ABSTRACT: Human acidic fibroblast growth factor (FGF-1) is a potent mitogen and angiogenic factor, with reportedly poor thermal stability and a relatively short in vivo half-life. However, certain mutants of FGF-1 have been described that exhibit a significant increase in half-life in tissue culture-based assays. FGF-1 contains three cysteine residues, two of which are highly conserved and buried within the protein core. Mutant forms of FGF-1 that substitute a serine residue at these cysteine positions have been reported to increase the protein’s half-life and specific activity as well as decrease the dependence upon heparin for full activity. However, the underlying physical basis for this increase in half-life has not been determined. Possible effects include stabilization of protein structure and elimination of sulfhydryl chemistry at these positions. Here we have used differential scanning calorimetry and isothermal equilibrium denaturation to characterize thermodynamic parameters of unfolding for individual, and combination, cysteine to serine mutations in human FGF-1. The results show that substitution by serine is destabilizing at each cysteine position in wild-type FGF-1. Thus, the increased half-life previously reported for these mutations does not correlate with thermal stability and is most likely due to elimination of sulfhydryl chemistry. The results also suggest a method by which protein half-life may be modulated by rational design.
MATERIALS AND METHODS

Mutagenesis and Expression. Wild-type FGF-1 was expressed in the pET21 vector (Invitrogen) by use of a synthetic gene for the 140 amino acid form of the protein (16, 21, 22). Mutations Cys16Ser, Cys83Ser, Cys117Ser, and His93Gly were constructed following the QuickChange site-directed mutagenesis protocol (Stratagene). Mutagenic oligonucleotides with a centrally located mutagenic codon were typically between 25 and 35 bases in length and had melting temperatures above 70 °C. Oligonucleotides were obtained from the Biomolecular Analysis Synthesis and Sequencing Laboratory at Florida State University. Combination mutants were constructed by the same method and starting with vector DNA for appropriate point mutants.

Protein Expression and Purification. Escherichia coli BL21(DE3) cells, transformed with the wild-type and mutant expression vectors, were grown at 37 °C in minimal medium (23) to an optical density of A600 = 1.0. At this point 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added and the incubation temperature was shifted to 25 °C. The cells were allowed to grow for an additional 4 h and were then harvested by centrifugation (5000g) for 10 min and stored frozen (−20 °C) prior to use. Wild-type and mutant FGF-1 protein was purified to apparent homogeneity, as judged by Coomassie Brilliant Blue-stained sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS—PAGE), by a combination of anionic, cationic, size-exclusion, and heparin affinity chromatography (21). The purified protein was exchanged into 20 mM N-(2-acetamido)iminodiacetic acid (ADA) and 0.1 M NaCl, pH 6.6 (ADA buffer), for all subsequent spectroscopic and calorimetric studies (21).

Isothermal Equilibrium Denaturation Studies. Isothermal equilibrium denaturation studies of wild-type and mutant FGF-1 were performed with guanidine hydrochloride (GuHCl) as a denaturant, and circular dichroism (CD) was used to monitor the extent of denaturation (21). Protein samples (0.04 mM), in various concentrations of GuHCl/ADA buffer, were allowed to equilibrate overnight at 298 K. CD spectroscopic measurements were performed on an Aviv 62 ADS circular dichroism spectrometer (Aviv, Lakewood, NJ), fitted with a thermoelectric cuvette holder and interfaced with a model CFT-33 refrigerated recirculator (NESLAB). Isothermal (298 K) CD spectra were acquired by scanning from 260 to 210 nm in 1-nm increments with a 1-nm bandwidth. Triplicate scans were recorded, averaged, and analyzed. Buffer scans were subtracted and the data was converted to molar ellipticity (deg cm² dmol⁻¹). The CD spectrum of FGF-1 exhibits a maximum at 227 nm (10, 21). Molar ellipticity data monitored at 227 nm were plotted against denaturant concentration. Analysis of the data was performed by the general-purpose nonlinear fitting program DataFit (Oakdale Engineering) with a six-parameter, two-state model (24):

\[
F = \frac{F_0N + (S_N[D]) + (F_0D + (S_D[D]))e^{-(\Delta G + m[D])/RT}}{1 + e^{-(\Delta G + m[D])/RT}}
\]

where the denaturant concentration is given by [D], the native state baseline intercept and slope are \(F_0N\) and \(S_N\), respectively, and the denatured state baseline intercept and slope are \(F_0D\) and \(S_D\), respectively. The Gibbs free energy of unfolding at 0 M denaturant is given by \(\Delta G_0\) and the slope of the free energy as a function of denaturant concentration is given by \(m\).

Differential Scanning Calorimetry. Calorimetric analyses were performed on a MicroCal model VP-DSC high-sensitivity differential scanning calorimeter (DSC) (Microcal, Inc., Northampton, MA). Previous studies have demonstrated that the thermal denaturation of FGF-1 is reversible and twostate in the presence of low concentrations (0.7 M) of added GuHCl (21). Protein samples (0.04 mM) in 0.7 M GuHCl/ADA buffer were degassed for 15 min prior to analysis. Nitrogen was used to keep the samples under constant pressure of 35 psi. The temperature range of the thermal scan was from 278 to 353 K, with a scan rate of 15 K/h. For each experiment, buffer and protein scans were run in triplicate, and each experiment was duplicated. Data were analyzed by using the nonlinear least-squares fitting program DataFit (Oakdale Engineering) and implementing a statistical mechanics-based two-state model (25–27):

\[
C_p(T) = C_N(T) + \Delta C_p(T)[1 - F_N(T)] + [\Delta H(T)^2F_N(T)(1 - F_N(T))]RT^2
\]

where \(C_p(T)\) is the linear heat capacity function of the native state, \(F_N(T)\) is the temperature-dependent native-state fractional component, \(\Delta H(T)\) is the temperature-dependent enthalpy of the system, and \(\Delta C_p(T)\) is the temperature-dependent difference heat capacity function between the denatured and native states. The value of \(\Delta C_p(T)\) at the \(T_m\) of the wild-type protein has previously been determined at 0.409 ± 0.033 J g⁻¹ K⁻¹ (21). This value was used as a fixed parameter in our nonlinear refinement to the statistical mechanics-based two-state model for all mutants (thus reducing the number of parameters during the fit). The good agreement between the DSC and isothermal equilibrium denaturation data (below) supports the use of a fixed \(\Delta C_p\) value in the present case. \(\Delta G\) as a function of temperature was calculated as follows:

\[
\Delta G(T) = -(DA)(T - T_m) - (DB)T \ln(T/T_m) + (DC)(1 - T/T_m)
\]

where \(DA\), \(DB\), and \(DC\) are terms of the second-order polynomial function describing \(\Delta H\) of unfolding and \(T_m\) is the melting temperature (26, 27).

RESULTS

Protein Purification. Natively folded FGF-1 elutes from heparin—Sepharose affinity resin at a buffer ionic strength of 1.2–1.4 M, whereas denatured FGF-1 elutes at approximately 0.6 M (28). All the cysteine mutants of FGF-1 that were soluble were observed to elute from heparin–Sepharose affinity resin at a buffer ionic strength of 1.2–1.4 M.

Isothermal Equilibrium Denaturation. The thermodynamic parameters deconvoluted from the differential CD spectrum at 227 nm for wild-type FGF-1 under isothermal conditions (298 K) yields an \(m\) value of −18.7 ± 0.4 kJ mol⁻¹ and a \(\Delta G\) value at 0 M GuHCl of 20.8 ± 0.5 kJ mol⁻¹ (21). The concentration of GuHCl at the transition point where \(\Delta G =
A point mutation of histidine to glycine at position 93 was made in an effort to produce a more stable FGF-1 molecule. This mutation has been identified from temperature-induced aggregation studies as a potentially stabilizing mutation (29). Thus, it could serve as a more stable background into which severely destabilizing mutations could be introduced to allow accurate thermodynamic analysis. The histidine to glycine mutant at position 93 resulted in the stabilization of FGF-1 by 5.5 kJ/mol (Table 1).

**Competition Mutants.** Double mutants were constructed to evaluate the potential for nonadditive behavior of the serine mutations at positions 16, 83, and 117. The double mutants involving a serine mutation at position 16 (Cys16Ser/Cys117Ser) could not be purified due to aggregation in ADA buffer. This aggregation suggests that these mutants may be partially or completely denatured. The Cys83Ser/Cys117Ser double mutant, however, could be purified. Deconvolution of the CD data shows that this double mutation destabilizes FGF-1 by 7.5 kJ/mol (Figure 2, Table 1).

A double mutant of Cys16Ser/His93Gly was constructed to allow more accurate analysis of the Cys16Ser point mutant. In contrast to the Cys16Ser point mutant, this double mutant was significantly folded at 298 K (Figure 1). Deconvolution of the CD data shows that this double mutant is more stable than wild type. ΔG0 refers to the value of ΔG at 0 M denaturant, and ΔGm0.7 is value of ΔG at 0.7 M GuHCl. The concentration of denaturant used in the DSC studies is 0.7 M, and these data are presented for direct comparison with the DSC data. Error values represent standard error of experimental repetitions.

### Table 1: Isothermal (298 K) Equilibrium Denaturation Data for Wild-Type and Mutant FGF-1

<table>
<thead>
<tr>
<th>mutant</th>
<th>ΔG0 (kJ/mol)</th>
<th>m (kJ/mol°)</th>
<th>ΔAG0 (kJ/mol)</th>
<th>ΔGNH7 (kJ/mol)</th>
<th>ΔGm0.7 (kJ/mol)</th>
<th>GuHCl at ΔG = 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>20.8 ± 0.5</td>
<td>−18.7 ± 0.4</td>
<td>7.7</td>
<td>7.7</td>
<td>7.7</td>
<td>1.11 ± 0.02</td>
</tr>
<tr>
<td>C83S</td>
<td>15.2 ± 0.2</td>
<td>−21.6 ± 0.1</td>
<td>5.5</td>
<td>0</td>
<td>7.7</td>
<td>0.70 ± 0.01</td>
</tr>
<tr>
<td>C117S</td>
<td>16.9 ± 0.1</td>
<td>−17.6 ± 0.1</td>
<td>3.9</td>
<td>4.6</td>
<td>3.1</td>
<td>0.95 ± 0.01</td>
</tr>
<tr>
<td>C83S/C117S</td>
<td>13.3 ± 0.3</td>
<td>−19.4 ± 2.3</td>
<td>7.5</td>
<td>−0.2</td>
<td>7.9</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>C16S</td>
<td>8.7 ± 0.9</td>
<td>−17.5 ± 0.8</td>
<td>12.1</td>
<td>−3.7</td>
<td>11.4</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>H93G</td>
<td>26.2 ± 2.0</td>
<td>−18.3 ± 2.0</td>
<td>−5.5</td>
<td>13.1</td>
<td>−5.4</td>
<td>1.45 ± 0.04</td>
</tr>
<tr>
<td>C16S/H93G</td>
<td>16.8 ± 1.4</td>
<td>−19.5 ± 1.3</td>
<td>3.9</td>
<td>3.2</td>
<td>4.5</td>
<td>0.86 ± 0.02</td>
</tr>
<tr>
<td>C83S/C16S</td>
<td>16.2 ± 0.8</td>
<td>−17.6 ± 0.4</td>
<td>7.5</td>
<td>12.1</td>
<td>7.7</td>
<td>1.11 ± 0.02</td>
</tr>
<tr>
<td>C83S/C16S/H93G</td>
<td>16.8 ± 0.9</td>
<td>−18.3 ± 1.4</td>
<td>3.9</td>
<td>3.2</td>
<td>4.5</td>
<td>0.86 ± 0.02</td>
</tr>
</tbody>
</table>

a ΔAG = ΔGWAT − ΔGSUT. A negative value for ΔAG indicates a mutant is more stable than wild type. ΔG0 refers to the value of ΔG at 0 M denaturant, and ΔG0.7 is value of ΔG at 0.7 M GuHCl. The concentration of denaturant used in the DSC studies is 0.7 M, and these data are presented for direct comparison with the DSC data. Error values represent standard error of experimental repetitions.
the triple serine mutant, was constructed in an attempt to evaluate the effects of the combination of serine mutations. However, this mutant also could not be isolated, due to aggregation.

**Differential Scanning Calorimetry.** The statistical mechanics-based model utilized for analysis of the thermal denaturation of FGF-1 has the underlying assumptions that the system is two-state, reversible, and in equilibrium. It has previously been demonstrated that in 20 mM ADA, 100 mM NaCl, and 0.7 M GuHCl, pH 6.60, and with a scan rate of 15 K/h, that these assumptions are true for wild-type FGF-1. However, the analysis using this model requires that there be some temperature at which the system is equally populated with the native and denatured states (i.e., the $T_m$ or melting temperature). Thus, if there is no temperature where this condition is true, the model cannot be applied.

**Point Mutants.** As determined from the isothermal equilibrium denaturation data, the Cys16Ser mutant is only partially folded at 298 K in 20 mM ADA and 100 mM NaCl, pH 6.60. Under the conditions required for DSC analysis (with the addition of 0.7 M GuHCl) there is no temperature at which the protein achieves at least 50% fraction folded (Figure 3). Thus, the DSC data for this point mutant cannot be accurately analyzed. However, DSC data for the Cys83Ser and Cys117Ser point mutations can be analyzed. The substitution of a serine residue for cysteine at position 83 decreases the melting temperature by 13 K, destabilizing the protein by 9.0 kJ/mol (Figure 4, Table 2). The serine mutation for cysteine at position 117 decreases the melting temperature by 2 K, destabilizing the protein by 1.1 kJ/mol (Figure 4, Table 2).

**Combination Mutants.** As described above, combination serine mutants involving position 16 (with the exception of the histidine 93 double mutant) could not be purified due to aggregation. The Cys83Ser/Cys117Ser double mutant decreased the melting temperature of the wild-type protein by 13.5 K, destabilizing the protein by 9.0 kJ/mol. The Cys16Ser/His93Gly double mutant decreased the melting temperature by 13.7 K, in comparison to the His93Gly point mutant, destabilizing the protein by 10.2 kJ/mol (Figure 4, Table 2).

**DISCUSSION**

The FGF family of proteins contains two highly conserved cysteine residues at positions 16 and 83 (140 amino acid numbering scheme). Despite early hypotheses that these positions form a disulfide bond, it is clear that these conserved residues are present as free cysteines within the solvent-excluded core of the protein. What are the structural and functional roles of these conserved cysteine residues? Substitution of these cysteine residues by the isosteric residue serine results in significantly increased half-life as determined by tissue culture-based assays (Figure 5). However, the results here demonstrate that serine substitutions, in each case, destabilize the protein structure.

The introduction of a novel hydroxyl group into the core region of a protein has been demonstrated to destabilize
The modest destabilizing effect of the serine substitution at position 117 suggests that solvent may readily participate as a hydrogen-bonding partner for this position (Tables 1 and 2) supports the role of solvent as a hydrogen-bonding partner for a serine at this position. The common feature of the cysteines at these positions is that they are free cysteines and are located in the core region of the protein. In the unfolded state, these residues would be accessible and could undergo chemical reactions unique to sulfhydryl groups. Formation of disulfide adducts, or oxidation to an s-sulfonate form, would have significant consequences for the folding equilibrium. As previously noted, formation of an intrachain disulfide bond between residues 16 and 83 renders the protein inactive. Interchain disulfide bond formation, or formation of disulfide bonds with other thiol-containing compounds, presents other problems with regard to the folding equilibrium. Specifically, the thiol adducts of these cysteine residues would need to be accommodated within the core region in order for FGF-1 to adopt a native conformation. Oxidation to the s-sulfonate form would result in a charged group that would have to be accommodated within the core region of the native structure. In the absence of an appropriately juxtaposed countercharge within the core, this type of chemical modification would result in significant destabilization of the native state. Therefore, it is plausible that the conserved cysteine residues in the FGF family of proteins are a structural feature contributing to an irreversible pathway from the denatured state. In conjunction with an inherently low thermal stability, the effective result is to reduce the half-life of the protein in vivo. FGF-1 is one of the broadest specificity human mitogens known (12). Overexpression of FGF-1 during development has been demonstrated to disrupt normal embryogenesis (32). Mutations that lead to an increased half-life would interfere with embryogenesis in a similar way. Thus, there would be a selective pressure to maintain the

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**Figure 5**: Half-life (in hours) for wild-type and mutant human FGF-1 from tissue culture-based assays (20, 29) (upper panel) versus the intrinsic thermal stability (ΔG values) derived from the equilibrium denaturation studies at 0 M GuHCl (lower panel). The ΔG value for the C16S/C83S mutant was determined from the ΔG values for the C16S point mutant and the C83S point mutant. The ΔG value for the C16S/C83S/C117S mutant was determined from the ΔG values for the C16S point mutant and the C83S/C117S double mutant. The ΔΔG values for these mutations, based on an assumption of additivity, are included for comparison with the published physiological half-life data.
conserved cysteines in the FGF-1 molecule. Finally, the introduction of buried free cysteine residues in other proteins may represent an effective de novo design to limit their in vivo half-life.

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REFERENCES