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Engineering a Cysteine-Free Form of Human Fibroblast Growth Factor-1 for "Second Generation" Therapeutic Application

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ABSTRACT

Human fibroblast growth factor-1 (FGF-1) has broad therapeutic potential in regenerative medicine but has undesirable biophysical properties of low thermostability and 3 buried cysteine (Cys) residues (at positions 16, 83, and 117) that interact to promote irreversible protein unfolding under oxidizing conditions. Mutational substitution of such Cys residues eliminates reactive buried thiols but cannot be accomplished simultaneously at all 3 positions without also introducing further substantial instability. The mutational introduction of a novel Cys residue (Ala66Cys) that forms a stabilizing disulfide bond (i.e., cystine) with one of the extant Cys residues (Cys83) effectively eliminates one Cys while increasing overall stability. This increase in stability offsets the associated instability of remaining Cys substitution mutations and permits production of a Cys-free form of FGF-1 (Cys16Ser/Ala66Cys/Cys117Ala) with only minor overall instability. The addition of a further stabilizing mutation (Pro134Ala) creates a Cys-free FGF-1 mutant with essentially wild-type biophysical properties. The elimination of buried free thiols in FGF-1 can substantially increase the protein half-life in cell culture. Here, we show that the effective cell survival/mitogenic functional activity of a fully Cys-free form is also substantially increased and is equivalent to wild-type FGF-1 formulated in the presence of heparin sulfate as a stabilizing agent. The results identify this Cys-free FGF-1 mutant as an advantageous "second generation" form of FGF-1 for therapeutic application.

Introduction

Cysteine (Cys) residues in proteins can have a pronounced effect on folding equilibrium. Oxidation of 2 Cys residues in close proximity, and with appropriate stereochemistry, in the native state can form a covalent bond (a cystine). Cystines prevent complete unfolding due to residual structure in the denatured state, thus substantially reducing the entropy of the denatured state (while having minimal consequences on the native state).[1,2] In such cases, the folding rate constant can be significantly increased, whereas the unfolding rate constant may be largely unperturbed (thereby shifting the folding equilibrium in favor of the native state).

In contrast, buried reduced Cys residues can have an opposite effect on the folding equilibrium. Cys residues are subject to chemical modification (e.g., oxidation) that can substantially alter their physical properties of size and charge. In the native state, a buried residue is largely protected from chemical modification by agents in bulk solvent; however, in the denatured state such buried residues are freely exposed and can readily participate in chemical modification. In the case of Cys residues, the alteration of size and charge associated with oxidative modification can preclude their accommodation within the buried environment of the native state. For example, exposed Cys residues can react to form a mixed disulfide with a thiol compound present in solution. Effective refolding would require that the added bulk of the thiol adduct be

Abbreviations used: FGF-1, fibroblast growth factor-1; WT, wild-type; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); GuHCl, guanidine hydrochloride; ANS, 1-anilino-8-naphthalene sulfonate; SLS, static light scattering; EPD, empirical phase diagram; HS, heparan sulfate; MHC, major histocompatibility complex.

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successfully accommodated within the core of the protein. Because most protein cores are efficiently packed,[3] it is unlikely that such accommodation is possible without significant structural and stability perturbation.[4,5] Cys residues can also oxidize to form various acidic forms (e.g., sulfenic, sulfinic, and cysteic acid); thus, refolding would require accommodation of a novel acidic charge within the core of the protein. However, the accommodation of an unpaired charge within the hydrophobic core region is associated with substantial destabilization—in the range of 20-24 kJ/mol.[6] Chemical modification of a buried Cys residue is therefore likely to result in an irreversible unfolding pathway. Such irreversibility shifts the folding equilibrium (by Le Chatelier's principle), continuously driving it toward the unfolded state, and substantially reducing functional half-life.[7-9]

Buried Cys residues are commonly found in proteins,[10,11] and their oxidation has long been known to lead to protein inactivation.[7,12-14] Fibroblast growth factor-1 (FGF-1) is a small protein (with a 140 amino acid "mature" form), with therapeutic potential in regenerative medicine,[15-18] and contains 3 buried Cys residues (at positions 16, 83, and 117).[19] A prior study of Cys positions in wild-type (WT) FGF-1 demonstrated that the functional half-life of FGF-1 in cell culture media can be increased as much as 40-fold by mutational substitution of Cys residues.[9] A detailed structural and thermodynamic study also showed that FGF-1 is structurally optimized to accept Cys residues at 2 of these buried positions (Cys16, Cys83)[20,21]; thus, their mutational substitution is associated with significant thermodynamic destabilization. Unfortunately, WT FGF-1 is characterized by very low overall thermostability[22,23]; thus, it is unable to accommodate any significantly destabilizing mutation and remain folded. Consequently, it is not feasible to generate a Cys-free form of FGF-1 by simple point mutation at each Cys position and retain a favorable folding equilibrium.[21,24]

Sequence and structure analysis of the FGF family indicates that one of the Cys residues (Cys83) in FGF-1 can be considered a vestigial half-cystine—such that an Ala66→Cys mutation (Ala66Cys; for facilitation of reading the single letter amino acid code will be used when describing combination mutations) can create a novel cystine with Cys83.[25] A query of the UNIPROT database indicates that this novel cystine is not present in any FGF-1 of any species. Although the Ala66Cys mutation introduces an additional Cys residue, the resulting Cys66-Cys83 cystine effectively eliminates one of the 3 native buried Cys residues in WT FGF-1 while simultaneously stabilizing the protein. Based on thermodynamic data for Ala, Val, Ser, and Thr point mutations at the remaining 2 buried Cys positions (Cys16 and Cys117)[21] it appeared feasible that point mutations that eliminate Cys residues at positions Cys16 and Cys117, although destabilizing overall, could potentially be combined with the stabilizing Ala66Cys disulfide mutation to generate a Cys-free form of FGF-1 with minimal overall thermodynamic perturbation. Such a mutation should entirely eliminate irreversible unfolding due to modification of buried Cys residues.

In the present report, we describe thermodynamic, biophysical, structural, and functional characterization of a Cys-free mutant form of FGF-1. We show the combination mutant C16S/A66C/C117A achieves an overall thermostability essentially indistinguishable from WT FGF-1, while effectively eliminating all Cys residues. This mutant does, however, manifest acidic (pH 5.0) sensitivity to unfolding that is not apparent in WT FGF-1. An additional point mutation, Pro134Ala, has only a modest increase in thermostability but less acidic pH sensitivity. Assays of cell survival and mitogenicity, using both a BaF3 cell system expressing FGF receptor-1c (FGFR-1c) and 3T3 fibroblasts, demonstrate up to a 3-order of magnitude increase in functional potency for various Cys-free mutants compared to WT FGF-1.

Materials and Methods

Mutant Design

Based on our previous studies,[25] the introduction of an Ala66Cys mutation yields a novel cystine with Cys83 and also provides ~10 kJ/mol gain in overall protein stability. However, no equivalent cystine-forming mutations were identified adjacent to positions Cys16 and Cys117. A systematic mutational analysis of substitutions of Cys16 in WT FGF-1 indicated that the least destabilizing amino acid substitution is Ser, albeit with a loss of ~10 kJ/mol in overall stability.[21,24] In contrast, position Cys117 is largely neutral to mutation, although Cys117Ala appears the best choice and provides ~2 kJ/mol increase in stability.[21,26] With simple additivity of mutational effects, the combination of Cys16Ser, Ala66Cys, and Cys117Ala offers the possibility of a completely Cys-free form of FGF-1 with minimal perturbation of WT FGF-1 stability. The C16S/A66C/C117A combination mutation was constructed to test this design strategy. Further stability gains were investigated by combining this Cys-free mutant design with additional stabilizing mutations Pro134Ala and Pro134Val.[27] Mutant pI values were calculated from the ExPASy Compute pI/Mw tool.[28] Major histocompatibility complex 1 (MHC-1) binding predictions were made using the IEDB analysis resource Consensus tool.[29] The average predicted affinity for a given nonapeptide centered at the site of mutation was calculated for all MHC alleles. The predicted aggregation potential for WT FGF-1 and the C16S/A66C/C117A/P134A mutant were calculated from the X-ray structures (WT FGF-1 PDB accession 1JQZ) using the AggreScan 3D server.[30]

Protein Expression and Purification

Recombinant protein expression used a pET21a(+) expression vector (EMD Millipore, Billerica, MA) with a codon-optimized synthetic gene encoding the 140 amino acid "mature" form of human WT FGF-1 and with an N-terminal 6× His tag. The QuickChange™ site-directed mutagenesis protocol (Agilent Technologies, Santa Clara, CA) was used to introduce all FGF mutations and were confirmed by DNA sequencing (Biomolecular Analysis Synthesis and Sequencing Laboratory, Florida State University). Recombinant WT FGF-1 protein was expressed from pET21a(+)/BL21(DE3) *Escherichia coli* as previously described.[31] Recombinant disulfide mutants were expressed from SHuffle T7 Express *E coli* (New England BioLabs, Ipswich, MA) and Luria broth media. The *E coli* culture was incubated at 30°C until $OD_{600} = 0.6$, at which point the temperature was shifted to 20°C and 1 mM isopropyl-β-D-thio-galactoside was added to induce protein expression with overnight incubation. The expressed protein was purified using sequential column chromatography on Ni-nitrilotriacetic acid affinity resin (Qiagen, Valencia, CA) and heparin Sepharose resin (GE Life Sciences, Pittsburgh, PA). Protein purity was evaluated by gel densitometry of Coomassie blue–stained SDS-PAGE. An extinction coefficient of E_{280nm} (0.1 %, 1 cm) = 1.26[31] was used for concentration determination of WT FGF-1 and all mutant proteins.

5,5′-Dithiobis-(2-Nitrobenzoic Acid) Assay

A 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay was used to determine the molarity of Cys in each purified mutant protein and thereby quantify the extent of cystine formation. Assays were performed by mixing 100 μL of 0.1-mM (1.6 mg/mL) purified protein with 400-μL DTNB reaction solution (0.75-mM DTNB, 0.1-M sodium phosphate, 7.5-M guanidinium hydrochloride [GuHCl], 1-mM EDTA, pH 8.0). The DTNB reaction mixtures were incubated