilizing agents (e.g. L-arginine) and temperature.

Refolding of hFGF-2 in the absence of reducing agents was monitored by measurement of the time-dependent decrease in the protein fluorescence at 355 nm (Figure 4). After constancy of protein fluorescence at 355 nm was reached, a protein emission spectrum was determined (Figure 4A). It was found that the emission spectrum of this hFGF-2 species was blue-shifted compared with the unfolded state of the protein, but was still dominated by tryptophan fluorescence. The dominance of the tryptophan fluorescence reveals that the native conformation of hFGF-2 was not achieved during refolding of the molecule in the absence of reducing agents. Subsequent addition of reducing agents such as 2-ME to the refolding mixture caused a further time-dependent decrease of the protein fluorescence at 355 nm (Figure 4B). The protein emission spectrum taken after constancy of protein fluorescence at 355 nm was reached after the addition of reducing agents, revealed the dominance of tyrosine fluorescence at this stage (Figure 4A). This result demonstrates that refolding of hFGF-2 only takes place after disulphide-bond reduction, which allows structural rearrangements within the molecule that move the internal quencher(s) of the tryptophan fluorescence into the correct position in relation to the single tryptophan. To prove further that reducing conditions are required for correct folding of hFGF-2, we analysed the mitogenic activity of hFGF-2 after refolding under non-reducing conditions and after subsequent addition of reducing agents (Figure 5). The results of the biological activity assays revealed that refolding under non-reducing conditions (conformation I in

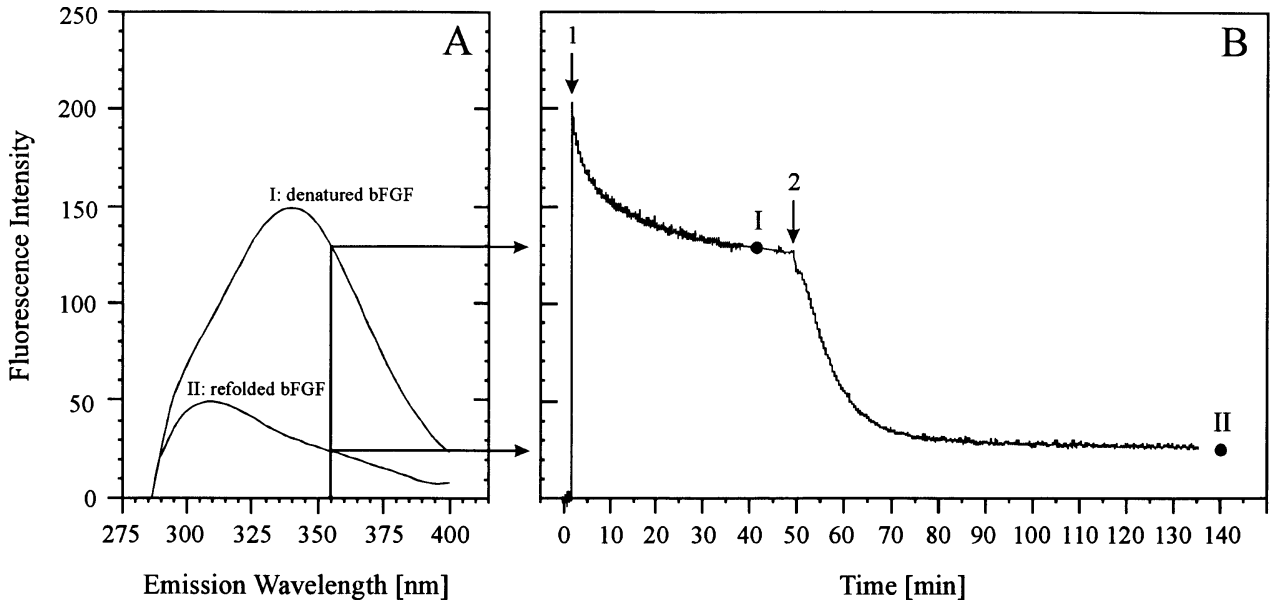

Figure 4 Refolding of hFGF-2 in the absence and presence of reducing agents

Unfolding of hFGF-2 was carried out for 1 h in 4 mol · l^{-1} GdmCl (0.1 mol · l^{-1} sodium phosphate buffer, pH 7.0). Refolding was initiated by a 10-fold dilution into 0.1 mol · l^{-1} sodium phosphate buffer (pH 7.0) at 25 °C. (**A**) The fluorescence emission spectra of refolded hFGF-2 before (I) and after (II) the addition of 0.1 mol · l^{-1} 2-ME to the refolding buffer are shown. (**B**) The refolding kinetics of hFGF-2 are represented as a time-dependent decrease in protein fluorescence emission at 355 nm. Arrows indicate the addition of unfolded hFGF-2 (1) and 0.1 mol · l^{-1} 2-ME (2) to the refolding buffer. The fluorescence emission spectra of refolded hFGF-2 before (I) and after the addition of 2-ME (II) were measured at the indicated times (●). Excitation was at 280 nm and the concentration of hFGF-2 was 0.5 μmol · l^{-1}.

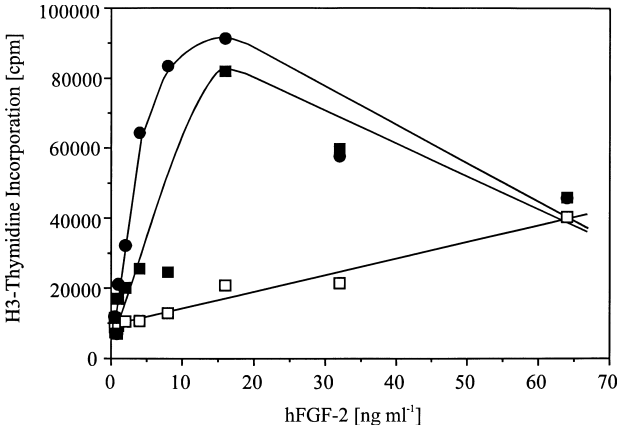

Figure 5 Stimulation of [^{3}H]thymidine incorporation into the DNA of serum-depleted BALB 3T3 cells by hFGF-2 refolded in the absence of reducing agents (□) and after addition of 10 mmol · l^{-1} 2-ME (■) to the refolding mixture, and by hFGF-2 (in 0.1 mol · l^{-1} sodium phosphate buffer, pH 7.0) not subjected to the unfolding and refolding procedure (●)

Unfolding of hFGF-2 was carried out for 1 h in 4 mol · l^{-1} GdmCl (0.1 mol · l^{-1} sodium phosphate buffer, pH 7.0, 25 °C) and refolding was initiated by a 10-fold dilution into 0.1 mol · l^{-1} sodium phosphate buffer (pH 7.0) at 25 °C. The concentration of hFGF-2 during refolding was 0.5 μmol · l^{-1}. Addition of 2-ME was carried out 3.5 h after initiation of refolding. Positive and negative controls for the mitogenic activity measurements were the stimulation of [^{3}H]thymidine incorporation into the DNA of serum-depleted BALB 3T3 cells by fetal calf serum (73742 c.p.m.) and medium without growth factors (7060 c.p.m.) respectively. The S.D. was less then 20%.

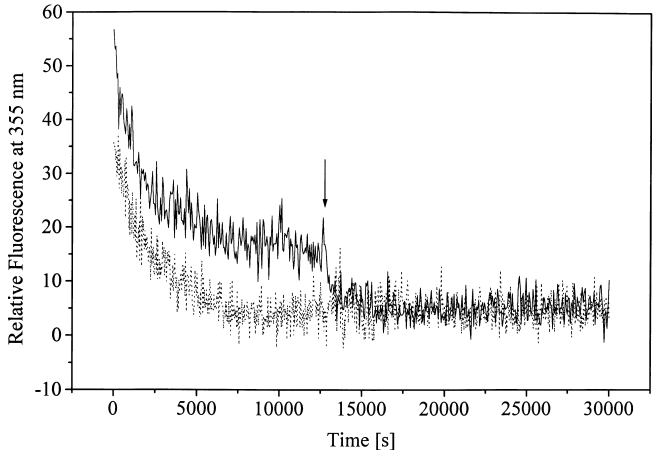

Figure 6 Comparative studies on the refolding of hFGF-2 (solid line) and FGF-2(C101S) (dotted line) in the absence and presence of reducing agents

Unfolding was carried out for 1 h in 5.7 mol · l^{-1} urea (0.1 mol · l^{-1} sodium phosphate buffer, pH 7.0, 25 °C) and refolding was initiated by a 10-fold dilution into 0.1 mol · l^{-1} sodium phosphate buffer (pH 7.0) at 25 °C. The refolding kinetics of hFGF-2 and FGF-2(C101S) are represented as a time-dependent decrease in protein fluorescence emission at 355 nm. The arrow indicates the addition of 10 mmol · l^{-1} 2-ME to the refolding mixtures. Excitation was at 280 nm and the concentration of hFGF-2 and FGF-2(C101S) in the refolding buffer was 0.5 μmol · l^{-1}.

Figure 4) did not regenerate the mitogenic activity of the native molecule. However, subsequent addition of 2-ME to hFGF-2, refolded in the absence of reducing agents (conformation II in Figure 4), regenerated a protein with approximately the same maximum stimulation of thymidine incorporation into the DNA of serum-depleted BALB 3T3 cells as observed for the native molecule not previously subjected to unfolding and refolding. However, maximum stimulation of thymidine incorporation by the refolded molecule was observed at slightly higher protein concentrations, indicating that unfolding and refolding of hFGF-2 using the described protocol did not result in complete renaturation of the entire protein fraction.

Incubation of hFGF-2 in chaotropic agents in the absence of reducing agents for different time periods revealed that the oxidation of the unfolded molecule proceeded rapidly under non-reducing conditions (Table 2). The decline in protein fluorescence observed during refolding of hFGF-2, before the addition of reducing agents to the refolding mixture, decreased with longer incubation times of hFGF-2 in chaotropic agents under non-reducing conditions. However, complete disappearance of tryptophan fluorescence, resulting from proper folding of hFGF-2, was achieved after addition of 2-ME to the refolding mixture, independent of the length of the pre-incubation time in chaotropic agents under non-reducing conditions. These results confirm that the rapid oxidation of hFGF-2 in the unfolded state under non-reducing conditions causes the formation of a disulphide-bonded species which is unable to fold into a native-like structure but which can refold after disulphide-bond reduction.

Refolding of FGF-2(C101S) does not require reducing conditions

The most likely candidates for the formation of an intramolecular disulphide bond are cysteines 96 and 101 (see Discussion section for details). Therefore, a mutant FGF-2, not able to form this

Table 2 Influence of the incubation time under non-reducing unfolding conditions on refolding of hFGF-2 before and after the addition of 2-ME to the refolding mixture

Unfolding was carried out in the absence of reducing agents in 4 mol · l^{-1} GdmCl (0.1 mol · l^{-1} sodium phosphate buffer, pH 7.0, 25 °C). Complete unfolding of hFGF-2 was achieved after 15 min incubation in 4 mol · l^{-1} GdmCl. Refolding was initiated by a 10-fold dilution into 0.1 mol · l^{-1} sodium phosphate buffer (pH 7.0) at 25 °C after the indicated incubation times in 4 mol · l^{-1} GdmCl. Refolding was monitored as the time-dependent decrease in protein fluorescence emission at 355 nm. Addition of 0.1 mol · l^{-1} 2-ME to the refolding mixture was carried out after constancy in the fluorescence emission was achieved. The fluorescence emission was corrected for the quenching effect of 2-ME and the decrease in the fluorescence emission before and after the addition of 2-ME are shown in relative units. Excitation was at 280 nm and the concentration of hFGF-2 was 0.5 μmol · l^{-1}.

Time (h) in GdmCl before refolding	Relative decline in fluorescence during refolding before the addition of 2-ME	Relative decline in fluorescence during refolding after the addition of 2-ME
0.25	70	15
1.00	54	39
2.00	21	64
3.00	13	78

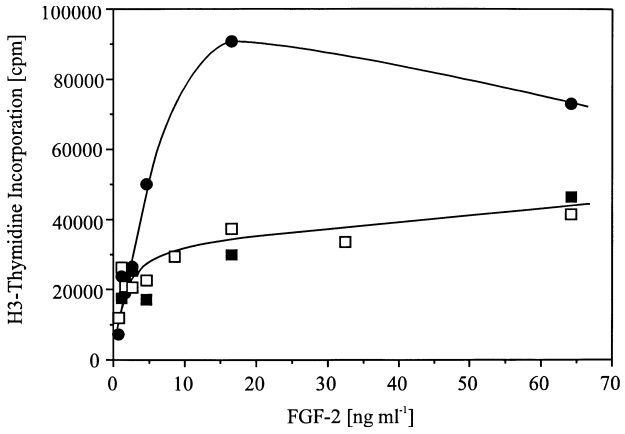

Figure 7 Stimulation of [^{3}H]thymidine incorporation into the DNA of serum-depleted BALB 3T cells by FGF-2(C101S) after refolding in the absence of reducing agents (□) and of FGF-2(C101S) not subjected to the unfolding and refolding procedure (■) (in 0.57 mol·l^{-1} urea/0.1 mol·l^{-1} sodium phosphate buffer, pH 7.0)

The mitogenic activity of hFGF-2 (in 0.1 mol·l^{-1} sodium phosphate buffer, pH 7.0) not subjected to the unfolding and refolding procedure is also shown (●). Unfolding of FGF-2(C101S) was carried out as described in the legend to Figure 6. Positive and negative controls for the mitogenic activity measurements were the stimulation of [^{3}H]thymidine incorporation into the DNA of serum-depleted BALB 3T3 cells by fetal calf serum (78284 c.p.m.) and medium (11391 c.p.m.) respectively. The S.D. was less than 20%.

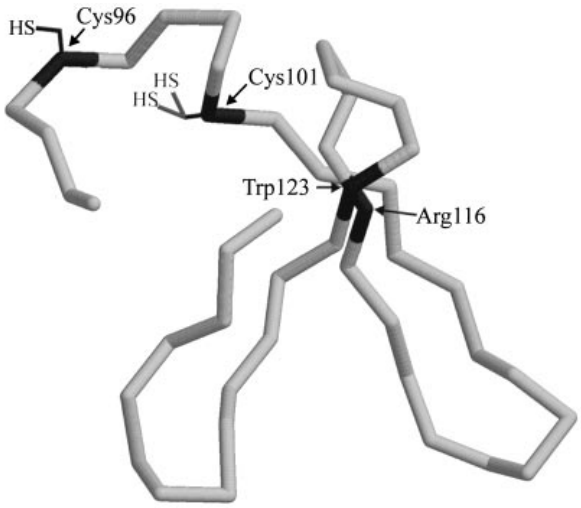

Figure 8 Three-dimensional structure of the peptide backbone of FGF-2 from leucine 92 to glycine 136

The positions of cysteines 96 and 101, arginine 116 and tryptophan 123 are indicated. The side-chain of cysteine 101 occupies two alternative positions. Cysteine 96 is located within a β-sheet barrel connecting loop. Arginine 116 and tryptophan 123 are located within two different anti-parallel β-sheets with their backbone atoms in van der Waals contact distance.

disulphide bond, was generated by replacing the cysteine at position 101 by a serine. As in native hFGF-2, the tryptophan fluorescence of native FGF-2(C101S) is completely quenched, but increases upon denaturant-induced unfolding (results not shown). After urea-induced unfolding of hFGF-2 and FGF-2(C101S) the refolding of both proteins in the absence of reducing agents was monitored until constancy of the protein fluorescence at 355 nm was reached (Figure 6). As shown before, addition of reducing agents to the refolding mixture containing hFGF-2 resulted in a further time-dependent decrease in the protein fluorescence at 355 nm. However, the mutant FGF-2(C101S) was able to refold completely without the addition of reducing agents. Addition of 2-ME to the refolding mixture did not result in a further decrease in protein fluorescence at 355 nm, indicating that the mutant protein did not require the presence of reducing agents for refolding. In addition, the protein emisssion spectrum taken before the addition of 2-ME to the refolding mixture revealed complete disappearance of the tryptophan fluorescence, indicative of the proper conformation of FGF-2(C101S) (results not shown). The conclusion that the mutant protein does not depend on reducing conditions for refolding was also verified by measuring the stimulation of thymidine incorporation into the DNA of serum-depleted BALB NIH 3T3 cells. These measurements revealed that FGF-2(C101S), refolded in the absence of reducing agents, exhibited the same mitogenic activity compared with the mutant protein not subjected to the unfolding and refolding procedure (Figure 7). However, in comparison with hFGF-2, only 30% of the maximum stimulatory effect was achieved by FGF-2(C101S), indicating that the mutation decreased the mitogenic potential of the protein.

DISCUSSION

The studies described above on the conformational stability and the folding properties of hFGF-2 using fluorescence emission spectroscopy complement previous studies on the stability of the protein using activity measurements. The easy loss of biological activity reported by several authors [8–12] is also reflected in a low conformational stability of the molecule. The low free-energy change during unfolding reveals that the conformational stability of the protein is also marginal under conditions guaranteeing the reversibility of the protein unfolding process. The stabilization of the native conformation by 21 kJ·mol^{-1} is at the lower margin of the free energies of unfolding observed for other globular proteins under physiological conditions (20–60 kJ·mol^{-1}) [35].

However, the conformational stability of hFGF-2 is particularly low under non-reducing conditions because hFGF-2 is highly susceptible to irreversible oxidative denaturation. The structural perturbations caused by oxidative disulphide-bond formation renders the molecule more susceptible to denaturant-induced unfolding, just as denaturant-induced unfolding renders the protein more susceptible to irreversible oxidative denaturation.

The presence or absence of disulphide bonds in native hFGF-2 has been discussed extensively in the past [20,22,30]. Thiol titration experiments suggested the presence of an intramolecular disulphide bond involving cysteines 34 and 101 [20]. However, the determination of the three-dimensional structure of hFGF-2 revealed that none of the four cysteines present in hFGF-2 is in sufficiently close proximity to form an intramolecular disulphide bond [13]. The two cysteines with the smallest distance to each other are cysteines 96 and 101, with their S^{γ} atoms being only 7.3 Å apart [14]. These cysteines have never been suggested to form a disulphide bond in native hFGF-2, and the orientation of their thiol groups would require a considerable conformational change within the molecule to allow disulphide-bond formation (Figure 8) [13,14]. However, there are several strong indications that these two cysteines are especially prone to intramolecular disulphide-bond formation.

Cysteine 96 is localized in a highly flexible loop with a very diffuse electron density in its vicinity and especially at the

position of its side-chain [13,18]. This cysteine can be easily altered by chemical modification [9,17,20]. Cysteine 101 is localized at the transition of this loop to a β-sheet strand, with its side-chain occupying two possible conformations (Figure 8) [18]. In one of the two positions 2-ME (an essential component of the crystallization medium) is bound to this cysteine [18]. Since cysteine 101 is otherwise inaccessible to chemical modification [9,17,20], the binding of 2-ME to this cysteine could be the result of thiol–disulphide exchange reactions involving these two cysteines and 2-ME. This interpretation is also in agreement with the results obtained by characterization of denatured hFGF-2 by MS. The mass spectroscopic analysis of denatured hFGF-2 revealed the presence of an intramolecular disulphide bond between cysteines 96 and 101 [9]. Other disulphide-bonded species of hFGF-2 were not detectable. In addition, it has been shown that intramolecular disulphide bonds are preferentially formed in hFGF-2 compared with intermolecular disulphide bonds, since disulphide-linked multimers of hFGF-2 are transformed by thiol–disulphide exchange reactions into monomers carrying intramolecular disulphide bonds under conditions causing unfolding of the polypeptide chain [19,36].

The reactivity of the thiol groups of these two cysteines should be high because of electrostatic influence from positive charges in their neighbourhood. Positive charges in the vicinity of thiol groups are known to decrease the pK_a values of cysteines, with the result that the reactive thiolate anion is present at neutral or even acidic pH [37–39]. There is a high concentration of positive charges close to cysteine 96, because the positively charged side-chain of the N-terminal neighbour amino acid, lysine 95, is nearly in van der Waals contact distance to the also positively charged side-chain of arginine 81.

The electrostatic influence of positive charges, the flexibility of the loop and their close proximity to each other render these two cysteines good candidates to form an intramolecular disulphide bond. In addition, in unfolded proteins and peptides with less than eight intervening amino acids between two cysteines, disulphide-bond formation is favoured when, as in the case of FGF-2, even numbers of intervening amino acids are present [40]. The hypothesis that irreversible oxidative denaturation of hFGF-2 is caused by the formation of an intramolecular disulphide bond involving cysteines 96 and 101 is also corroborated by the observation that the mutant protein FGF-2(C101S), which is not able to form this disulphide bond, does not require the presence of reducing agents for proper refolding.

The ease with which an intramolecular disulphide bond can be formed between cysteines 96 and 101 renders hFGF-2 very sensitive to irreversible oxidation under conditions resulting in the unfolding of the molecule. Consequently, the complex β-sheet barrel structure of native hFGF-2 cannot be attained when refolding of hFGF-2 occurs in the absence of reducing agents. Correct folding of hFGF-2 can be determined by the disappearance of the tryptophan fluorescence. This very unusual total quenching of the tryptophan fluorescence can be taken as a sensitive indicator of perturbations within the native structure of hFGF-2. In the native molecule, the single tryptophan is localized at the end of a very tight loop connecting two anti-parallel β-sheet elements (Figure 8). The formation of a cystine bond between cysteines 96 and 101 would certainly distort the complex β-sheet arrangement and result in considerable changes within the local environment of the single tryptophan.

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