Thermodynamic Characterization of Mutants of Human Fibroblast Growth Factor 1 with an Increased Physiological Half-Life†

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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.

Human acidic fibroblast growth factor (FGF-1)[1] is a member of the FGF growth factor family, which currently comprises 18 members and whose mitogenic and chemotactic properties are mediated through tyrosine kinase receptors (*1*, *2*). Several members of the FGF family are known to be potent angiogenic factors that have potential in treating ischemia via "angiogenic therapy" (*3*−*9*). FGF-1 exhibits relatively poor thermal stability; however, binding of heparin protects FGF-1 from denaturation by heat, acid, proteolysis, and mild oxidation (*10*−*13*). Furthermore, tissue culture studies indicate that interaction with heparin results in an increase in the in vivo half-life of FGF-1 by as much as 10-fold (*14*, *15*).

FGF-1 comprises a single, nonglycosylated polypeptide of either 154 or 140 amino acids (*16*). In human FGF-1 there are three cysteine residues, located at positions 16, 83, and 117 (using the numbering scheme of the 140 amino acid form). When comparing all of the members in the FGF family, the cysteine residue at position 16 is conserved in 11 members, the cysteine at position 83 is conserved in every member so far identified, and cysteine 117 is unique to human FGF-1. Highly conserved cysteine residues often participate in disulfide bond formation, and residue positions 16 and 83 were originally hypothesized to form such a bond (*17*). Studies of cysteine to serine mutations in FGF-1 demonstrated that disulfide bond formation between residues 16 and 83 resulted in complete inactivation of protein function, and thus, these conserved cysteine residues must be present as free cysteine residues (*12*). Subsequent X-ray structure analysis has unambiguously shown that the three cysteine residues in FGF-1 are present as free cysteine residues (*18*, *19*). Furthermore, the X-ray crystal structure has shown that residue positions 16 and 83 are buried in the interior of the protein and are inaccessible to solvent, while position 117 has partial accessibility.

Functional studies of cysteine to serine mutations in FGF-1 indicate that a combination mutant, with serine substitutions at residue positions 16 and 83, increases the protein half-life (in serum-free medium and in the absence of heparin) from 0.26 to 13 h (*20*). These studies also showed that a combination mutant, with serine substitutions at positions 16, 83, and 117, increased the protein half-life even further to 73 h. The specific activity of FGF-1 also was observed to increase in response to these serine substitutions, with the triple serine mutant being 2.5 times as active as the wild-type protein (*20*). The underlying basis for the observed increases in protein half-life and specific activity were postulated to be due to either a stabilizing effect of the serine substitutions or elimination of sulfhydryl chemistry unique to the cysteine residues. We report here a determination of the thermodynamic parameters of unfolding for cysteine to serine mutations in human FGF-1, using a combination of isothermal equilibrium denaturation and differential scanning calorimetry.

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[1] Abbreviations: FGF-1, human acidic fibroblast growth factor; IPTG, isopropyl β-D-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ADA *N*-(2-acetamido)iminodiacetic acid; GuHCl, guanidine hydrochloride; CD, circular dichroism; DSC, differential scanning calorimetry.

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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 $A_{600} = 1.0$. 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 (5000*g*) 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 cm^2 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*):

$$F = \frac{F_0N + (S_N[D]) + (F_0D + (S_D[D]))e^{-(\Delta G_0 + m[D])/RT}}{1 + e^{-(\Delta G_0 + m[D])/RT}}$$

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 ΔG_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 two-state 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*):

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

where $C_N(T)$ 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 ΔC_p 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 ΔC_p value in the present case. ΔG as a function of temperature was calculated as follows:

$$\Delta G(T) = -(DA)T(T - T_m) - (DB)T\ln\left(\frac{T}{T_m}\right) + (DC)\left(1 - \frac{T}{T_m}\right)$$

where *DA*, *DB*, and *DC* are terms of the second-order polynomial function describing ΔH 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^{-2} and a ΔG value at 0 M GuHCl of 20.8 ± 0.5 kJ mol^{-1} (*21*). The concentration of GuHCl at the transition point where $\Delta G =$

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

mutant	ΔG_0 (kJ/mol)	m (kJ/mol^2)	$\Delta\Delta G_0$ (kJ/mol)	$\Delta G_{0.7}$ (kJ/mol)	$\Delta\Delta G_{0.7}$ (kJ/mol)	GuHCl at $\Delta G = 0$
wild-type	20.8 ± 0.5	−18.7 ± 0.4		7.7		1.11 ± 0.02
C83S	15.2 ± 0.2	−21.6 ± 0.1	5.5	0	7.7	0.70 ± 0.01
C117S	16.9 ± 0.1	−17.6 ± 0.1	3.9	4.6	3.1	0.95 ± 0.01
C83S/C117S	13.3 ± 0.3	−19.4 ± 2.3	7.5	−0.2	7.9	0.69 ± 0.02
C16S	8.7 ± 0.9	−17.5 ± 0.8	12.1	−3.7	11.4	0.49 ± 0.03
H93G	26.2 ± 2.0	−18.3 ± 2.0	−5.5	13.1	−5.4	1.45 ± 0.04
C16S/H93G	16.8 ± 1.4	−19.5 ± 1.3	3.9	3.2	4.5	0.86 ± 0.02

[a] $\Delta\Delta G = \Delta G_{WT} - \Delta G_{MUT}$. A negative value for $\Delta\Delta G$ indicates a mutant is more stable than wild type. ΔG_0 refers to the value of ΔG at 0 M denaturant, and $\Delta G_{0.7}$ 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.

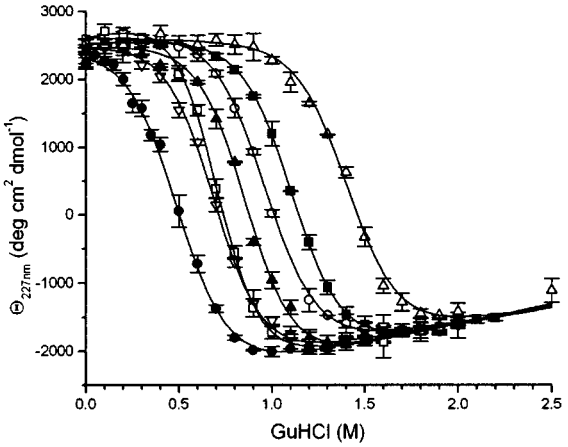

FIGURE 1: Isothermal (298 K) equilibrium molar ellipticity at 227 nm as a function of [GuHCl] for wild-type FGF-1 (■), Cys117Ser (○), Cys16Ser/His93Gly (▲), Cys83Ser (□), Cys83Ser/Cys117Ser (▽), Cys16Ser (●), and His93Gly (△) mutants. Included with the data are the fits to a six-parameter two-state model.

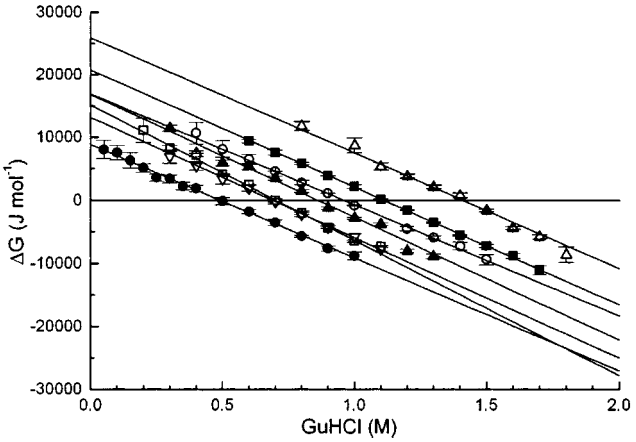

FIGURE 2: Deconvoluted isothermal (298 K) equilibrium ΔG values as a function of [GuHCl] for wild-type FGF-1 (■), Cys117Ser (○), Cys16Ser/His93Gly (▲), Cys83Ser (□), Cys83Ser/Cys117Ser (▽), Cys16Ser (●), and His93Gly (△) mutants. Included with the data are the ΔG functions derived from the fit of the six-parameter two-state model.

0 is 1.11 ± 0.02 M GuHCl. The m values for the different mutants vary from −17.5 kJ/mol^2 (Cys16Ser) to −21.6 kJ/mol^2 (Cys83Ser). The increased m value for the Cys83Ser mutant may reflect the interior location of this position and destabilizing effects upon the core region. However, there is no observed correlation between m values and the observed effects upon physiological half-life.

Point Mutants. The CD spectrum of the cysteine to serine mutation at position 16, in comparison to the wild-type protein, indicates that this mutant is only partially folded at 298 K (Figure 1). Deconvolution of the CD data for this mutant is therefore subject to error due to a lack of a native-state baseline. However, the mole fraction of the native state of this mutant as a function of denaturant can be determined by comparison to the known molar ellipticity for the native state of the wild-type protein. The isothermal equilibrium data indicates that a serine mutation at position 16 destabilizes FGF-1 by 12.1 kJ/mol (Table 1). The CD data for the serine mutation at position 83 indicate a relatively short, but identifiable, native-state baseline at 298 K (Figure 1). Deconvolution of the CD data shows that a serine mutation at position 83 destabilizes FGF-1 by 5.5 kJ/mol (Figure 2, Table 1). A serine mutation at position 117 destabilizes FGF-1 by 3.9 kJ/mol (Figure 2, Table 1).

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).

Combination 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/Cys83Ser and 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 298K (Figure 1). Deconvolution of the CD data shows that this double mutant is 9.4 kJ/mol less stable that the His93Gly point mutant and 3.9 kJ/mol less stable than wild-type FGF-1 (Figure 2, Table 1).

A triple serine mutant (at positions 16, 83, and 117) was also constructed to evaluate the potential for nonadditive behavior among these positions. However, it was not possible to isolate pure protein due to aggregation. A quadruple mutant, introducing the stabilizing His93Gly mutation into

Table 2: DSC Data for Wild-Type and Mutant FGF-1

mutant	T_m (K)	ΔT_m[a] (K)	ΔH (kJ/mol)	σ[b] (kJ mol^{-1} K^{-1})	$\Delta\Delta G$ (kJ/mol)	$\Delta\Delta G_{298}$ (kJ/mol)
FGF-1	312.6		257.3	0.6		
C83S	299.6 ± 0.6	13	137.5 ± 12	0.6	8.7 ± 0.1	8.0 ± 0.2
C117S	310.6 ± 0.7	2	219.4 ± 11	0.9	1.1 ± 0.3	2.6 ± 0.4
C83S/C117S	299.1 ± 1.3	13.5	127.6 ± 26	1.6	9.0 ± 0.9	7.6 ± 0.3
H93G	319.9 ± 0.3	−7.3	332.6 ± 9	1.4	−6.7 ± 0.2	−2.3 ± 0.9
C16S/H93G	306.2 ± 0.8	6.4	169.9 ± 24	0.2	3.5 ± 0.2	5.8 ± 0.5

[a] ΔT_m = (T_m of wild type) − (T_m of mutant). A negative value for ΔT_m indicates a mutant is more stable than wild-type FGF-1. Error values represent standard error of experimental repetitions. [b] Standard error for the fit of the two-state model to the observed data. [c] $\Delta\Delta G = \Delta G_{WT} - \Delta G_{MUT}$ determined at the T_m of wild type. A negative value for $\Delta\Delta G$ indicates a mutant is more stable than wild type. $\Delta\Delta G_{298}$ values are determined from the ΔG curves at 298 K, the temperature utilized in the isothermal equilibrium denaturation studies.

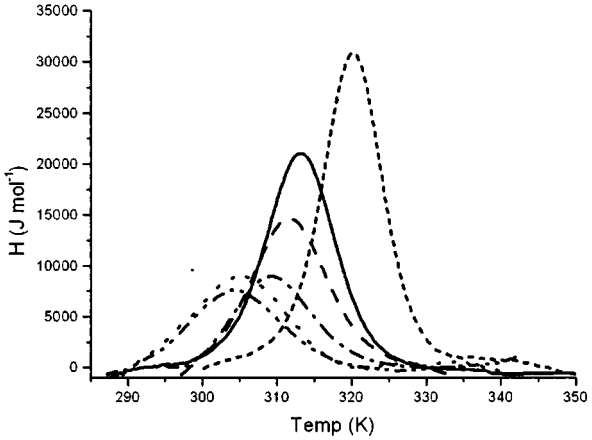

FIGURE 3: DSC excess enthalpy data, in 0.7M GuHCl, for wild type FGF-1 (—), Cys117Ser (− − −), Cys16Ser/His93Gly (−•−), Cys83Ser (•••), Cys83Ser/Cys117Ser (−••−), and His93Gly (---) mutants.

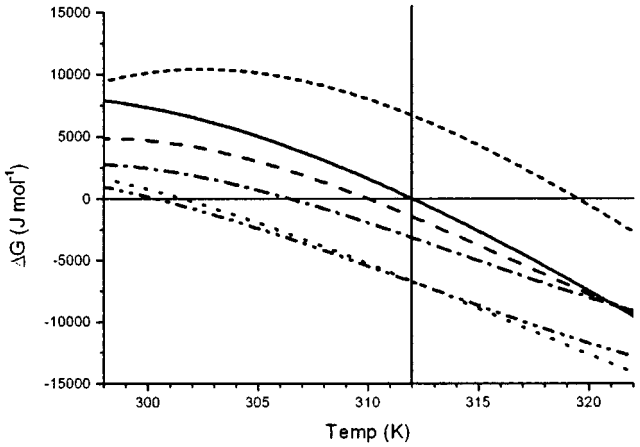

FIGURE 4: Deconvoluted DSC data ΔG curves, in 0.7 M GuHCl, for wild type FGF-1 (—), Cys117Ser (− − −), Cys16Ser/His93Gly (−•−), Cys83Ser (•••), Cys83Ser/Cys117Ser (−••−), and His93Gly (---) mutants. The vertical line indicates the T_m for the wild-type protein.

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 (*21*). 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 8.7 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

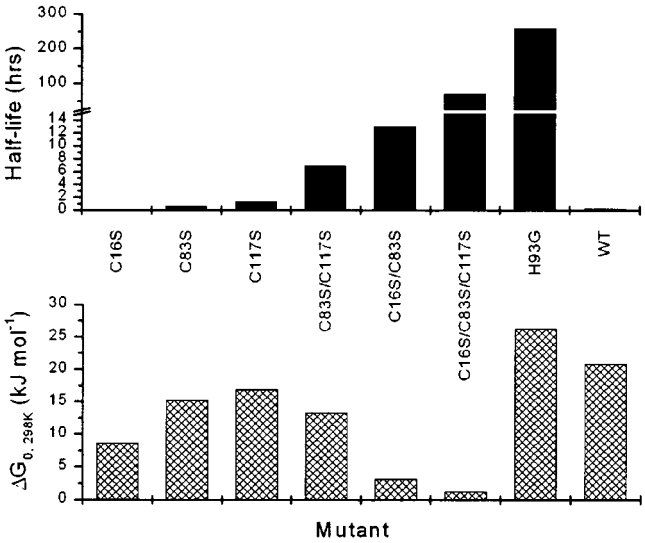

FIGURE 5: Half-life (in hours) for wild-type and mutant human FGF-1 from tissue culture-based assays (*20*, *29*) (upper panel) versus the intrinsic thermal stability (ΔG values) derived from the equilibrium denaturation studies at 0 M GuHCl (lower panel). The ΔG value for the C16S/C83S mutant was determined 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 $\Delta\Delta G$ values for these mutations, based on an assumption of additivity, are included for comparison with the published physiological half-life data.

proteins by approximately 4−12 kJ/mol (*30*). The range of destabilization is affected by the ease with which an appropriate hydrogen-bonding partner can be provided for the introduced hydroxyl. Analysis of the X-ray crystal structure of wild-type FGF-1 (*18*) indicates that there are no obvious hydrogen-bonding partners near position 16 to directly interact with the introduced serine hydroxyl. The nearest potential hydrogen-bonding partners would be atoms O 129 (3.7 Å distal) and N 18 (3.8 Å distal). Thus, distortion of the local structure appears to be necessary to satisfy the hydrogen-bonding requirements of a serine mutation at position 16. A similar analysis of position 83 indicates that the main-chain amide of residue position 80 would be approximately 3.5 Å distal to the introduced serine hydroxyl and would form an NH···O−C_β angle of 90°. A potential hydrogen bond could therefore be formed between these groups, requiring only a modest adjustment of the local structure or the χ_1 angle of the serine residue. Thus, when comparing positions 16 and 83, much more significant structural rearrangements appear to be required to provide an appropriate hydrogen-bonding partner for position 16, and this is the most destabilizing mutant. Partial accessibility of position 117 suggests that solvent may readily participate as a hydrogen-bonding partner for a serine at this position. The modest destabilizing effect of the serine substitution at this position (Tables 1 and 2) supports the role of solvent as a hydrogen-bonding partner in this case.

Increased half-life was reported for a serine double mutant (at positions 16 and 83) and a serine triple mutant (at positions 16, 83, and 117) (*20*). This raises the possibility that combination mutants may interact in some way and be more stable than predicted by the sum of the respective point mutations. Although such mutants have been isolated by Thomas and co-workers (*20*), we were unable to isolate any serine double mutants involving position 16, or the triple serine mutant, in a soluble folded form in ADA buffer. One possible reason for this discrepancy relates to the choice of buffer conditions. Thomas and co-workers utilized purification buffers containing phosphate and sulfate ions. FGF-1 has subsequently been demonstrated to bind both types of ions (*18, 19*), which causes an increase in the apparent melting temperature of FGF-1 (*31*). The studies presented here utilized ADA buffer to determine the effects upon stability in the absence of such bound ions.

In an attempt to characterize severely destabilizing mutations, we constructed them in a mutant form of FGF-1 (His93Gly) with enhanced thermostability (*29*). However, we were unable to isolate double mutants involving position 16, and the triple serine mutant, in soluble form (even after refolding attempts). We conclude that even if the combination serine mutants exhibit nonadditive effects upon protein stability, they are still significantly destabilized in comparison to the wild-type protein. Based on the results from the point mutations, the triple serine mutation would cause a reduction in ΔG in excess of 20 kJ/mol, with a corresponding decrease in T_m of approximately 30 K. Therefore, the triple serine mutant is significantly destabilized in comparison to the wild-type protein.

The destabilization of FGF-1 by the substitution of serines for cysteine residues suggests that the cysteine residues at positions 16 and 83 may be highly conserved due to considerations of protein stability. However, while substitution of cysteine residues at these positions may destabilize FGF-1, it is clear that the half-life of the protein can be dramatically increased by such mutations. 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 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

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

1. Johnson, D. E., Lu, J., Chen, H., Werner, S., and Williams, L. T. (1991) *Mol. Cell. Biol. 11*, 4627−4634.
2. Jaye, M., Schlessinger, J., and Dionne, C. A. (1992) *Biochim. Biophys. Acta 1135*, 185−199.
3. Unger, E. F., Banai, S., Shou, M., Lazarous, D. F., Jaklitsch, M. T., Scheinowitz, M., Correa, R., Klingbeil, C., and Epstein, S. E. (1994) *Am. J. Physiol. 266*, H1588−H1595.
4. Landau, C., Jacobs, A. K., and Haudenschild, C. C. (1995) *Am. Heart J. 129*, 924−931.
5. Pu, L.-Q., Sniderman, A. D., Brassard, R., Lachapelle, K. J., Graham, A. M., Lisbona, R., and Symes, J. F. (1993) *Circulation 88*, 208−215.
6. Schumacher, B., Pecher, P., von Specht, B. U., and Stegmann, T. (1998) *Circulation 97*, 645−650.
7. Nabel, E. G., Yang, Z.-Y., Plautz, G., Forough, R., Zhan, X., Haudenschild, C. C., Maciag, T., and Nabel, G. J. (1993) *Nature 362*, 844−846.
8. Schlaudraff, K., Schumacher, B., Specht, B. U. v., Seitelberger, R., Schlosser, V., and Fasol, R. (1993) *Eur. J. Cardiothoracic Surg. 7*, 637−644.
9. Thomas, K. A., Rios-Candelore, M., Gimenez-Gallego, G., DiSalvo, J., Bennett, C., Rodkey, J., and Fitzpatrick, S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6409−6413.
10. Copeland, R. A., Ji, H., Halfpenny, A. J., Williams, R. W., Thompson, K. C., Herber, W. K., Thomas, K. A., Bruner, M. W., Ryan, J. A., Marquis-Omer, D., Sanyal, G., Sitrin, R. D., Yamazaki, S., and Middaugh, C. R. (1991) *Arch. Biochem. Biophys. 289*, 53−61.
11. Gospodarowicz, D., and Cheng, J. (1986) *J. Cell. Physiol. 128*, 475−484.
12. Linemeyer, D. L., Menke, J. G., Kelly, L. J., Disalvo, J., Soderman, D., Schaeffer, M.-T., Ortega, S., Gimenez-Gallego, G., and Thomas, K. A. (1990) *Growth Factors 3*, 287−298.
13. Rosengart, T. K., Johnson, W. V., Friesel, R., Clark, R., and Maciag, T. (1988) *Biochem. Biophys. Res. Commun. 152*, 432−440.
14. Damon, D. H., Lobb, R. R., D'Amore, P. A., and Wagner, J. A. (1989) *J. Cell. Physiol. 138*, 221−226.
15. Mueller, S. N., Thomas, K. A., Di Salvo, J., and Levine, E. M. (1989) *J. Cell. Physiol. 140*, 439−448.
16. Gimenez-Gallego, G., Conn, G., Hatcher, V. B., and Thomas, K. A. (1986) *Biochem. Biophys. Res. Commun. 128*, 611−617.
17. Fox, G. M., Schiffer, S. G., Rohde, M. F., Tsai, L. B., Banks, A. R., and Arakawa, T. (1988) *J. Biol. Chem. 263*, 18452−18458.
18. Blaber, M., DiSalvo, J., and Thomas, K. A. (1996) *Biochemistry 35*, 2086−2094.
19. Romero, A., Lucena-Pineda, and Gimenez-Gallego, G. (1996) *Eur. J. Biochem. 241*, 453−461.
20. Ortega, S., Schaeffer, M.-T., Soderman, D., DiSalvo, J., Linemeyer, D. L., Gimenez-Gallego, G., and Thomas, K. A. (1991) *J. Biol. Chem. 266*, 5842−5846.
21. Blaber, S. I., Culajay, J. F., Khurana, A., and Blaber, M. (1999) *Biophys. J. 77*, 470−477.
22. Linemeyer, D. L., Kelly, L. J., Menke, J. G., Gimenez-Gallego, G., DiSalvo, J., and Thomas, K. A. (1987) *Bio/Technology 5*, 960−965.
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning: A laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24. Eftink, M. R. (1994) *Biophys. J. 66*, 482−501.
25. Freire, E., and Biltonen, R. L. (1978) *Biopolymers 17*, 463−479.
26. Kidokoro, S.-I., and Wada, A. (1987) *Biopolymers 26*, 213−229.
27. Kidokoro, S.-I., Uedaira, H., and Wada, A. (1988) *Biopolymers 27*, 271−297.
28. Adamek, D., Popovic, A., Blaber, S., and Blaber, M. (1998) in *Biocalorimetry: Applications of calorimetry in the biological sciences* (Ladbury, J. E., and Chowdhry, B. Z., Eds.) pp 235−241, John Wiley & Sons, Ltd., London.
29. Arakawa, T., Horan, T. P., Narhi, L. O., Rees, D. C., Schiffer, S. G., Holst, P. L., Prestrelski, S. J., Tsai, L. B., and Fox, G. M. (1993) *Protein Eng. 6*, 541−546.
30. Blaber, M., Lindstrom, J. D., Gassner, N., Xu, J., Heinz, D. W., and Matthews, B. W. (1993) *Biochemistry 32*, 11363−11373.
31. Blaber, M., Adamek, D. H., Popovic, A., and Blaber, S. I. (1997) *Biophysical and structural analysis of human acidic fibroblast growth factor*, Vol. VIII, Academic Press, San Diego, CA.
32. Slack, J. (1994) *Mol. Reprod. Dev. 39*, 118−124.

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