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CHARACTERIZATION OF A CYSTEINE-FREE ANALOG OF RECOMBINANT HUMAN BASIC FIBROBLAST GROWTH FACTOR

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Using oligo site-directed mutagenesis, we have modified our synthetic gene for human basic fibroblast growth factor (bFGF) to replace all four cysteine codons with serine codons. The corresponding protein was expressed in Escherichia coli and purified from inclusion bodies by solubilization in urea followed by a series of column chromatographies and a folding step. The resulting protein, having no cysteine residues, is unable to form either intramolecular or intermolecular disulfide bonds. The secondary and tertiary structures of the purified analog, as determined by circular dichroism and fluorescence spectroscopy, were identical within experimental error to recombinant bovine and human bFGF with unaltered amino acid sequences. Reflecting the similar conformation, the analog protein exhibited mitogenic activity on NIH 3T3 cells which was indistinguishable from the natural sequence molecule.

Basic fibroblast growth factor (bFGF) is a potent mitogen for a wide variety of cell types of mesodermal and neuroectodermal origin (1-3). Although bFGF contains four cysteine residues, there is no conclusive evidence that disulfide bonds are necessary for maintenance of the tertiary structure or for mitogenic activity. Neither has it been established that free sulfhydryls are required for proper structure or for biological activity. In a previous paper, we described the production and characterization of recombinant natural sequence bFGF in E. coli and suggested that the cysteines at positions 70 and 88 are exposed to the solvent in free form while the

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Abbreviations used: bFGF, basic fibroblast growth factor; E. coli, Escherichia coli; CD, circular dichroism; ATP, adenosine triphosphate; dNTP, dinucleotide triphosphate; DNA, deoxyribonucleic acid.

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cysteines at positions 26 and 93 are probably joined by a disulfide bond (4). Consistent with this hypothesis, we reported that an analog with serine residues substituted for the cysteines at positions 70 and 88 had biological activity indistinguishable from that of the natural sequence molecule, but not its tendency to form intermolecular disulfide bonds (4). Seno et al. have also reported a serine-70,88 analog with biological activity equivalent to that of natural sequence bFGF (5). It is, therefore, interesting to examine the properties of a molecule in which all four cysteine residues have been replaced with serine. In this paper, we describe the production of the cysteine-free analog of bFGF in E. coli by recombinant techniques and its characterization by circular dichroism and fluorescence spectroscopy. The properties of this analog are then compared with those of recombinant natural sequence bFGF.

MATERIALS AND METHODS

A previously constructed bFGF analog gene in which codons for cysteines 70 and 88 had been converted to serine codons was used as the starting template for the oligo site-directed mutagenesis (4). The oligonucleotide primers shown below were each designed to change a single nucleotide in order to convert the cysteine codons at positions 26 and 93 to serine codons:

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                                        C
cys-26 to ser-26)    5' ACCGTTTTTGGAGTACAGACG  3'

                                        C
cys-93 to ser-93)    5' GAAAAAGAAAGATTCGTCAGT  3'
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In each case, the primer corresponds exactly to the antisense strand of the template DNA except in the position of the desired change - the base originally present at the altered position is shown directly above the primer sequence. Approximately 0.5 μg of template DNA was mixed with 5 pmol universal M13 sequencing primer and 5 pmol of each of the oligonucleotide primers shown above, heated to 65°C for 3 minutes and allowed to slow cool. The annealed template-primer was then mixed with ATP, a dNTP mix, DNA polymerase I large fragment, and ligase followed by incubation at 15°C overnight. Aliquots of this reaction mixture were transformed into competent JM101 cells and plated in 0.7% L-agar. Plaques containing mutant phage were selected by hybridization of replica nitrocellulose filters with each ^{32}P-labeled mutagenic primer. Single strand DNA was prepared from plaques which scored positive in both hybridization screens. DNA sequencing by the dedeoxy chain-termination method verified that the resulting gene did indeed code for an analog of bFGF in which all four cysteine codons had been replaced by serine codons. Following sequence verification, the altered bFGF gene was excised as an XbaI/HindIII fragment and transferred to the E. coli expression plasmid pCFM1156 (4).

E. coli cells harboring the plasmid containing the mutant bFGF gene were grown in 2X Luria broth at 30°C to approximately 0.5 A_{600}, then shifted to 42°C to induce expression of bFGF. After growth overnight, the cells were harvested

by centrifugation and lysed by three passages through a French press at 10,000 psi. The insoluble material containing the bFGF trapped in inclusion bodies was collected by low speed centrifugation. The inclusion bodies were solubilized in urea and subjected to cation exchange and silica column chromatographies. The partially purified analog was folded by dilution and then purified to apparent homogeneity, judged by sodium dodecylsulfate polyacrylamide gel electrophoresis, by cation-exchange column chromatography. Recombinant human bFGF without serine for cysteine substitutions (natural sequence human bFGF) was purified from the soluble fraction of lysed E. coli cells by a series of chromatographic steps as previously described (4). A folding step was not required to generate bioactive material.

Protein concentrations were determined spectrophotometrically on a Hewlett-Packard Model 8451A diode array spectrophotometer. The extinction coefficient used was 1.3 at 278 nm and 1 cm path length for 0.1% protein solution. Circular dichroic spectra were determined at room temperature on a Jasco Model J-500C spectropolarimeter equipped with an Oki If 800 model 30 computer. Measurements were carried out using cuvettes of 1 and 0.02 cm for near and far UV ranges, respectively. The data were expressed as the mean residue ellipticity, [θ], calculated using the mean residue weight of 112 for both human and analog bFGF. Fluorescence spectra were determined on a Perkin-Elmer LS-5 spectrofluorimeter at room temperature. Slit widths were adjusted to 10 and 3 mm for excitation and emission, respectively. A cuvette with a 1 cm path length was used. The protein concentration was adjusted to 0.1 mg/ml.

NIH 3T3 cells were obtained from ATCC. The cells were grown in DME supplemented with 10% calf serum, 10 μ/ml penicillin and 10 μ/ml streptomycin. Cells were passed at a ratio of 1:40 two times per week. On day 1 of the assay, subconfluent cultures were trypsin dispersed and plated into 24-well plates at a concentration of 20,000 cells/ml, 1 ml per well in the above media. On day 5, the media was replaced with DME containing penicillin, streptomycin, and 5% human platelet poor plasma, 1 ml/well. On day 6, experimental samples were added to the media in volumes no greater than 100 μl. Eighteen hours later, cells were pulsed for 1 hour with 1 ml DME containing 5% calf serum and 2 μCi of tritiated thymidine at 37°C. The cells in each well were then washed with 1 ml DME without calf serum. One milliliter of 0.25 M sucrose, 10 mM sodium phosphate, 1 mM EDTA, pH 8 was added to each well and allowed to incubate for 10 minutes at 37°C. Cells were harvested with a Skatron harvester and filters were dried and counted in Aquasol-2 (Dupont) on a Beckman liquid scintillation counter.

RESULTS AND DISCUSSION

The bFGF analog lacking cysteine residues was produced in E. coli at an expression level similar to that of natural sequence recombinant bFGF. Since the analog protein was found in the insoluble inclusion bodies, it first had to be solubilized by a denaturing agent such as urea, partially purified, then allowed to refold by dilution of the denaturant. In order to examine the folding process, the secondary and tertiary structure of the analog was compared with that of the natural sequence human recombinant bFGF by CD and fluorescence spectroscopy.

Figure 1 shows the fluorescence spectra of recombinant analog and natural sequence human bFGF at 0.1 mg/ml in 20 mM sodium citrate, 0.1 M NaCl, pH

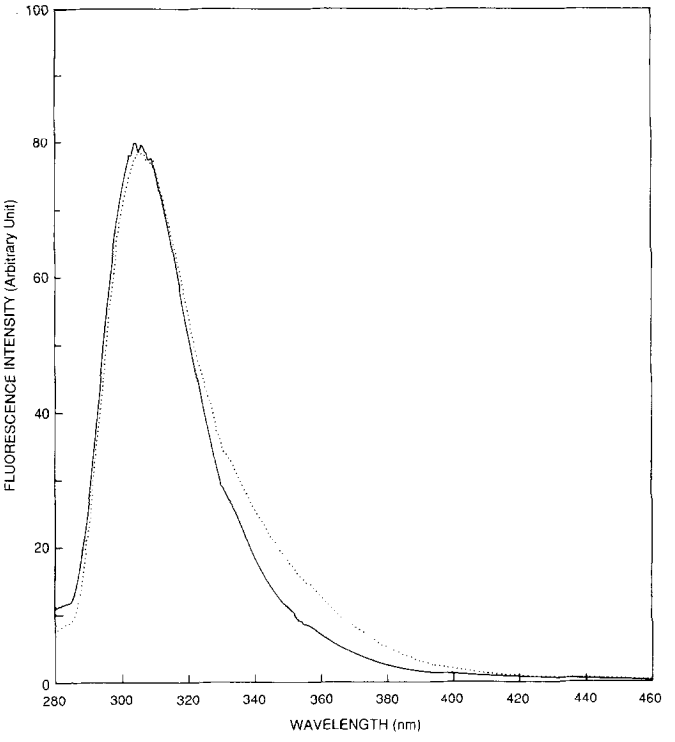

Figure 1. Fluorescence spectra of analog (solid line) and natural human sequence (dotted line) bFGF in 20 mM sodium citrate, 0.1 M sodium chloride, pH 5.0. Protein concentration was 0.1 mg/ml. The excitation wavelength used was 280 nm. The slit widths were adjusted to 10 and 3 nm for excitation and emission. All measurements were performed at room temperature using a cuvette of 1 cm path length.

5.0. This pH was chosen to minimize intermolecular disulfide formation for the natural sequence human protein. Both spectra are characterized by a peak at 306 nm and a shoulder around 340 nm. This spectral profile indicates that the single tryptophan is largely quenched and that the efficient energy transfer from tyrosine to tryptophan does not occur in either the natural sequence or analog protein. The similarity of the spectra indicates that the fluorescent tyrosine and tryptophan residues of the two proteins are in similar environments, suggesting that the analog is folded into a conformation closely resembling that of the natural sequence human bFGF. Both proteins exhibited similar spectra at pH 7.5, although prolonged exposure of the human natural sequence protein to this pH caused formation of disulfide-linked oligomers as judged by non-reducing sodium dodecylsulfate polyacrylamide gel electrophoresis. There was no apparent effect of pH on the conformation of either product.

Figure 2 shows the CD spectra of the two proteins in 20 mm sodium citrate, 0.1 M sodium chloride, pH 5.0. The two spectra are almost superimposable in both the far (Figure 2A) and near (Figure 2B) UV region, suggesting that these two proteins have very similar secondary and tertiary structures. As for the fluorescence spectra, no differences were observed when the CD was examined at pH 7.5. Thus, all the spectroscopic data indicate that the cysteine residues (and hence disulfide bonding) are not required for folding the protein into a native conformation.

The biological activity of the analog bFGF was tested by its ability to stimulate ^{3}H-thymidine uptake in confluent cultures of NIH 3T3 cells. Figure 3 shows the dependence of mitogenic activity on concentration for the analog and for natural sequence human bFGF. Within experimental error, the two profiles are indistinguishable, with the half-maximal mitogenic effect observed at a dose of about 150 pg/ml. These results indicate that the conformation required for receptor-mediated mitogenic activity is not altered by the substitution of the four cysteine residues, in agreement with our spectroscopic analysis. Further, free sulfhydryl groups are apparently not required for the mitogenic activity of bFGF. Comparison of the amino acid sequences of known FGF-related proteins [acidic FGF, bFGF, and the oncogene products int-2 and hst; (6-10)] reveals that the cysteines at positions 26 and

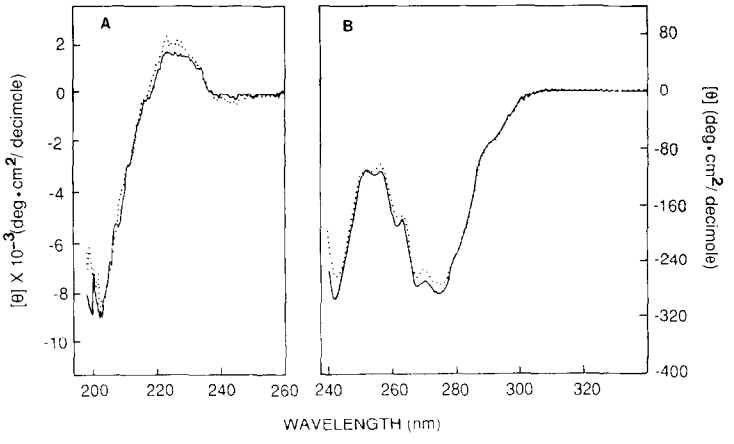

Figure 2. CD spectra of analog (solid line) and natural human sequence (dotted line) bFGF in the far (A) and near (B) ultraviolet region. Both proteins were in 20 mM sodium citrate, 0.1 M sodium chloride, pH 5.0.

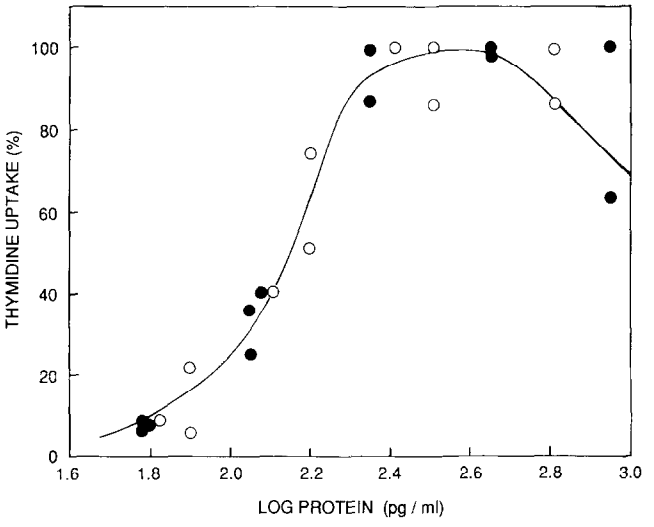

Figure 3. Mitogenic activity of human and analog bFGF on 3T3 cells. Uptake of ^{3}H-thymidine by confluent cultures of NIH 3T3 cells is plotted against protein concentration for both analog (○) and human natural sequence (●) bFGF.

93 are always conserved while those at positions 70 and 88 in bFGF are absent or at different positions in the other proteins. If, as our results indicate, no disulfide bonds are required for bFGF activity, these conserved cysteine residues may be important structurally for other reasons. Since serine is chemically and physically very close to cysteine, this change may not have affected such interactions. Alternatively, a disulfide bond may be present in tissue-derived bFGF but not be absolutely required for folding of the recombinant material into a biologically active form. It would be of interest to examine the effect of substituting different amino acids on the structure of the protein.

In contrast to our results, Seno et al. (5) have reported that their analog of human bFGF in which all four cysteines have been replaced by serine shows only 10% of the biological activity of their natural sequence recombinant bFGF. This discrepancy may be explained by differences in the method of preparation of the two proteins. Whereas the material of Seno et al. (5) was purified by heparin affinity chromatography of a relatively crude bacterial extract, our bFGF analog has been subjected to several purification steps plus a step designed to fold the protein into a biologically active form. It is possible that the analog bFGF is manufactured by E. coli in a biologically

inactive form or is unstable inside the cell. In either case, the majority of the bFGF in the bacterial extract could be biologically inactive without a refolding step. Baird et al. (11) have presented evidence that the heparin binding property of bFGF is probably due to the cumulative effect of several small peptide binding regions rather than a specific locus or a particular tertiary structure. If this is the case, heparin affinity chromatography would not be expected to efficiently distinguish active from inactive material and the preparation of Seno et al. (5) may contain a significant amount of improperly folded, biologically inactive conformers. Separation of active from inactive forms could require additional chromatographic steps that are more sensitive to the protein's conformation.

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