

Increased Functional Half-life of Fibroblast Growth Factor-1 by Recovering a Vestigial Disulfide Bond

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Abstract: The fibroblast growth factor (FGF) family of proteins contains an absolutely conserved Cys residue at position 83 that is present as a buried free cysteine. We have previously shown that mutation of the structurally adjacent residue, Ala66, to cysteine results in the formation of a stabilizing disulfide bond in FGF-1. This result suggests that the conserved free cysteine residue at position 83 in the FGF family of proteins represents a vestigial half-cystine. Here, we characterize the functional half-life and mitogenic activity of the oxidized form of the Ala66 → Cys mutation to identify the effect of the recovered vestigial disulfide bond between Cys83 and Cys66 upon the cellular function of FGF-1. The results show that the mitogenic activity of this mutant is significantly increased and that its functional half-life is greatly extended. These favorable effects are conferred by the formation of a disulfide bond that simultaneously increases thermodynamic stability of the protein and removes a reactive buried thiol at position 83. Recovering this vestigial disulfide by introducing a cysteine at position 66 is a potentially useful protein engineering strategy to improve the functional half-life of other FGF family members.

Keywords: FGF-1; protein engineering; disulfide-bond; half-cystine; protein evolution; free cysteine.

I INTRODUCTION

Fibroblast growth factors (FGFs) are a family of polypeptide exhibiting diverse biological roles in multiple developmental and metabolic processes. The 22 members of the human FGF family have a conserved ~120 amino acid core, however, they exhibit significant difference in size (17–25kDa) and sequence (13–71% sequence similarity) (Itoh and Ornitz 2004, 2008). Despite of the variations, three positions are absolutely conserved in the entire human FGF family members including Gly71, Cys83 and Phe132 (based on the 140 amino acid numbering scheme of FGF-1). Cys83 has brought more attention because highly conserved cysteine residues in proteins are often involved in functionally important sites. Cys83 was initially believed to be a half-cystine of a disulfide bond that played a key role in stabilizing the FGF structure (Burgess, et al. 1985, Strydom, et al. 1986). However, subsequent sequence and structural information revealed that only a few members of FGFs (at least 3 members, and possibly as many as 6) have this residue participating as a half-cystine to form a disulfide bond with adjacent cysteine at position 66 (Harmer, et al. 2004, Olsen, et al. 2006, Goetz, et al. 2007). Cys83 in the other 16 members, including FGF-1 and FGF-2, is present as a free cysteine (and adjacent position 66 is a noncysteine residue) (Zhang, et al. 1991, Blaber, et al. 1996, Bellosta, et al. 2001, Plotnikov, et al. 2001, Ye, et al. 2001, Yeh, et al. 2003, Olsen, et al. 2006).

In a previous study, we explored the origin of the conserved nature of Cys83 in FGF-1 (where Cys83 is present as a buried free cysteine and position 66 is Ala) by constructing a series of point mutations at position 83 and 66 (Lee and Blaber 2009b). Notably, introduction of cysteine at position 66 induces disulfide bond formation with Cys83 under oxidized condition and results in ~10 kJ/mol of increased thermostability. X-ray structure of the oxidized form of Ala66 → Cys mutant confirms a near-optimal disulfide bond between Cys66 and Cys83 without exhibiting apparent perturbation in the local structure (Fig. 1). These results suggest that the conserved Cys83 in FGF-1 is a vestigial half-cystine and the disulfide can be recovered by “backmutation” of alanine 66 to cysteine. A buried free cysteine in FGFs has been known to limit functional stability of the protein due to reactive thiol chemistry (Ortega, et al. 1991, Culajay, et al. 2000, Lee and Blaber 2009a). Therefore, the absence of a cysteine residue at position 66 of some members of FGF family proteins may ensure that the cysteine residue at position 83 remains a free cysteine that functions as a buried reactive thiol to regulate (*i.e.*, reduce) the functional half-life of the protein. Also, giving up the disulfide bond at this position costs significant destabilization of the protein that may contribute to functional regulation of the protein. Limiting the functional half-life of FGF proteins by maintaining a free cysteine at position 83 may be important for some FGF proteins considering that FGF proteins are generally potent mitogens.

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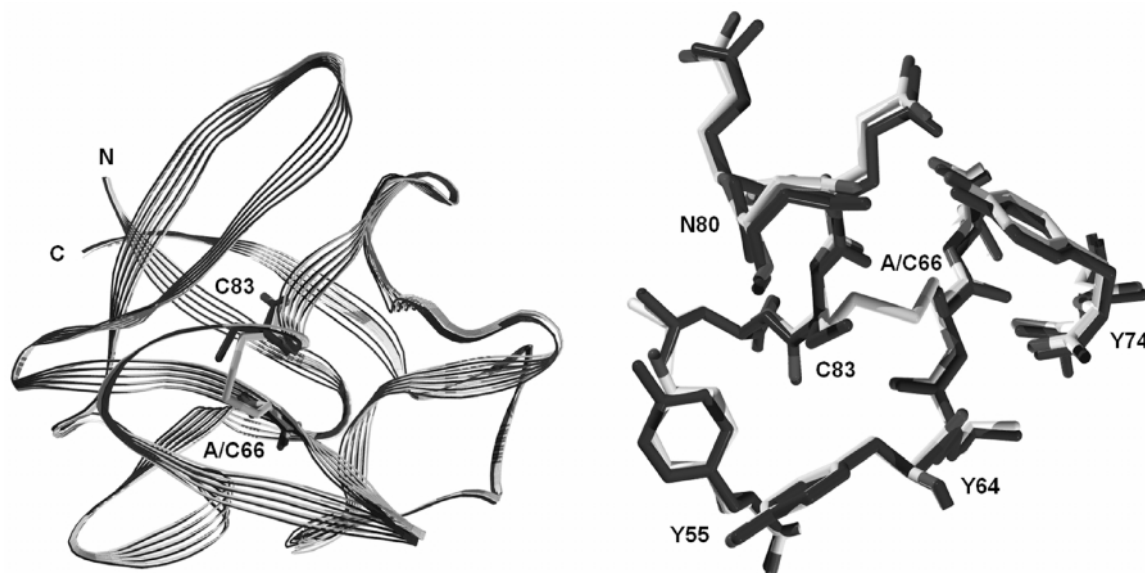


Figure 1: X-ray Structure of the Oxidized form of Ala66 → Cys Mutant (PDB code 3HOM; light gray) Overlaid with Eild-type FGF-1 (PDB code 1JQZ; darker gray). (Left) Ribbon Diagrams of the Wild-type and the Mutant Structures show the Location of Position 66 and 83. These two Positions are Located in Solvent Excluded Region of the Protein. (Right) The Local Structure in the Region of Position 66 and 83 of the Wild-type and Mutant FGF-1 Protein. In the Ala66 → Cys Mutant Structure, Cys Residues at both Position 66 and 83 Adopt a *gauche+* rotamer in Forming a Disulfide Bond. The Local Structure is almost Identical in Comparison to wild-type FGF-1 Indicating the Disulfide Bond is Accommodated without any Apparent Structural Perturbation.

In this report, we characterized the functional activity of Ala66 → Cys mutant FGF-1 to identify the effect of recovered vestigial disulfide bond. The Ala66 → Cys mutant with intermolecular disulfide bond exhibits substantially increased mitogenic activity and functional half-life against NIH 3T3 fibroblast cells. The results confirm that effective elimination of the buried reactive thiol at position 83 by disulfide bond formation indeed improves the functional stability of the protein. This observation supports the regulatory role of a buried free cysteine at position 83 in FGF-1. Furthermore, the results suggest that other members of the FGF family with a free cysteine at position 83 may similarly be stabilized by the introduction of a novel disulfide bond involving a cysteine at position 66, with an associated favorable impact on functional half-life. This mutation form provides a useful strategy to improve pharmaceutical or therapeutic application of FGF proteins.

II MATERIALS AND METHODS

Mutagenesis, Expression and Purification

Details of expression and purification of the wild-type and mutant proteins were described previously (Brych, et al. 2001, Lee and Blaber 2009b). Briefly, Ala66 → Cys

point mutation was introduced using QuikChange™ site directed mutagenesis protocol (Stratagene, La Jolla CA) on a synthetic gene for the 140 amino acid form of human FGF-1 containing an additional amino-terminal His tag (Gimenez-Gallego, et al. 1986, Linemeyer, et al. 1990, Cuevas, et al. 1991, Blaber, et al. 1996). pET21a(+) plasmid/BL21(DE3) *Escherichia coli* host expression system (Invitrogen) was utilized for expression of the proteins as described (Blaber, et al. 1999, Culajay, et al. 2000). The protein was purified by Nickel-nitrilotriacetic acid (Ni-NTA) (QIAGEN) and subsequent heparin Sepharose CL-6B affinity column chromatography (GE Healthcare). The purified Ala66 → Cys mutant protein contains mixture of reduced and oxidized forms. To isolate fully oxidized form of Ala66 → Cys, the purified protein was air-oxidized at room temperature for 3 weeks. The oxidized mutant protein was subsequently equilibrated in 0.14 M NaCl, 5.1 mM KCl, 0.7 mM Na₂HPO₄, 24.8 mM Tris base, pH 7.4 ("TBS buffer") for 3T3 stimulation assay.

Mitogenic Activity and Functional Half-life in Unconditioned Medium

The mitogenic activity of the protein was evaluated by a cultured fibroblast proliferation assay as previously

described (Dubey, et al. 2007). Briefly, NIH 3T3 fibroblasts were plated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad CA) supplemented with 0.5% (v/v) newborn calf serum (NCS) (Sigma-Aldrich Corp., St. Louis MO) for 48 hr at 37 °C with 5% (v/v) CO₂. The quiescent serum-starved cells were stimulated with fresh medium supplemented with FGF-1 protein (0–10 ng/ml) and incubated for an additional 48 hr. After this incubation period the cells were counted using a hemacytometer (Hausser Scientific, Horsham PA). Experiments were performed in quadruplicate and cell densities were averaged. The protein concentration yielding one-half maximal cell density (EC₅₀ value) was used for quantitative comparison of mitogenicity. To evaluate the effect of exogenous heparin on mitogenic potency, 10 U/ml of heparin sodium salt (Sigma-Aldrich Corp., St. Louis MO) was added to the protein prior to cell stimulation. Heparin is known to stimulate the activity of FGF-1 by forming a thermostable complex (Copeland, et al. 1991)

For functional half-life studies the wild-type and mutant FGF-1 proteins were pre-incubated in

unconditioned DMEM/0.5% NCS at 37 °C for various time periods (spanning 0–72 hr depending on the mutant) before being used to stimulate the 3T3 fibroblast mitogenic response as described above.

III RESULTS

Mitogenic Activity in Response to the Presence/Absence of Exogenous Heparin

The mitogenic response of NIH 3T3 cells to wild-type and oxidized Ala66 → Cys mutant protein in the presence and in the absence of heparin is shown in Fig. 2 and Table 1. The EC₅₀ value of wild-type FGF-1 in the absence of heparin is 58.4 ng/ml, while the Ala66 → Cys mutant exhibits an EC₅₀ value of 5.43 ng/ml. Thus, the Ala66 → Cys mutant exhibits ~10-fold increase in mitogenic activity relative to wild-type FGF-1 in the absence of added heparin. In the presence of heparin the wild-type FGF-1 and Ala66 → Cys mutants are essentially indistinguishable, with EC₅₀ values of 0.48 ng/ml and 0.36 ng/ml, respectively. These results show that the Ala66 → Cys mutant has enhanced mitogenic activity in the absence of added heparin.

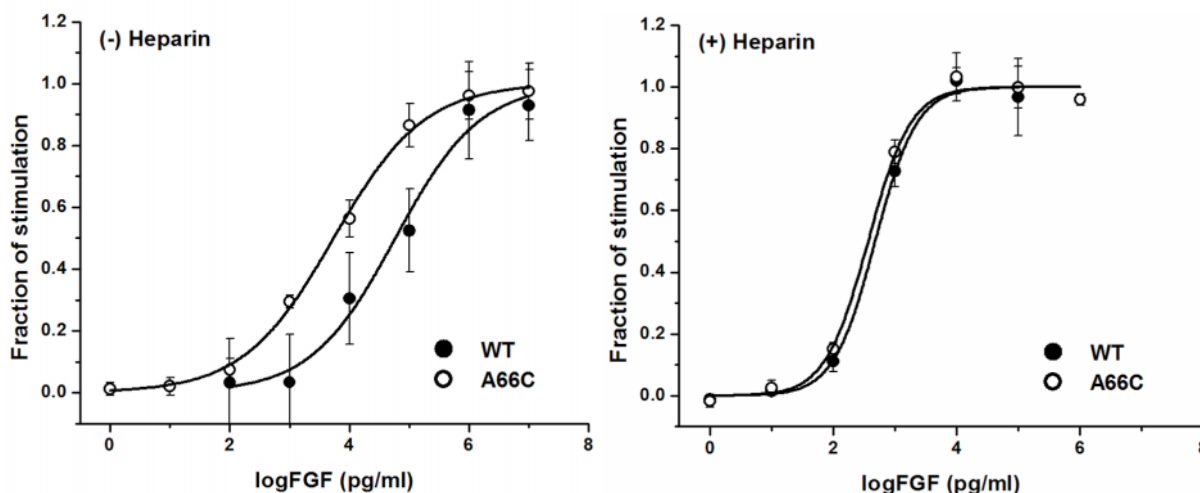


Figure 2: 3T3 Fibroblast Mitogenic Assay of Wild-type FGF-1 (filled circles) and Ala66 → Cys Mutant (open circles) in the Absence (top) and in the Presence (bottom) of 10U/ml Added Heparin.

Table 1: Summary of the Mitogenic Activity and Functional Half-life of the Ala66 → Cys (oxidized form) Mutant of FGF-1

Protein	EC ₅₀ (ng/ml)		Unconditioned medium half-life (hr)
	(-) Heparin	(+) Heparin	
Wild-type FGF-1	58.4 ± 25.4	0.48 ± 0.08	1.0
Ala66 → Cys	5.43 ± 3.96	0.36 ± 0.20	14.2

Functional Half-life

The residual mitogenic activity of wild-type and oxidized Ala66 → Cys mutant protein as a function of pre-incubation period in unconditioned DMEM/0.5% NCS medium at 37 °C is shown in Fig. 3 and Table 1. Under

half-life of 1.0 hr; however, the Ala66 → Cys mutant exhibits ~14-fold increase yielding a functional half-life of 14.2 hr.

IV DISCUSSION

The human FGFs are one of broadest specific human mitogens involved in variety of cellular events such as cell proliferation, differentiation, angiogenesis, morphogenesis, and wound healing (Zakrzewska, et al. 2008). FGF-1 is unique among 22 human FGF family members because it is the only FGF that can bind to all four FGF receptors as a universal ligand. FGF-1 exhibits strong angiogenic, osteogenic and tissue-injury repair properties (Burgess and Maciag 1989, Landgren, et al.

1998, Barnes, et al. 1999) and has been suggested for use as a protein biopharmaceutical in treating a wide range of diseases and conditions. For example, FGF-1 as a protein biopharmaceutical is currently in phase II clinical trials (NCT00117936) for pro-angiogenic therapy in coronary heart disease. FGF-1 has been also suggested for use in regenerating nervous system tissue following spinal cord injury or trauma, such as brachial plexus injury, neuroimmunologic disorders, such as acute or idiopathic transverse myelitis (TM), or any other disease or condition where regeneration and/or protection of neurons or neural tissue is desired (Cheng, et al. 2004, Lin, et al. 2005, Lin, et al. 2006)

Despite its tremendous potential, pharmaceutical or therapeutic, administration of FGF-1 is limited by the fact that wild-type FGF-1 exhibits poor thermostability. For example, the melting temperature of FGF-1 is only

marginally above physiological temperature (Copeland, et al. 1991). Furthermore, FGF-1 contains three buried free-cysteine residues that severely limit functional stability due to reactive thiol chemistry (Ortega, et al. 1991, Engleka and Maciag 1992, Estape, et al. 1998, Culajay, et al. 2000) resulting in considerably short functional half-life; in unconditioned DMEM, the half-life of wild-type FGF-1 is only about 1.0 hour according to a cultured fibroblast proliferation assay (Lee and Blaber 2009a). Because of its intrinsic property of instability, FGF-1 is prone to both aggregation and proteolysis, which may cause immunogenicity. Accordingly, various efforts have been made to increase the thermodynamic stability and/or half-life of proteins that are intended for use as biopharmaceuticals, while reducing their aggregation and/or immunogenicity.

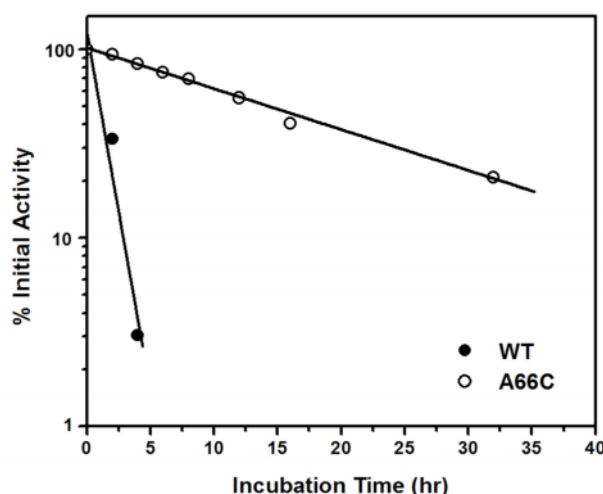


Figure 3: Functional Half-life Assay for Wild-type FGF-1 (filled circles) and Ala66 → Cys Mutant (open circles) in Unconditioned DMEM/0.5% NCS at 37 °C. The Log of the Percent initial Mitogenic Activity is Plotted as a Function of Incubation Time Prior to Mitogenic Assay.

Several mutant forms of FGF-1 which provide a noticeable increase in thermostability have been reported in last several years (Arakawa, et al. 1993, Brych, et al. 2001, Zakrzewska, et al. 2005, Dubey, et al. 2007, Lee and Blaber 2009b). These mutant forms of FGF-1 typically improve the functional stability and protease resistance of the protein by shifting the denaturation equilibrium to the native state. Another useful approach to improve functional activity of the protein is elimination of a buried free cysteine in FGF-1 by mutation (Perry and Wetzel 1987, Ortega, et al. 1991, Engleka and Maciag 1992, Estape, et al. 1998, Santiveri, et al. 2004, Fomenko, et al. 2008, Lee and Blaber 2009a). Although this type of

mutation often reduces thermodynamic stability, the functional half-life may be considerably increased because mutation of these reactive free cysteine residues diminishes thiol mediated irreversible denaturation. Additionally, we reported a tight interaction between thermodynamic stability and buried free cysteine in regulating the functional half-life of FGF-1 by combining separate mutations that eliminate free cysteines with other mutations that increase the thermostability of the protein (Lee and Blaber 2009a). This type of combination mutation has a synergetic effect on the half-life of the protein by avoiding the irreversible denaturation pathway resulting from thiol reactivity of the free cysteine (*e.g.*, disulfide

formation) while simultaneously increasing the thermodynamic stability of the protein. This is especially true considering that protein unfolding, which is dependent on protein stability, is often a necessary first step for the irreversible denaturation pathway resulting from exposure of the reactive free cysteine.

In this paper we report the functional half-life of the Ala66 → Cys mutant form of FGF-1. Replacement of alanine to cysteine at position 66 can induce formation of a disulfide bond with a free cysteine at position 83 and simultaneously provide a significant increase in thermodynamic stability (Lee and Blaber 2009b). These results provide the evidence of vestigial half-cystine for the conserved cysteine at position 83 of FGF-1. Here, we show a more than 10 fold increase in mitogenic activity and functional half-life of the protein in the absence of heparin, which unambiguously confirms that a newly formed disulfide bond in the Ala66 → Cys mutant provides favorable impact on functional stability of the protein. The results support a regulation role of a buried free cysteine at position 83 of FGF-1. From the protein engineering point of view, Ala66 → Cys is a unique mutant form of FGF-1 because enhancement of thermostability and elimination of buried free thiol can be achieved by the *addition* of a novel cysteine that effectively recovers a vestigial disulfide bond of FGF-1. Furthermore, X-ray structure of the oxidized form of Ala66 → Cys mutant shows that the disulfide bond is formed with minimal structural perturbation and in a solvent excluded region of the protein. Therefore, it is expected that Ala66 → Cys may be generally immune-permissive. Considering there are 15 other members of FGF containing a buried free cysteine at position 83, adding half-cystine at position 66 might be a useful strategy to improve the protein stability as well as functional half-life with minimal immunogenic potential.

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