Crystal Structure of Human pFGE, the Paralog of the Co-formylglycine-generating Enzyme*

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In eukaryotes, sulfatases are degraded by sulfatases, which possess a unique Co-formylglycine residue in their active site. The defect in post-translational formation of the Co-formylglycine residue causes a severe lysosomal storage disorder in humans. Recently, FGE (formylglycine-generating enzyme) has been identified as the protein required for this specific modification. Using sequence comparisons, a protein homologous to FGE was found and denoted pFGE (paralog of FGE). pFGE binds a sulfatase-derived peptide bearing the FGE recognition motif, but it lacks formylglycine-generating activity. Both proteins belong to a large family of pro- and eukaryotic proteins containing the DUF323 domain, a formylglycine-generating enzyme domain of unknown three-dimensional structure. We have crystallized the glycosylated human pFGE and determined its crystal structure at a resolution of 1.86 Å. The structure reveals a novel fold, which we denote the FGE fold and which therefore serves as a paradigm for the DUF323 domain. It is characterized by an asymmetric partitioning of secondary structure elements and is stabilized by two calcium cations. A deep cleft on the surface of pFGE most likely represents the sulfatase polypeptide binding site. The asymmetric unit of the pFGE crystal contains a homodimer. The putative peptide binding site is buried between the monomers, indicating a biological significance of the dimer. The structure suggests the capability of pFGE to form a heterodimer with FGE.

Sulfatases hydrolyze various N- and O-sulfate esters of steroids, glycopetides, glycolipids, and other compounds. In higher eukaryotes, they are engaged in the remodeling of heparin sulfate located at the cell surface or in lysosomal degradation, whereas in bacteria and lower eukaryotes, sulfatases are involved in sulfur scavenging (1–4). Sulfatases hydrolyze various N- and O-sulfate esters of steroids, glycopetides, glycolipids, and other compounds. In higher eukaryotes, they are engaged in the remodeling of heparin sulfate located at the cell surface or in lysosomal degradation, whereas in bacteria and lower eukaryotes, sulfatases are involved in sulfur scavenging (1–4).

In vitro studies by Mariappan et al (39) demonstrate that pFGE, which together with FGE resides in the endoplasmic reticulum, has no FGLy-generating activity, although it binds in the catalytic site, Co-formylglycine (FGly)1 (5). In mammals, the modification of a specific cysteine to FGly occurs during transition of the nascent polypeptide into the lumen of the endoplasmic reticulum (6–9). FGly, in the form of an aldehyde hydrate, is the nucleophile for the hydrolysis of sulfate esters. Recently, the FGLy-generating enzyme (FGE) has been identified independently by biochemical purification (10) and by functional complementation experiments (11) as the sole enzyme responsible for the generation of FGly from cysteines in human sulfatases.

The failure of mutated forms of FGE to generate FGly results in catalytically inactive sulfatases and causes multiple sulfatase deficiency, a rare disorder leading to death in infancy or childhood (10–12). Recently, the peptide binding site of FGE has been identified by chemical cross-linking of a photoreactive peptide (38).

Data base searches of the human genome revealed a coding region highly similar to FGE (48% sequence identity, 62% similarity), therefore termed paralog of FGE (pFGE) (10, 11, 13). pFGE and FGE belong to a large protein family currently comprising 164 members sharing a common domain of unknown function and unknown three-dimensional structure (DUF323). These DUF323 domains exhibit a mean average identity of 26% over the average length of 246 amino acids and are found in prokaryotic and eukaryotic proteins. Even more proteins, ~300, containing this domain are suggested by the COG1262 (clusters of orthologous groups of proteins (14). Interestingly, the domain resides mostly on its own, presumed to fulfill divergent functions such as an oxygenase involved in the synthesis of a β-lactam antibiotic (Erwinia carotovora) (15, 16) in toxoflavin (Burkholderia glumae), as transcriptional regulator (enhancement of xylanase A production in Bacillus halodurans), as an enhancer of xylanase production (Bacillus stearothermophilus), and as FGLy-generating enzyme FGE (in mammals) (10, 11). In ~12% of the proteins, the DUF323 domain has been found to be fused to other domains that are thought to function as a serine/threonine kinase in bacteria (Chlamydia trachomatis, Trichodesmium erythraeum), as cytosine/adenosine deaminases in fungi, as a peptidase in archaea and bacteria (Nostoc sp. and Methanosarcina barkeri), or as methyltransferase (Nitrosomonas europaea) or as Hsp60 chaperonin (Homo sapiens).

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The atomic coordinates and structure factors (code 1Y4J) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 The abbreviations used are: FGly, Co-formylglycine; FGE, FGLy-generating enzyme; pFGE, paralog of FGE; DUF323, domain of unknown function number 323; MIRAS, multiple isomorphous replacement with anomalous scattering.
Crystallization of pFGE

Crystal Structure of pFGE

Data collection

| Data set | Native | Mercury | Platinum | Xenon |
|----------|--------|---------|----------|-------|
| Wavelength [Å] | 1.5418 | 1.5418 | 1.5418 | 1.5418 |
| Resolution range (Å)* | (50.00–2.14) | (50.00–2.14) | (50.00–2.14) | (50.00–2.14) |
| Space group | P2_1 | P2_1 | P2_1 | P2_1 |
| Cell dimensions [Å]* | a = 47.1 | a = 47.3 | a = 47.0 | a = 47.2 |
| b = 100.8 | b = 101.1 | b = 100.1 | b = 100.9 |
| c = 62.7 | c = 62.9 | c = 62.6 | c = 62.7 |
| β = 101.7 | β = 101.8 | β = 102.0 | β = 101.4 |
| Unique reflections | 47,438 (1411) | 21,578 (1670) | 16,941 (1061) | 28,347 (2240) |
| Completeness [%] | 98.7 (87.3) | 97.5 (76.4) | 94.7 (60.2) | 97.8 (77.6) |
| R_{sym} | 5.5 (58.6) | 7.0 (19.4) | 5.5 (19.4) | 7.6 (24.6) |
| Average I/σ(I) | 22.7 (1.8) | 37.4 (7.9) | 22.7 (8.6) | 83.4 (14.9) |
| Multiplicity | 3.6 (3.0) | 5.3 (5.2) | 4.7 (3.7) | 27.0 (21.8) |
| Disallowed regions | 0.97 | 0.65 | 1.76 | 0.96 |
| Overall figure of merit | 0.102 | 0.102 | 0.102 | 0.102 |

MIRAS Phasing

| Heavy atom sites | 2 | 2 | 2 |
| Phasing Power | | | |
| Isomorphous | 0.541 | 0.432 | 1.152 |
| Anomalous | 0.320 | 0.253 | 0.940 |
| R-cu(λ) | 0.920 | 0.974 | 0.740 |
| Isomorphous | 0.694 | 0.986 | 0.593 |
| Anomalous | | | |

After solvent flattening

| Before solvent flattening | 0.30 | 0.30 | 0.30 |
| After solvent flattening | 0.83 | 0.83 | 0.83 |

Refinement statistics

| R_{cryst} [%]/R_{free} [%] | 17.2 (20.5) |
| Coordinate error (Å) | 0.102 |
| No. of protein atoms | 8475 |
| No. of ligand atoms | 149 |
| No. of water molecules | 562 |
| Ramachandran plot‡ | | |
| Most favorable regions [%] | 89.7 |
| Additional allowed regions [%] | 8.3 |
| Generously allowed regions [%] | 1.1 |
| Disallowed regions [%] | 0.9 |
| Root mean square deviations from ideality | | |
| Bond lengths (Å) | 0.01/1.34 |
| Average B values (Å²) | 28.3 |
| Protein residues/waters | 536/562 |

* Numbers in parenthesis refer to the highest resolution shell.

‡ R_{cryst} = 100ΣΣ[I(h)] / (I(h))/ΣΣ[I(h)] where I(h) is the hth measurement of the h reflection and (h) is the average value of the reflection intensity.

§ Calculated using PROCHECK (28).
FIG. 1. Structure of the pFGE monomer. A, the ribbon model of the pFGE monomer is rainbow-colored from the N terminus in blue to C terminus in red (compare Fig. 2A). Secondary structure motifs (η denotes a 3₁₀ helix) are numbered consecutively from the N terminus to the C terminus. Calcium cations are depicted as red spheres. B, an experimental electron density map of the pFGE reveals the N-glycosylation site at Asn-191. Electron density maps were calculated using MIRAS phases modified by solvent flattening. From the γ-amino group of Asn-191, additional electron density emanates that fits a trisaccharide comprising GlcNAc(-Fuc)-GlcNAc. The refined model of the branched trisaccharide is superimposed as a stick representation with the residues colored according to the atom type.

Structure Determination and Refinement—Initial phases were obtained with AUTOshARP (Global Phasing Ltd., Cambridge, UK) using data sets obtained from heavy atom derivatized crystals with platinum, xenon, and mercury. The initial phases were improved by solvent flattening as implemented in AUTOshARP and extended to 1.9 Å, which resulted in overall figure of merits of 0.83 (see Table I for details). No noncrystallographic symmetry averaging was necessary due to the high quality of the initial electron density map. A partial model (452 residues out of 568 residues, 54 of which were built as alanines) was automatically built using the auto-build as implemented in RESOLVE (17) and completed by manual fitting into χ-weighted 2Fo − DFc and Fo − Fc difference electron density maps (18) using the programs XTALVIEW (19) and O (20). The model was refined against the native data set (maximum resolution, 1.86 Å) with REFMAC5 (21) using standard parameters. A random set of 5% of reflections was excluded from refinement to monitor Rfree (22, 23). Water molecules were assigned automatically for >3σ peaks in Fo − Fc difference maps by cycling the REFMAC5 refinement with ARP/wARP (24) and retained if they obeyed hydrogen bonding criteria according to HBPLUS (25) and returned >1σ 2Fo − Fo density after refinement. The final model consists of two molecules (A and B) encompassing residues 28–294 with Thr-109 missing in molecule A. No electron density was visible for the N-terminal residue 26 of molecule A and the C-terminal residues 295–312, which were therefore not included in the model. Residues Gln-106 of molecule A and Lys-107 of molecule B were modeled as alanines due to lack of side chain density. Alternate side chain conformations were modeled for Arg-79, Arg-84, Met-89, Arg-127, Arg-253, and Arg-268 of molecule A and Arg-84, Gln-138, Lys-205, and Arg-250 of molecule B. The refinement statistics are summarized below (see Table I). Surface complementarity coefficients and solvent-accessible surface areas were calculated with SC using a 1.7 Å radius probe (26). Possible hydrogen bonds, salt bridges, and van der Waals contacts were detected with HBPLUS and CONTACSYM (27) using default parameters. Surface potentials were calculated with PyMol using the implemented function of vacuum electrostatics. The quality of the model was checked using PROCHECK (28) and WHATCHECK (29). The Ramachandran plots (30) showed that 89.7% are in the most favorable regions and 8.3% are in the additionally allowed region, whereas 1.1% are in the generously allowed region. Only 0.9% of the residues are in the disallowed region, namely Leu-116 and Asn-233. Leu-116 of both molecules has dΨ combinations (chain A, φ = 47.2°, ψ = −118.3°; chain B, φ = 47.5°, ψ = −117.5°) that lie in the disallowed regions of the Ramachandran plot, but the residues are very well defined in the electron density map. Leucine 116 is part of the contact area between the two molecules in the asymmetric unit. The distortion appears to be a consequence of this interaction. Asn-233 is located between 2 residues that are involved in calcium cation binding via their carbonyl oxygens. The calcium cation probably induces force on the peptidyl chain, resulting in the observed forbidden conformation of the Asn-233 residue chain: A, φ = 72.2°, ψ = −87.3°; chain B, φ = 69.7°, ψ = −85.0°. The monomer contains two cis-prolines at positions 53 and 201.

RESULTS AND DISCUSSION

Structure Determination—The crystal structure of pFGE was determined by means of MIRAS using several heavy atom derivatives (Table I). The initial electron density map obtained from experimental phases after solvent flattening was of excellent quality and allowed autotracing of ~450 out of 560 amino acids of the polypeptide chains (Fig. 1A). The quality of the initial MIRAS electron density map is exemplified by the presence of density for three sugar moieties attached to Asn-191 (Fig. 1B). A native data set was used for refinement to a resolution of 1.86 Å (Table I). The pFGE structure was refined to a free R-value of 17.2%, exhibiting good stereochemistry (Table I). The asymmetric unit of the crystal contains two pFGE molecules. Due to the high resolution, no noncrystallographic symmetry restraints were applied during refinement, allowing meaningful comparison of the two monomers. An overlay of both monomers reveals a root mean square deviation of 0.32 Å, indicative for the lack of significant structural differences between the two monomers (data not shown).

The attached trisaccharide, consisting of 1 fucose and 2...
GlcNAc residues, was modeled into the electron density for molecule A (Fig. 1B), but only the 2 GlcNAc residues could be modeled in the molecule B of the asymmetric unit. These GlcNAc residues protrude from the molecule into a solvent channel in the crystal. By contrast, the fucose residue of molecule A is involved in the crystal packing by interaction with a molecule.
of the neighboring asymmetric unit, thereby stabilizing the crystal structure. Usually, the flexibility of carbohydrate side chains is detrimental to ordered crystal packing, but not so in pFGE.

**Overall Fold**—The fold of pFGE consists of five \( \alpha \)-helices ranging from 5 to 11 amino acids in length and two short \( \beta \)-helices (Figs. 1A and 2). The 11 \( \beta \)-strands are 2-7 amino acids in length and form four antiparallel \( \beta \)-sheets (Fig. 2A). The first two sheets, formed by \( \beta \)-strands 1/4/11 and 2/3, respectively, engulf a helix 5, which on the opposing side is flanked by the third sheet (\( \beta \)-strands 7/8/9/10). The arrangement of sheet 3 and sheet 4 (\( \beta \)-strands 5/6) allows the formation of a deep cleft (see below). The surface of sheet 4 opposing this cleft is shielded from the solvent by two short helices, \( \alpha \)-helix 3 and a \( \beta \)-helix, \( \eta \). With the exception of the remaining two longer helices of perpendicular arrangement (helices 1 and 4) and one short helix (helix 2), the structure is void of secondary structure elements (Fig. 1A). Indeed, pFGE exhibits a surprising lack of secondary structure elements, 37% in total with 17.7% helices (2.3% \( \alpha \)-helices and 15.4% \( \beta \)-helices), and 19.2% \( \beta \)-strands. Remarkably, the few secondary structure elements present are distributed in a nonuniformly fashion on one side of the molecule, which in the following will be referred to as the "motif-rich" side (Fig. 1A). The pFGE monomer is of almost ellipsoid shape (50 × 30 × 30 Å) and compact with two larger protrusions, one at Asn-191, where the trisaccharide is attached ("sugar side"), and a bulge formed by \( \beta \)-helix \( \eta \) on the opposing side of the molecule (Fig. 1A).

The crystal structure of pFGE reveals a novel fold with an interesting partitioning of the molecule in two halves, distinguishable by the amount of secondary structure elements they contain (see above). Thus, it was not surprising that no structures with a significant homology were found when performing a search using DALI (36). The structurally closest protein is a fragment of a sugar-binding protein, the C-type carbohydrate recognition domain (Crd4) from the macrophage mannose receptor (Legg-A) with a DALI score of 5.5 (a score of ~2.5 is structurally dissimilar, whereas the self-comparison score of pFGE is 51). However, this homology extends over only 98 residues (of a total of 268) and shares a sequence identity of 8%. Additionally these homologous 98 residues are dispersed over the complete pFGE as well as the Crd4 domain, which comprises 132 residues. The low DALI score and arrangement of the structurally similar arranged residues prompted us to conclude that pFGE does not share any structural homology with known proteins.

**The FGE Fold as a Paradigm for the DUF323 Domain**—The unusual paucity of secondary structure elements and their uneven distribution within the molecule are the main hallmarks of this new fold that has paradigmatic character for 300 other proteins. The pFGE and FGE structures represent the first three-dimensional models of a DUF323/COG1262 domain-containing protein. Due to the close relationship of pFGE to the functionally well characterized FGE, the novel fold of the DUF323 domain is denoted "FGE fold." Sequence comparison of all known DUF323 domains suggests that all of them adopt the FGE fold (Fig. 2B).

The manifold functions associated with the DUF323 domain, such as oxygenase, transcriptional regulator, or as an enhancer of xylanase production (see the Introduction), indicate that this fold harbors an extremely versatile set of functions. This raises the question of how the fold of the DUF323 domain can fulfill this multitude of functions. Examples in the data base showing that the DUF323/COG1262 domain is found in combination with other domains, like a serine/threonine kinase, a peptidase, or a chaperonin domain (see the Introduction), suggest that it has a predominant function in polypeptide binding/modification. The few biochemical data that are available on these proteins are insufficient to point to a common active site pres-
ent in all FGE fold proteins. Thus, further biochemical and structural data are needed to clarify this question.

**Fold-determining and Structure-stabilizing Features of pFGE**—The tertiary structure of pFGE is stabilized by a conserved disulfide bond formed by the two cysteines present in the molecule (Fig. 3A). The bridge between Cys-156 located in helix 4 to the Cys-290 in close proximity to the last β-strand (β-11) arrests this β-strand in close proximity to β-strand 4 to become part of the same β-sheet (Fig. 2A). This disulfide bridge is also conserved in FGE, underscoring its stabilizing function. Interestingly, FGE has 6 additional cysteines, 4 of which form two additional disulfide bridges, and the remaining 2 cysteines play a role in catalytic activity (13, 38, 40).

Two metal binding sites could clearly be identified in the electron density maps of pFGE. Both metal ions are buried in the core of pFGE and display low B-values, indicative of their structure-stabilizing function. As the presence of calcium cations is required for crystallization, and the endoplasmic reticulum, where pFGE is naturally localized, contains elevated calcium cation concentrations, calcium cations must be the natural metal ion in pFGE. Calcium binding site 1 (Fig. 3C) is located in the center of a bipyramidal arrangement with the corners of the pyramid base formed by the carbonylate group of the side chain of Asn-194, the Asp-208 side chain carbonylate group, and two water molecules located in 2.3-2.6 Å distance to the calcium cation. Asp-204 coordinates these two water molecules, and its side chain conformation depends on a sharp kink in the polypeptide chain due to cis-Pro-201 (Fig. 3C). The tips of the bipyramid are located off center with respect to the pyramid base and are occupied by the carbonyl groups of Leu-195 and Phe-210 in a distance of 2.3 Å to the calcium cation (Fig. 3C). A much more irregular coordination is observed for the other calcium site. The coordination of the calcium cation 2 (Fig. 3B) is achieved by the carbonyl groups of Asp-229, Gly-232, Val-234, as well as the side chain of Glu-236, showing comparable distances between 2.5 and 2.9 Å. The calcium cation is shifted from the central plane of the bipyramid toward the tip occupied by Leu-230. The second tip is harboring a water molecule (Fig. 3B). The distances from the calcium cation to the pyramid tips are similar, 2.5 Å to the water molecule and 2.6 Å to the Leu-230 (Fig. 3B). Only Leu-230 is off center when compared with the axis defined by the water molecule and the calcium cation. The carbonyl group of Leu-230 is oriented almost perpendicular to this axis, which is in contrast to the other residues involved in the coordination of both calcium cations. The sequence comparison of DUF323 domains reveals that the two calcium cation binding sites are conserved, but the cis-proline and the disulfide bridge in pFGE are predominantly found in those of mammalian origin (Fig. 2A).

**Peptide Binding and Dimer Formation**—It has been shown that human pFGE lacks formylglycine-generating activity (39). Nevertheless, the function of pFGE is likely to be related to FGE function for four reasons. (i) The comparison of expression levels revealed an equal pFGE/FGE ratio in all tissue types studied; (ii) the compromising effect of pFGE on FGE mediated generation of catalytically active sulfatases in coexpression studies; (iii) pFGE has been shown to bind peptides bearing the recognition motif required for the generation of FGly in arylsulfatase A in vitro; and (iv) the three-dimensional structures of pFGE and FGE are closely related (39, 40).

The active site of FGE has recently been identified by the analysis of the FGE crystal structure (40) and confirmed by chemical cross-linking studies (38), Pro-182 of FGE, which was shown to chemically cross-link with the bound substrate related peptide, is structurally conserved in pFGE (Pro-120). These prolines border one end of a cleft thought to be involved in peptide binding (Fig. 4A, Pro-120 in blue). This cleft is
108 van der Waals interactions, dimer, the monomers interact by three H-bonds, one salt bridge, and highlighted in representation with the residues involved in formation of the pocket to the C terminus in second pFGE of the homodimer is colored in red. The loop in pFGE (indicated by an arrow) is missing in FGE, as it has been removed by limited proteolysis prior to crystallization. A compelling notion with respect to the function of pFGE and FGE, the formation of a heterodimer appears also to be possible. A model of the pFGE/FGE heterodimer, obtained by superimposing the FGE structure on a monomer of the pFGE dimer, reveals heterodimer formation to be stereochemically feasible (Fig. 5B). The low root mean square deviation value of 1.3 Å for 249 common Ca atoms of pFGE and FGE indicates that the proposed heterodimer could form, exhibiting a similar interaction surface and cavity size. The heterodimer formation would enhance binding of FGE to the substrate polypeptide and form a protected reaction environment. In line, a channel remains at the heterodimer interface that would still be able to accommodate the unfolded polypeptide chain of the sulfatases. The formation of a heterodimer would explain the observation of a decrease in FGE activity when pFGE is overexpressed in cells and also the constant expression ratio of endogenous mRNA levels found across different tissues (39). Indeed, using the yeast two-hybrid system, co-expression of pFGE and FGE led to the induction of three reporter genes, suggesting a physical interaction of pFGE and FGE (39). Furthermore, for FGE, homodimers and larger complexes with a sulfatase could be identified (38, 39). With respect to the structures, pFGE and FGE have the capability to form homo- and heterodimers, respectively, although this oligomerization might only occur upon binding to the unfolded polypeptide chain of the sulfatases. The formation of a heterodimer would explain the observation of a decrease in FGE activity when pFGE is overexpressed in cells and also the constant expression ratio of endogenous mRNA levels found across different tissues (39). Indeed, using the yeast two-hybrid system, co-expression of pFGE and FGE led to the induction of three reporter genes, suggesting a physical interaction of pFGE and FGE (39). Furthermore, for FGE, homodimers and larger complexes with a sulfatase could be identified (38, 39). With respect to the structures, pFGE and FGE have the capability to form homo- and heterodimers, respectively, although this oligomerization might only occur upon binding to the unfolded polypeptide chain of the sulfatase substrates.

Conclusion—The crystal structures of pFGE and FGE represent the first three-dimensional structures of the DUF323 domain, which therefore was termed the FGE fold. Due to the high sequence similarity within the proteins belonging to the DUF323 family, we can expect that all DUF323 domains adopt the FGE fold. Despite the fact that pFGE exhibits no FGly-generating activity, it is expressed in an equal pFGE/FGE ratio in many tissue types, indicating an important cellular function. Interestingly, pFGE crystallizes as a dimer, with the peptide binding site buried between the monomers, indicating a biological significance of dimerization. In addition, the formation of a pFGE/FGE heterodimer appears to be possible, pointing toward a function of pFGE in assisting or regulating FGE activity.

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