Disulfide Isoform Intermediates in the Reoxidation of Recombinant Human Basic Fibroblast Growth Factor†

Margherita Ruoppolo,‡ Gianpaolo Nitti,§ Barbara Valsasina,§ Antonio Malorni,‡ Gennaro Marino,‡,∥ and Piero Pucci*,‡,⊥

Servizio di Spettrometria di Massa del CNR, via Pansini 5, 80131 Napoli, Italy, Dipartimento di Biotecnologie, Farmitalia Carlo Erba, Milano, Italy, CEINGE, s.r.l., Napoli, Italy, and Dipartimento di Chimica Organica e Biologica, Università di Napoli, Napoli, Italy

Received October 30, 1992; Revised Manuscript Received March 1, 1993

ABSTRACT: The reoxidation of human recombinant basic fibroblast growth factor was investigated following treatment of the protein with a mixture of reduced and oxidized glutathione, both in the absence and in the presence of protein disulfide isomerase. The oxidative process took place throughout the formation of two transient intermediates and yielded a stable bFGF derivative, GS_2-bFGF. All of these components were separated by HPLC and accurately characterized at the molecular level by advanced mass spectrometric procedures. When the reoxidation was carried out in the presence of PDI, a 4-fold increase in the reaction rate was estimated. A mixed disulfide with a single glutathione molecule was shown to occur in the two transient intermediates, each of which has different cysteine residues involved in the linkage. The final product GS_2-bFGF was structurally different from other bFGF derivatives previously described [Thompson, S. A. (1992) *J. Biol. Chem. 267*, 2269–2273; Caccia et al. (1992) *Eur. J. Biochem. 204*, 649–655]. The four cysteine residues are all involved in disulfide bridges; Cys 34 and Cys 78 are linked to exogenous glutathione, whereas Cys 91 and Cys 101 form an intramolecular S–S bridge.

Basic fibroblast growth factor (bFGF)[1] belongs to the heparin-binding factor family, which at present consists of seven structurally related proteins (Folkman & Klagsburn, 1987; Burgess & Maciag, 1989; Marics et al., 1989; Finch et al., 1989). bFGF shows potent mitogenic activity toward a wide variety of cells of mesodermal and neuroectodermal origin *in vitro* and can induce angiogenesis *in vivo*.

As isolated from pituitary tissues, bovine bFGF is a single-chain, nonglycosylated protein which consists of 146 amino acids with a molecular mass of 16.5 kDa (Esch et al., 1985). Thereafter, the analysis of bovine and human cDNAs revealed the existence of higher molecular weight forms and suggested that the primary translation product is a 155-residue protein (including the putative initial methionine) (Abraham et al., 1986a,b); in fact, a mature form of bFGF consisting of 154 amino acids was purified in the presence of protease inhibitors (Klagsburn et al., 1987; Veno et al., 1986).

The oxidation states of the cysteine residues in native bFGF as well as the relevance of these residues to the functions of the protein are still under investigation. When bFGF was first purified from pituitary tissues, six cysteine residues were inferred on the basis of amino acid analysis (Esch et al., 1985), whereas only four cysteines are predicted from the nucleotide sequence. This observation led to the speculation that native bFGF might be present in the cells with two SH groups involved in mixed disulfides with cysteine or glutathione. More recently, it has been demonstrated that natural bFGF contains all four cysteine residues in the reduced form; it has been suggested that the presence of the S-thiolated form of bFGF is due either to a reaction with glutathione occurring *in vivo* or to an artifact during purification of the protein (Thompson, 1992).

Expression of the bFGF gene in *Escherichia coli* led to the production of a recombinant active protein containing all four cysteines in the reduced form (Iwane et al., 1987; Squires et al., 1988; Barr et al., 1988). In the absence of stabilizing agents, rhbFGF displayed chemical instability due to the rapid oxidation of the thiol groups. Attempts to reduce this instability were performed by site-directed mutagenesis experiments where all four cysteine residues were in turn replaced by serine; some of these modified bFGF molecules fully retained their biological activity and heparin-binding capability (Fox et al., 1988; Seno et al., 1988; Arakawa et al., 1989).

Thiolation of cysteines 78 and 96 of rhbFGF by direct reaction *in vitro* with oxidized glutathione did not result in increased chemical stability since the thiolated adducts undergo reshuffling of the S–S bridges following storage at room temperature or incubation at alkaline pH (Caccia et al., 1992). A stable derivative of rhbFGF was recently obtained by irreversibly blocking cysteines 78 and 96 with iodoacetic acid (Caccia et al., 1992).

This article describes the investigation of the reoxidation of rhbFGF following treatment of the protein with an appropriate redox system under different experimental conditions. A single, stable bFGF derivative, GS_2-bFGF, which is different from other modified bFGF molecules previously described, was obtained. Both the stable product and two transient intermediates formed along the reoxidation pathway were characterized using mass spectrometric procedures. The

† Supported by the CNR Target Project "Biotecnologie e Biostrumentazione" and by grants from the Ministero Università e Ricerca Scientifica (MURST 40% and British Council–MURST bilateral program).

* Author to whom correspondence should be addressed.
‡ Servizio di Spettrometria di Massa del CNR.
§ Farmitalia Carlo Erba.
∥ CEINGE.
⊥ Università di Napoli.

[1] Abbreviations: bFGF, basic fibroblast growth factor; rhbFGF, recombinant human basic fibroblast growth factor; PDI, protein disulfide isomerase; GSH reduced glutathione; GSSG, oxidized glutathione; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol, TFA, trifluoroacetic acid; PITC, phenyl isothiocyanate; ES/MS, electrospray mass spectrometry; FAB/MS, fast atom bombardment mass spectrometry.

0006-2960/93/0432-4991$04.00/0

four cysteine residues in the GS_2-bFGF molecule were all involved in disulfide bridges; two of them, namely, Cys 34 and 78, are linked to exogenous glutathione molecules, whereas Cys 96 and Cys 101 are joined by an intramolecular S–S bridge.

MATERIALS AND METHODS

Human recombinant bFGF was expressed in *E. coli* and purified as described previously (Caccia et al., 1992). Amino acid numbering throughout this article refers to the 155 amino acid form of bFGF where the initial methionine is residue 1. Protein disulfide isomerase (PDI) was prepared from bovine liver according to Hillson et al. (1984).

GSH, GSSG, Tris base, DTNB, DTT, pepsin, glycerol, and thioglycerol were obtained from Sigma Chemical Co.; endoproteinase Glu-C was purchased from Boheringer. All other chemicals were HPLC grade from Carlo Erba.

Glutathione stock solutions were generally made fresh daily in 0.1 M Tris-HCl (pH 8.00) at a concentration of 50 mM GSSG and 50 mM GSH. All of the solutions were deoxygenated in order to avoid unwanted air oxidation of the thiol groups, and the reoxidation experiments were carried out under nitrogen; 1 mM EDTA was added to the buffers to prevent oxidation catalyzed by trace heavy metals.

Sample Preparation and HPLC Analysis. Aliquots of a stock solution of rhbFGF containing 1.2 mg of protein were used in the reoxidation studies. The pH of the solution was adjusted to 8.0 with Tris base, and the desired amounts of GSH and GSSG were added. Typically, final concentrations of the glutathione species in the incubation mixture were 4 mM GSH and 0.4 mM GSSG. A portion of the protein solution was withdrawn before the addition of GSH/GSSG and analyzed by HPLC to ensure that rhbFGF was still present in the reduced form. When the reaction took place in the presence of PDI, the enzyme was added at a final concentration of 0.1 mg/mL (enzyme to substrate ratio 1/10).

The reoxidation of rhbFGF was monitored on a time-course basis by sampling aliquots of the incubation mixture at appropriate intervals. The aliquots were quenched by lowering the pH to about 2 with the addition of 0.1% TFA and quickly vortexing; after acid quenching, the samples were analyzed by HPLC. When the reoxidation of rhbFGF led to the formation of a single HPLC peak which did not show any further modifications, the reaction was stopped by acid quenching using 0.5% TFA and the bFGF derivative was purified by HPLC.

The S-thiolated form of rhbFGF (GS-FGF) was prepared by direct thiolation of the protein with GSSG following essentially the same procedure. The pH of the rhbFGF solution was adjusted to 8.0, and GSSG was added to a final concentration of 0.4 mM. The extent of the reaction was monitored by HPLC analysis; within 1 h the modification of rhbFGF was completed and the S-thiolated derivative was purified by HPLC.

A Vydac TP 214 reversed-phase C_4 column, 0.46 × 25 cm, was used both to separate the different forms of bFGF and to recover the final product. The elution system consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). Native and modified rhbFGF samples were eluted with a linear gradient of solvent B from 30% to 45% at a flow rate of 1 mL/min. Protein monitoring was carried out at 220 nm. The retention time for rhbFGF was 22.5 min, whereas the stable GS_2-bFGF derivative was eluted after 13.5 min. The S-thiolated derivative GS-FGF showed the same retention time as GS_2-bFGF under these experimental conditions, but was eluted with a slightly higher retention time on a shallower gradient.

Structural Characterization of rhbFGF Isoforms. The free SH content of native rhbFGF, the S-thiolated GS-FGF, and the stable GS_2-bFGF derivatives was estimated by DTNB titration in 0.3 M Tris-HCl and 1 mM EDTA (pH 7.5) containing 6 M guanidinium chloride, and the formation of the 2-nitro-5-thiobenzoate dianion was measured at 412 nm (extinction coefficient 13 600 M^{-1} cm^{-1}). The protein concentration of the different bFGF samples was measured either by amino acid analysis after vapor-phase HCl hydrolysis for 90 min at 150 °C and automated precolumn derivatization with PITC followed by identification of the modified amino acids by HPLC or by using the Bradford method (Bradford, 1976).

The identification and structural characterization of all of the bFGF isoforms formed during the reoxidation reaction were carried out using mass spectrometric procedures. The stable GS_2-bFGF product and the two transient intermediates identified along the reoxidation pathway were collected from an analytical HPLC run and directly submitted to ES/MS analysis using a VG BIO Q triple quadrupole mass spectrometer. Protein samples (10 μL) at concentrations ranging from 3 to 7 pmol/μL were injected into a fused silica capillary tube through a Rehodyne injection valve and introduced into the ion source at a flow rate of 2 μL/min. The spectra were scanned from 600 to 1400 m/z at 10 s/scan. Mass-scale calibration was performed by means of the multiply charged ions from a separate injection of myoglobin (average molecular mass 16 950.6 Da); all molecular masses shown are average values.

Assignments of the disulfide bridges present in the GS_2-bFGF molecule were performed by FAB/MS analysis of the peptide mixture generated by a double proteolytic digest of the protein using pepsin and endoproteinase Glu-C (Morris & Pucci, 1985). Peptic hydrolysis was carried out in 5% formic acid at 37 °C for 2 h using an enzyme to substrate ratio of 1/50; digestion of the peptic peptides with endoproteinase Glu-C was performed in 0.4% ammonium acetate (pH 4.0) at 40 °C overnight (E/S = 1/50, w/w). FAB mass spectra of the resulting peptide mixture were recorded on a VG ZAB 2SE double-focusing mass spectrometer equipped with a VG cesium gun operating at 25 kV (2 μA). Samples (1–3 nmol) were dissolved in 5% acetic acid and loaded onto a glycerol-coated probe tip; thioglycerol was added just before insertion into the ion source. Spectra were recorded on UV-sensitive paper and manually counted; assignments of the mass signals to the corresponding native or modified peptides within the bFGF sequence were accomplished on the basis of their molecular weights with the aid of a computer program (Pucci & Sepe, 1988).

RESULTS

Reoxidation of rhbFGF. The reoxidation of rhbFGF in the presence of the appropriate GSH/GSSG redox system was followed by HPLC analysis of aliquots of the incubation mixture withdrawn at different intervals. Figure 1 shows the HPLC time-course analysis of the reoxidation process which took place when rhbFGF was incubated with 4 mM GSH and 0.4 mM GSSG. Native rhbFGF exhibited a single symmetric peak with a retention time of 22.5 min (Figure 1A); following incubation with the redox system, an oxidative process took place leading to the formation of three new molecular species eluting at lower retention times (Figure 1B). All of these

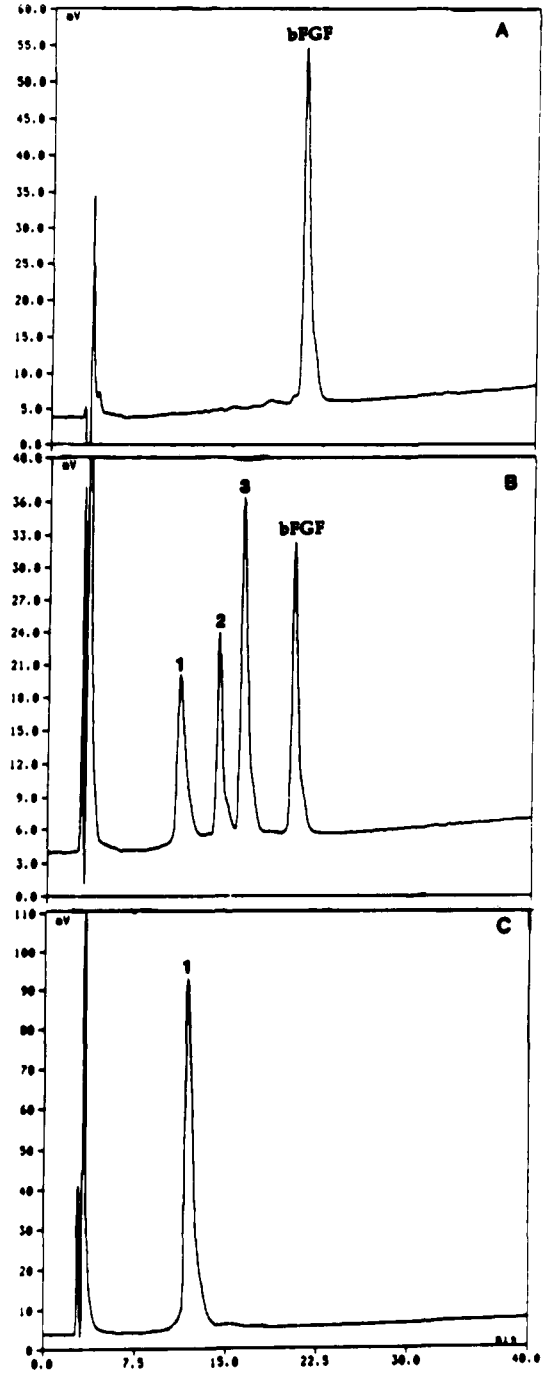

FIGURE 1: HPLC time-course analysis of the reoxidation process following incubation of rhbFGF with GSH/GSSG: (A) native rhbFGF; (B) aliquot of the reaction mixture withdrawn after 10 min; (C) aliquot of the reaction mixture withdrawn at day 4.

species were separated with a base-line resolution and collected for subsequent structural characterization. As the reoxidation process proceeded, native rhbFGF tended to disappear, and the two transient intermediates 2 and 3 were slowly converted to form a single product indicated as component 1 in Figure 1 and hereafter referred to as GS_2-bFGF. This bFGF derivative did not undergo any further modification and it accumulated at the end of the reaction, as shown in Figure 1C.

The oxidative process occurs within a few minutes after the addition of the GSH/GSSG redox system, as demonstrated by the appearance of three new components in the HPLC analysis of the 10-min aliquot (see Figure 1B). The complete conversion of native rhbFGF into the stable derivative GS_2-bFGF, however, is a much slower process which needs 3–4 days to be completed.

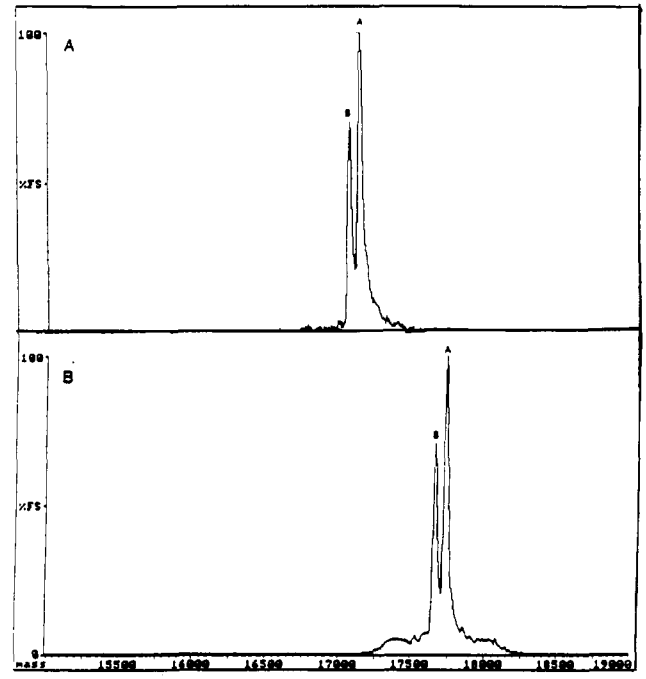

FIGURE 2: Electrospray mass spectrometric analysis of (A) native rhbFGF and (B) the final reoxidation product GS_2-bFGF. Both samples consist of two molecular species differing in the N-terminal Ala. The measured molecular masses are 17 122.7 ± 2.6 and 17 052.9 ± 3.4 Da for native rhbFGF and 17 732.9 ● 2.2 and 17 661.9 ± 2.3 Da for GS_2-bFGF, respectively.

When the reoxidation process was carried out in the presence of PDI, an identical reaction pathway was observed. At the early stages of the process, native rhbFGF was oxidized leading to the formation of the transient intermediates 2 and 3, which were slowly converted into GS_2-bFGF. However, approximately a 4-fold increase in the reaction rate was estimated on the basis of the time-course analysis.

Identification of the HPLC Peaks. All of the components present at the early stages of the reoxidation process were separated by HPLC and directly submitted to electrospray mass spectrometric analysis in order to provide an identification of the individual molecular species. The same analysis was also performed on the native rhbFGF peak as a reference.

The electrospray mass spectra of the native rhbFGF showed the characteristic bell-shaped distribution of multiply charged ions, from which the molecular mass of the protein could easily be calculated. This multiply charged ion spectrum can be transformed on a real mass scale, as shown in Figure 2A. Two molecular species could be distinguished in the rhbFGF sample whose molecular masses were measured as 17 122.7 ± 2.6 and 17 052.9 ± 3.4 Da, respectively.

The mass value of the component with higher molecular weight completely agrees with that predicted for the mature form of rhbFGF in which the initial methionine residue had been removed (17 122.6 Da). The second protein component was identified as a truncated form of rhbFGF where the N-terminal Met-Ala dipeptide had been proteolytically processed (theoretical mass value 17 051.5 Da) according to previous observations on the *E. coli* recombinant bFGF (Caccia et al., 1992).

Figure 2B shows the electrospray mass spectrum of the stable derivative GS_2-bFGF transformed on a real mass scale. Again, two components were detected in the protein sample; their molecular masses were measured as 17 732.9 ± 2.2 and 17 661.9 ± 2.3 Da, respectively. Both mass values are 610

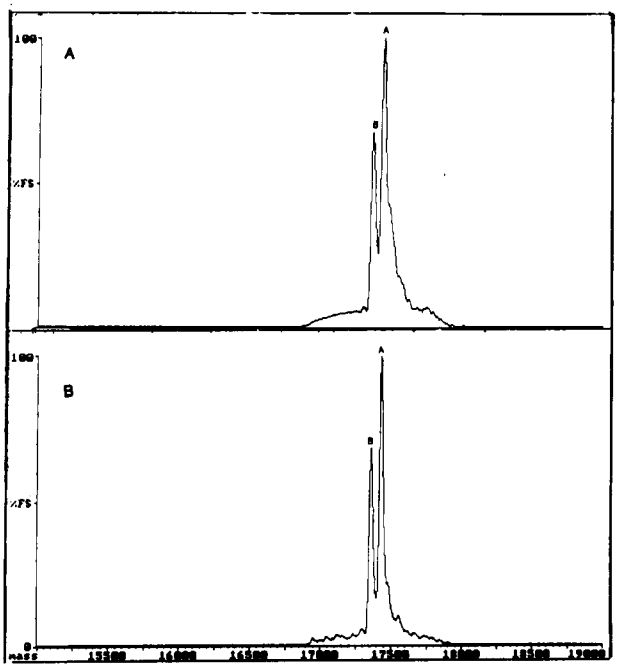

FIGURE 3: Electrospray mass spectrometric analysis of reoxidation intermediates (A) 2 and (B) 3. Both samples display identical molecular masses for the two protein species; the measured molecular weights are 17 427.7 ± 2.28 and 17 357.2 ± 2.38 Da for intermediate 2 and 17 427.7 ± 1.2 and 17 357.7 ± 1.8 Da for intermediate 3, respectively.

mass units higher than those obtained for the corresponding native rhbFGF forms (see Figure 2A); on the addition of 1000-fold molar excess of DTT, the two components were reconverted to the native rhbFGF form. On the basis of the mass difference and the DTT experiment, GS_2-bFGF was tentatively identified as a modified form of rhbFGF in which two cysteine residues had been involved in mixed disulfides with exogenous glutathione molecules.

The ES/MS spectra of transient intermediates 2 and 3, transformed on a real mass scale, are shown in Figure 3. As expected, both intermediates consist of two molecular species which differ in the alanine residue at the N-terminus. Both intermediates displayed identical molecular masses for the two protein species: 17 427.7 Da for the first component and 17 357.7 Da for the truncated one. Since DTT treatment reverts these molecular species to native rhbFGF, these mass values were associated with a single glutathione molecule linked to each form of native rhbFGF. Therefore, both intermediates contain a single cysteine residue involved in a mixed disulfide with exogenous glutathione. Since the two intermediates are separated by HPLC, they must be structurally different; these differences could be due to the glutathione molecule, which might be linked to a different cysteine residue in each sample, thus conferring different hydrophobic properties upon them. Unfortunately, due to the instability of the transient intermediates, it was impossible to define which cysteine is linked to glutathione in each component. These species, in fact, do not accumulate during the reoxidation process and, when concentrated after HPLC collection, tend to interconvert one into the other.

Structural Characterization of GS_2-bFGF. The final product of the reoxidation process, GS_2-bFGF, was purified from the incubation mixture by HPLC and submitted to an accurate structural characterization. A portion of the protein sample was lyophilized several times and reanalyzed by HPLC; GS_2-bFGF was fully recovered as a single peak with the expected retention time. This result demonstrated that GS_2-bFGF is stable upon lyophilization and storage at room temperature, whereas native rhbFGF can only be lyophilized and stored in the presence of stabilizing agents.

The free SH group content of GS_2-bFGF was estimated by DTNB titration under denaturing conditions, showing that no thiol groups are present in the protein sample. Since the assessment of the oxidation state of the cysteine residues of bFGF has often been misleading due to artifacts, rearrangements, and heterogeneity, a control experiment was carried out to demonstrate that the conditions used to isolate GS_2-bFGF had prevented the occurrence of any reshuffling of the S–S bridges.

Samples of both native rhbFGF and its derivative GS-FGF with Cys 78 and Cys 96 S-thiolated with glutathione were passed through HPLC under the same conditions. The proteins were recovered from the column and submitted to DTNB titration, yielding four SH groups and two SH groups, respectively. This result indicated that scrambling of the disulfide bridges did not take place under the conditions used for isolating GS_2-bFGF; therefore, all four of the cysteine residues in the GS_2-bFGF molecule had to have been involved in disulfide bridges. The ES/MS analysis of GS_2-bFGF showed that the protein contained two mixed disulfides with glutathione; the two remaining cysteine residues must then be involved in an intramolecular disulfide bridge.

In order to confirm this hypothesis and to ascertain which cysteines are linked to the glutathione molecules and which pair forms the S–S bond, the GS_2-bFGF was analyzed via peptide mapping using FAB/MS (Morris et al., 1983). The protein was doubly digested with pepsin and endoproteinase Glu-C at acidic pH to avoid any reshuffling of the S–S bonds due to the possible presence of free thiol groups. The resulting peptide mixture was analyzed directly by FAB/MS (Figure 4); most of the mass signals could be assigned to the corresponding peptides along the bFGF sequence on the basis of their molecular weights. These assignments were confirmed by a single step of manual Edman degradation followed by mass spectrometric reexamination of the truncated peptide mixture.

Two anomalous signals were detected in the FAB spectra at m/z 1826 and 1213, respectively. The signal at m/z 1213 occurred 2 mass units lower than that expected for fragment 92–102 and shifted to m/z 1215 following incubation of the peptide mixture with DTT, thus demonstrating the existence of an intramolecular S–S bridge joining Cys 96 and Cys 101 (Morris & Pucci, 1985). Accordingly, this signal shifted to m/z 1100 following a single step of manual Edman degradation due to the removal of Leu 92.

The mass signal at m/z 1826 was identified as peptide 69–82 in which the cysteine residue at position 78 was involved in a mixed disulfide bond with exogenous glutathione. Following reduction with DTT, in fact, the mass signal was shifted to m/z 1521, which corresponds to the unmodified peptide 69–82 with Cys 78 in the thiol form; the analysis of the truncated peptide following an Edman degradation step confirmed the assignment.

Due to the absence of any free SH groups and on the basis of electrospray mass spectral data, the remaining cysteine residue, Cys 34, must be linked to a second glutathione molecule via a mixed disulfide bond. However, no fragments containing such an S–S bond were detected in the FAB mapping of GS_2-bFGF, possibly due to the well-known suppression phenomena occurring during FAB analysis of complex peptide mixtures (Naylor et al., 1986; Pucci et al., 1992).

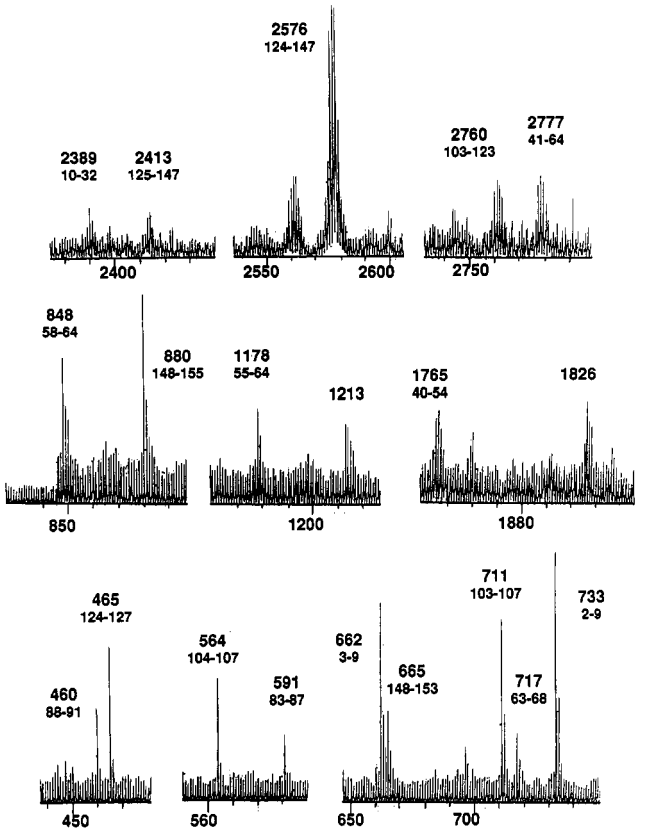

FIGURE 4: FAB/MS analysis of the peptic and endoproteinase Glu-C double digest of GS_2-bFGF. The recorded mass signals are associated with the corresponding peptide along the protein sequence on the basis of their molecular weights.

The accurate characterization of GS_2-bFGF demonstrated that the four cysteine residues present in the structure of native rhbFGF are all involved in disulfide bridges; Cys 34 and Cys 78 are, in fact, linked to exogenous glutathione, whereas Cys 96 and Cys 101 form an intramolecular S–S bond. The absence of free thiol groups in the molecule might explain the increased chemical stability shown by GS_2-bFGF as compared to native rhbFGF. The biological activity of this stable bFGF derivative was evaluated in comparison to that of the native rhbFGF; GS_2-bFGF was found to be equipotent to rhbFGF in stimulating the proliferation of endothelial cells and DNA synthesis in 3T3 fibroblasts (data not shown).

DISCUSSION

Native rhbFGF displays chemical instability due to the rapid oxidation of the SH groups, giving rise to a series of molecular species which can be reconverted into the native molecule following treatment with a reducing agent (Caccia et al., 1992). Nevertheless, upon incubation with an appropriate redox system, this random oxidation process can be driven toward the formation of a new, stable bFGF derivative, GS_2-bFGF, in which all four cysteine residues are involved in disulfide bridges.

A mixture of reduced and oxidized glutathione in the appropriate ratio (10/1, mol/mol) was used as the redox system; the most favorable conditions for promoting the reoxidation process were found when bFGF was incubated with 4 mM GSH and 0.4 mM GSSG, whereas lower glutathione concentrations resulted in much longer reaction times. The reoxidation process takes place through the formation of two transient intermediates which are slowly converted into the final product, GS_2-bFGF. Both the intermediates and the final product could be separated by HPLC; the reaction process could then be monitored on a time-course basis. When PDI was added to the incubation mixture, the reaction pathways remained unchanged but a 4-fold increase in the reaction rate was estimated. This result is consistent with previous reports on the catalytic activity of PDI on the formation of native disulfide bridges and strongly suggests the involvement of a thiol–disulfide exchange mechanism taking place during the reoxidation process. It is well-known, in fact, that PDI is able to catalyze disulfide bond formation during the folding/refolding process both *in vivo* and *in vitro* (Freedman, 1984).

The structural characterization of GS_2-bFGF showed that all four cysteine residues are involved in disulfide bridges. Two of them, Cys 34 and Cys 78, form mixed disulfides with glutathione molecules, whereas the remaining two cysteines, at positions 96 and 101, are involved in an intramolecular S–S bridge. These structural features make GS_2-bFGF rather unique amonge the bFGF derivatives described so far. Direct thiolation of bFGF with oxidized glutathione, in fact, results in the production of GS-FGF, a derivative having Cys 78 and Cys 96 S-thiolated with glutathione. When the GS-FGF is submitted to DTNB titration, two SH groups per molecule can be detected, whereas no thiol groups are titratable in the GS_2-bFGF molecule. Moreover, the analysis of the peptic digest of GS_2-bFGF shows the presence of an intramolecular S–S bridge joining Cys 96 and Cys 101; such a disulfide bridge could not be observed in the same digest of GS-FGF (data not shown). Therefore, the formation of this S–S bond, which makes GS_2-bFGF different from the bFGF derivatives described so far, occurs during the reoxidation reaction and is not due to artifacts during manipulation of the sample.

Direct alkylation with iodoacetic acid (Caccia et al., 1992) or *N*-ethylmaleimide (Thompson, 1992) carried out under nondenaturing conditions also resulted in the modification of Cys 78 and Cys 96. It has been suggested that these cysteines are located on the surface of the protein molecule and therefore are particularly amenable to sulfhydryl reagents (Caccia et al., 1992). The cysteine residues at positions 34 and 101 were shown to be buried inside the bFGF molecule, and they cannot be modified unless the protein is in the unfolded state (Thompson, 1992).

The analysis of the three-dimensional structure of human recombinant bFGF confirmed the location of the four cysteine residues and ruled out any possibility of an intrachain S–S bridge joining Cys 34 and Cys 101. Despite the strict conservation of these residues in the bFGF family, which suggests the presence of an intramolecular disulfide bond, the two cysteines are too far apart to form an S–S bond (Zhang et al., 1991; Ago et al., 1991). However, it has been observed that the formation of a disulfide bridge between Cys 96 and Cys 101 occurs under particular conditions. When the thiolated adduct of rhbFGF, with Cys 78 and Cys 96 involved in mixed disulfides with glutathione, is exposed to alkaline pH, a rapid reshuffling of S–S bridges takes place, leading to the formation of an intramolecular disulfide bond between Cys 96 and Cys 101 (Caccia et al., 1992). The two S^{γ} atoms of these cysteine residues are only 7.3 Å apart, although only a substantial conformational change within the six amino acid loops containing the two cysteine residues would have allowed the formation of the S–S bridge.

Such a conformational change has probably occurred during the reoxidation of native rhbFGF with glutathione. Two pieces of experimental evidence support this hypothesis; firstly, mass spectrometric analysis showed that both intermediates formed

along the reaction pathway contain a cysteine residue linked to exogenous glutathione, thus confirming that stable, covalent glutathione–bFGF adducts had been formed. Secondly, Cys 34, which is thought to be totally inaccessible to the solvent, was modified by glutathione in the GS_2-bFGF molecule, thus suggesting the occurrence of a conformational change somewhere in the reaction pathway leading to the migration of this residue to the surface of the protein.

The analysis of the biological properties of GS_2-bFGF showed that this derivative displays the same activity as the native rhbFGF in stimulating the proliferation of endothelial cells as well as in promoting DNA synthesis. These results are consistent with previous reports on other modified bFGF molecules; the bFGF derivatives with the cysteine residues at positions 78 and 96, either chemically blocked or replaced by serines, were shown to retain fully their biological activities (Fox et al., 1988; Seno et al., 1988; Arakawa et al., 1989; Caccia et al., 1992).

A possible explanation is that the SH groups of these two cysteines are not involved in recognition mechanisms with other macromolecules. Furthermore, the role of the intracellular glutathione redox system in promoting the conversion of the reversibly modified bFGF derivatives into a putative active form should also be considered. Previous data described by Esch and co-workers (Esch et al., 1985) and recent results published by Thompson (1992) lead to the hypothesis that endogenous bFGF might exist in the active form as a mixed disulfide with glutathione molecules, although the involvement of this derivative in regulatory and/or protection mechanisms cannot be ruled out. Further work is needed to define which is the actual active form of bFGF and, possibly, which region of the protein molecule is responsible for binding with receptors.

ACKNOWLEDGMENT

The authors thank Prof. R. B. Freedman for his generous gift of protein disulfide isomerase and Ms. G. Lisboa for reviewing the manuscript.

REFERENCES

Abraham, J., Mergia, A., Whang, J., Tumolo, A., Friedman, J., Hjerrild, K., Gosporadowicz, D., & Fiddes, J. (1986a) *Science 233*, 545–548.

Abraham, J., Whang, J., Tumolo, A., Friedman, J., Gospodarowicz, D., & Fiddes, J. (1986b) *EMBO J. 5*, 2523–2528.

Ago, H., Kitagawa, Y., Fujishima, A., Matsura, Y., & Katsube, Y. (1991) *J. Biochem. 110*, 360–363.

Arakawa, T., Hsu, Y. R., Schiffer, S. G., Tsai, L. B., Curless, C., & Fox, G. M. (1989) *Biochem. Biophys. Res. Commun. 161*, 335–341.

Barr, P., Consens, L., Lee-Ng, C., Medina-Selby, A., Masiarz, F., Hollewell, R., Chamberlain, S., Bradley, J., Lee, D., Steimer, K., Poulter, L., Burlingame, A., Esch, F., & Baird, A. (1988) *J. Biol. Chem. 263*, 16471–16478.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–253.

Burgess, W. H., & Maciag, T. (1989) *Annu. Rev. Biochem. 5*, 575–606.

Caccia, P., Nitti, G., Cletini, O., Pucci, P., Ruoppolo, M., Bertolero, F., Valsasina, B., Roletto, F., Cristiani, C., Canet, G., Sarmientos, P., Malorni, A., & Marino, G. (1992) *Eur. J. Biochem. 204*, 649–655.

Esch, F., Baird, A., Ling, N., Veno, N., Hill, F., Denoroy, L., Klepper, R., Gospodarowicz, D., Bohlen, P., & Guillemin, R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6507–6511.

Finch, P. W., Rubin, J. S., Miki, T., Ron, D., & Aaronson, S. (1989) *Science 245*, 752–755.

Folkman, J., & Klagsburn, M. (1987) *Science 235*, 442–447.

Fox, G., Schiffer, S., Rohde, M., Tsai, L., Banks, A., & Arakawa, T. (1988) *J. Biol. Chem. 263*, 18452–18458.

Freedman, R. B. (1984) *Trends Biochem. Sci 9*, 438–441.

Gospodarowicz, D., Neufeld, G., & Schweigerer, L. (1987) *J. Cell. Phys. Suppl. 5*, 15–26.

Hillson, D. A., Lambert, N., & Freedman, R. B. (1984) *Methods Enzymol. 107*, 281–294.

Iwane, M., Kurokawa, T., Sasada, R., Seno, M., Nakagawa, S., & Igarashi, K. (1987) *Biochem. Biophys. Res. Commun. 146*, 470–477.

Klagsburn, M., Smith, S., Sullivan, R., Shing, Y., Davidson, S., Smith, J., & Sasse, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1839–1843.

Marics, I., Adelaide, J., Raybaud, F., Mattei, M. G., Coulier, F., Planche, J., Lapeyriere, O., & Birnbaum, D. (1989) *Oncogene 4*, 335–340.

Morris, H. R., & Pucci, P. (1985) *Biochem. Biophys. Res. Commun. 126*, 1122–1728.

Morris, H. R., Panico, M., & Taylor, G. W. (1983) *Biochem. Biophys. Res. Commun. 117*, 299–305.

Naylor, S., Findeis, A. F., Gibson, B. W., & Williams, D. H. (1986) *J. Am. Chem. Soc. 108*, 6359–6363.

Pucci, P., & Sepe, C. (1988) *Biomed. Environ. Mass Spectrom. 17*, 287–291.

Pucci, P., Sepe, C., & Marino, G. (1992) *Biol. Mass Spectrom. 21*, 22–26.

Seno, M., Sasada, R., Iwarne, M., Sudo, K., Kurokawa, T., Ito, K., & Igarashi, K. (1988) *Biochem. Biophys. Res. Commun. 151*, 701–708.

Squires, C., Childs, J., Eisemberg, S., Polverini, P., & Sommer, A. (1988) *J. Biol. Chem. 263*, 16297–16302.

Thompson, S. A., (1992) *J. Biol. Chem. 267*, 2269–2273.

Veno, N., Baird, A., Esch, F., Ling, N., & Guillemin, R. (1986) *Biochem. Biophys. Res. Commun. 138*, 580–588.

Zhang, J., Cousens, L. S., Barr, F. J., & Sprang, S. R. (1991) *Proc. Natl. Acad. Sci. 88*, 3446–3450.