

Article

Structural and Biophysical Analyses of Human Prostamide/Prostaglandin F Synthase with Two Active Form-Mimicking Mutations

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Abstract

Human prostamide/prostaglandin F synthase (PGFS) catalyzes the NADPH-dependent conversion of prostaglandin H₂ (PGH₂) to prostaglandin F₂α that plays a key role in regulating intraocular pressure and labor. Despite its physiological importance, structural and biochemical information of the human PGFS has been limited because of difficulties in obtaining sufficient quality of PGFS wild-type crystal and short half-life of PGH₂. Here, we report the crystal structure of human PGFS with two active site mutations, C44S/C47S double mutant (DM), which mimics the reduced active form of the CXXC motif of human PGFS. Structural analysis revealed that PGFS DM adopts a typical thioredoxin (Trx)-like fold. Analysis of B-factors and MD simulations reveals that Tyr108–Asp124 is an intrinsically flexible region, devoid of any stabilizing crystal contacts. Unlike canonical Trx-like proteins, Pro167 in PGFS adopts a trans-conformation, inducing a specific Arg40 side chain localization that creates a positive charge near the CXXC motif. Activation of PGFS by reduction of disulfide bond in the CXXC motif enhanced the thermal stability via core stabilization, yet an unexpected increase in the structural disorder was detected with CD spectroscopy, especially upon ligand binding. These findings collectively establish PGFS as a structurally distinct and redox-regulated enzyme. Our results provide novel molecular insights into PGFS as an underexplored but promising therapeutic target.

Keywords: prostamide/prostaglandin F synthase; CXXC motif; prostaglandin F₂α; thioredoxin fold-containing protein



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1. Introduction

Prostamide/prostaglandin F synthase (PGFS) generates prostamide F₂ and prostaglandin F₂α (PGF₂α) from prostamide H₂ and prostaglandin H₂ (PGH₂), respectively, using NADPH as a proton donor [1–3]. PGFS is also known as family 213 Member B (FAM213B) or Peroxiredoxin-like 2B (PRXL2B), indicating its antioxidant properties. The antioxidant activity of PGFS is attributed to the CXXC motif, which is also frequently found in the thioredoxin (Trx) family, where the cysteine thiol-disulfide link is involved in redox

processes as an oxidoreductase [4–7]. In the case of PGFS, the generated free cysteine residues reduce the cyclic endoperoxide of PGH₂, thereby producing PGF₂α (Figure 1) [1].

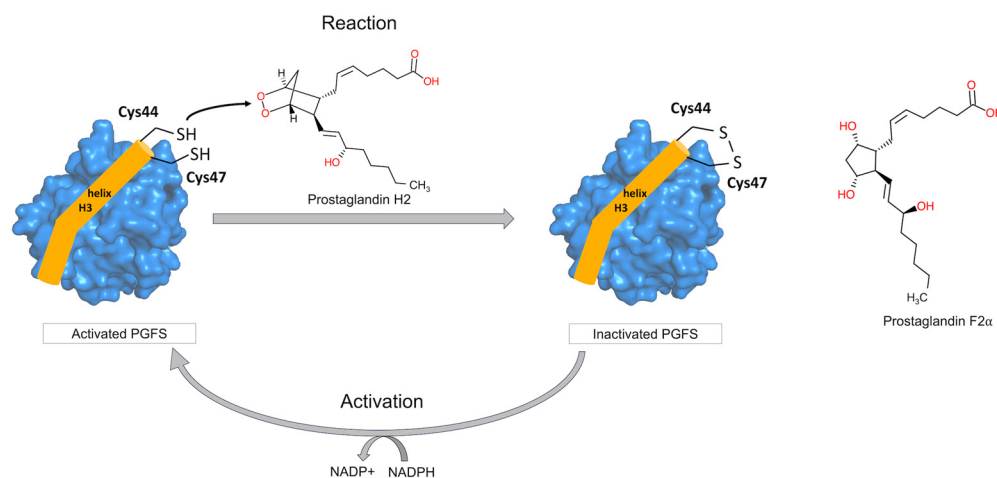


Figure 1. Catalytic mechanism of human prostamide/prostaglandin F synthase (PGFS). PGFS contains two cysteine residues (Cys44 and Cys47) in its active form (reduced state) that constitute the conserved CXXC motif. These residues play a key role in the conversion of prostaglandin H₂ (PGH₂) into prostaglandin F₂α (PGF₂α). During catalysis, PGH₂ is reduced to form PGF₂α, resulting in the formation of disulfide bond between Cys44 and Cys45 of PGFS (oxidated or inactive state). NADPH acts as a cofactor for PGFS by reducing disulfide bond of the CXXC motif, thereby maintaining the enzyme in its catalytically active state.

PGF₂α, one of the earliest identified prostaglandins, is widely produced in mammals and plays diverse physiological roles in humans [8–10]. PGF₂α exerts its biological effects by binding to the prostaglandin F receptor (PTGFR), which activates IP₃-mediated signaling and increases intracellular Ca²⁺ levels. This Ca²⁺ elevation subsequently induces smooth muscle contraction [11]. In particular, PGF₂α stimulates uterine contraction to initiate labor [12], promotes luteolysis in the ovary, and lowers intraocular pressure by facilitating aqueous humor outflow [13]. Accordingly, synthetic analogs of PGF₂α are clinically used to manage intraocular pressure, while native PGF₂α is medically applied to induce labor. Beyond its clinical applications, PGF₂α has been extensively investigated for many years due to its potential functions in the brain and various other organs [14,15]. These studies highlight their importance as a key endogenous molecule in human physiology.

Moreover, previous studies on the highly conserved regions of Trx have elucidated the essential function of a cis-proline residue adjacent to the CXXC motif [16]. Research showed that most conserved prolines are located close to the CXXC motif and are thought to participate in structural stabilization or substrate release [16–19]. Furthermore, research has shown that the proline-minus-one (P-1) residue located next to the conserved proline residue has a significant function. Due to the lack of conservation at this site, the P-1 residue is composed of a wide range of amino acids. Although the hydrophilicity varies, structural analyses have revealed that the side chain and backbone carbonyl group of the P-1 residue are located within 3–4 Å of the disulfide bond in the CXXC motif. In addition, comparative analyses of the oxidoreductase activity between various P-1 mutants and the wild-type (WT) protein revealed differences in enzymatic performance [16].

Numerous proteins with Trx folds have been identified, and numerous studies have been conducted to investigate their structural features. Nonetheless, the structure of the PGFS has remained unresolved, and its characteristics have not been thoroughly examined. Herein, we report the crystal structure of human PGFS and compare it with other proteins containing a Trx-like fold. In addition, in silico analyses and various spectroscopic

experiments revealed a novel feature of recognizing substrate with a disordered region, which has not been discovered in the other thioredoxin family proteins.

2. Materials and Methods

2.1. Cloning and Protein Expression

The cDNA for human PGFS WT was acquired from the Korean Human Gene Bank. The sequence of human PGFS was amplified by PCR and inserted into the pET28a vector. The recombinant vector served as a template for the creation of the C44S C47S PGFS mutant (PGSF DM), which was produced using a site-directed mutagenesis method. The cloned vector was validated through sequencing and subsequently transformed into SoluBL21 competent cells. The transformed cells were incubated at 37 °C in Luria-Broth medium until the optical density at 600 nm (OD₆₀₀) reached 0.6–0.8, followed by induction with 0.5 mM IPTG (1-thio-β-D-galactopyranoside) for 18–21 h at 20 °C. The induced cells were harvested by centrifugation at 6000× *g* for 10 min at 4 °C and sonicated after adding buffer A (20 mM Tris-HCl pH 7.5, 500 mM NaCl, and 35 mM imidazole) and 1 mM PMSF (phenylmethylsulfonyl fluoride). The lysate was centrifuged at 30,000× *g* for 1 h at 4 °C to eliminate cellular debris. The supernatant obtained from centrifugation was filtered through a 0.45 μm syringe filter, and the resulting filtrate was applied to a Ni²⁺ charged HiTrap chelating HP column (GE Healthcare, Chicago, IL, USA). The protein-loaded column was equilibrated with buffer A, and the bound PGFS was eluted using buffer B (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 M imidazole) with a linear gradient. The bound PGFS was eluted with 150–250 mM imidazole, and the eluent was subsequently purified using ion exchange chromatography using HiTrap Q HP (GE Healthcare, Chicago, IL, USA). Ion exchange chromatography (IEC) was performed using buffer C (20 mM Tris-HCl pH 7.1, 100 mM NaCl) for column loading, followed by elution of the bound protein with buffer D (20 mM Tris-HCl pH 7.1, 1 M NaCl) through a steady increase in the proportion of buffer D. The eluted fractions were finally purified using size-exclusion chromatography using HiLoad 16/600 Superdex 200 pg (GE Healthcare, Chicago, IL, USA), pre-equilibrated with a storage buffer consisting of 20 mM MES at pH 6.5, 200 mM NaCl, and 5 mM 1,4-dithiothreitol (DTT). Each step of purification was validated by SDS-PAGE analysis of the fractions. Consequently, WT and double mutant PGFS were highly purified with the same protocol as above.

2.2. Crystallization

The crystallization conditions for PGFS DM were screened with commercial screening kits (Hampton Research, Aliso Viejo, CA, USA). Crystals for PGFS DM were formed at 0.2 M ammonium sulfate and 20% (*w/v*) polyethylene glycol 3350 (PEG 3350) and incubated at 22 °C with vapor diffusion methods, both the sitting drop method and the hanging drop method.

2.3. X-Ray Diffraction Data Collection, Phasing, and Refinement

Prior to collecting diffraction data, all crystals were cryoprotected with 20% (*w/v*) glycerol adding to reservoir solution and flash-frozen into liquid nitrogen. The X-ray diffraction data of PGFS DM was collected on the BL-11C beamline at the Pohang Accelerator Laboratory, Pohang, Republic of Korea. The collected data were indexed, integrated, and scaled using the HKL2000 package (v.721.3) [20]. The PGFS DM crystal belongs to the P₃121 space group with unit cell parameters of *a* = *b* = 55.2 Å and *c* = 107.7 Å with one asymmetric unit. Statistics of all X-ray diffraction data are summarized in Table 1. For the PGFS DM datasets, 5% of the total data were randomly excluded to serve as the *R*_{free} set, and the remaining data were employed to calculate *R*_{work}. The phasing problem for PGFS

DM was solved by creating a template from sequence-similar proteins using MrBump from the CCP4i suite (v8.0.019) and Phaser in the Phenix suite (v1.21.2), then refining the primary model manually by fitting it into the electron density map using COOT (v0.9.8.93) [21,22]. The manually refined model is then further refined with Phenix until the resulting structure satisfies validation criteria from MolProbity (v4.5.2) [23]. The statistics of the refinement are summarized in Table 1.

Table 1. Crystallographic data collection and refinement statistics.

Data Collection	PGFS DM (C44S/C47S)
Beam line	PAL-7A
Wavelength (Å)	0.97933
Space group	P3 ₁ 21
a, b, c (Å)	55.2, 55.2, 107.7
α, β, γ (°)	90.0, 90.0, 120.0
Resolution (Å) ^a	28.69–2.30 (2.37–2.30)
Unique reflections	8662 (705)
Completeness	90.02 (81.69)
I/σ (I)	19.18 (4.49)
Redundancy	12.4 (10.9)
R _{meas} (%) ^b	15.1 (82.0)
R _{pim} (%)	4.2 (24.2)
CC _{1/2}	99.8 (91.7)
R _{work} /R _{free} (%) ^c	21.19/24.93
Bond lengths (Å)	0.003
Bond angles (°)	0.577
Protein	1327
Water	86
Average B-factor (Å ²)	35.58
Protein	35.40
Water	38.18
Favored/Outliers (%)	99.37/0.00
Poor rotamers (%)	0.00
PDB codes	9USA

^a Values in parentheses refer to the highest resolution shell. ^b $R_{meas} = \frac{\sum_{hkl} \left(\frac{N_{hkl}}{N_{hkl}-1} \sum_{i=1}^{N_{hkl}} |I_{hkl,i} - \langle I_{hkl} \rangle| \right)}{\sum_{hkl} \sum_{i=1}^{N_{hkl}} I_{hkl,i}}$, where I_{hkl} is the intensity of reflection h, and $\sum_{hkl}$ is the sum over all reflections. ^c $R_{work} = \frac{\sum_{hkl \in work} ||F_{obs}(hkl)| - |F_{calc}(hkl)||}{\sum_{hkl \in work} |F_{obs}(hkl)|}$, $R_{free} = \frac{\sum_{hkl \in free} ||F_{obs}(hkl)| - |F_{calc}(hkl)||}{\sum_{hkl \in free} |F_{obs}(hkl)|}$.

2.4. Docking Simulation

Docking simulations were performed using the crystal structure of the PGFS double mutant (DM) deposited in the Protein Data Bank (PDB ID: 9USA). The protein structure was prepared using AutoDockTools by removing water molecules, adding polar hydrogens, and assigning Kollman charges to the entire protein [24]. The coordinate file of PGH₂ was also prepared with AutoDockTools by adding polar hydrogens and assigning Gasteiger charges. The docking grid was defined around the CXXC motif with dimensions of 20 × 20 × 20 Å, and the X, Y, and Z coordinates were specified accordingly. Docking calculations were conducted using AutoDock Vina version 1.1.2 with the prepared input files [25]. MM/GBSA calculations were performed using the MMPBSA.py script, employing the generalized Born (GB) implicit solvent model to estimate polar solvation energies after secondary energy minimization of the PGH₂ docked pose using GROMACS (v2025.4) (Table S2) [26].

2.5. Molecular Dynamics Simulations

The MD simulations were implemented using the Desmond simulation package distributed by Schrodinger LLC (New York, NY, USA). All simulations were carried out using the OPLS4 force field [27]. The refined PGFS DM structure was prepared by using the Protein Preparation Workflow (PDB ID: 9USA) [28]. The prepared protein was solvated in a TIP4P water box, and the size of the box was minimized properly. After solvation, the simulation box was neutralized and supplemented with NaCl to a final concentration of 150 mM, corresponding to physiological ionic strength (pH 7.4). Energy minimization steps were conducted before simulations for 20 ns separately. Each system was simulated in three independent runs, each with a duration of 200 ns, under NPT ensemble conditions at 300 K and 1 bar.

2.6. Protein Thermal Stability Using DSF

Protein thermal stability was assessed using Differential Scanning Fluorimetry (DSF), and measurement was performed on the QuantStudio™ real-time PCR (ThermoFisher Scientific, Waltham, MA, USA). The concentration of PGFS samples was optimized to 10 µM in the reaction buffer (10 mM phosphate buffer, pH 7.5, 50 mM NaCl). Protein Thermal Shift™ dye used for the measurements was added at 1:1000 dilution from a concentrated stock solution. The reaction mixture was transferred into a black 384-well plate, ensuring minimal air bubbles to avoid inconsistencies in fluorescence detection. To compare the thermal stability of PGFS WT, PGFS DM, and their reduced forms, samples were prepared by adding 5 mM of either DTT or TCEP as reducing agents. The samples were scanned from 25 °C to 90 °C at a rate of 0.05 °C per second, and all measurements were repeated five times. Data were normalized for protein concentration and baseline corrected using QuantStudio™ real-time PCR software 1.3 (ThermoFisher Scientific). The melting temperature (T_m) of the protein, which is a midpoint of the protein's unfolding transition, was calculated from the fluorescence data using the Boltzmann equation.

2.7. Circular Dichroism

PGFS WT and DM were individually purified using size-exclusion chromatography in reaction buffer composed of 10 mM phosphate buffer (pH 7.5) and 50 mM NaCl. To prepare reduced WT, purified PGFS WT was incubated in reaction buffer supplemented with 0.5 mM TCEP for 1 hour at room temperature, followed by re-purification using the reaction buffer and size-exclusion chromatography. All PGFS samples were adjusted to a final protein concentration of 10 µM using the reaction buffer. To examine the PGFS–ligand interaction, PGH2 was added to DM PGFS at a molar ratio of 1:10 and incubated for 10 min at room temperature prior to data collection. The final DMSO concentration was maintained at 0.1% to minimize any solvent-related effects. To further reduce PGH2-derived background signals, baseline correction was performed by subtracting data collected from PGH2 alone under identical conditions. Circular dichroism (CD) spectra were acquired using a 1 mm pathlength quartz cuvette (Hellma Analytics, Müllheim, Germany) on a Chirascan Plus spectrometer (Applied Photophysics Ltd., Leatherhead, UK). Measurements were performed at 25 °C across a wavelength range of 180–260 nm, using a step size of 1 nm. All measurements were repeated three times separately and averaged. Data processing was performed using the DichroWeb server [29], and secondary structure content was estimated based on the SMP180 reference dataset.

2.8. Ellman's Assay

Highly purified PGFS WT, reduced WT, and DM—prepared using the same protocol as above—were used to quantify free cysteine residues. All protein samples were adjusted

to 5 μM in reaction buffer. Measurements were performed five times using a SpectraMax ID3 multi-mode microplate reader (Molecular Devices Ltd., San Jose, CA, USA) to ensure reproducibility. Free cysteine content was determined from a calibration curve generated with L-cysteine, which showed a high linearity ($R^2 \approx 1$).

2.9. Intrinsic Tryptophan Fluorescence Spectroscopy

Purified PGFS WT, reduced WT, and DM were prepared for intrinsic tryptophan fluorescence analysis. The concentration of each sample was adjusted to 5 μM using the reaction buffer. Tryptophan excitation was performed at 280 nm, and fluorescence emission was measured at 330 nm and 350 nm, with three replicates collected at each wavelength. To minimize background noise from the buffer, measurements were also taken five times using the buffer alone, and the averaged buffer signal was subtracted from each protein sample measurement. For comparative analysis, fluorescence intensity at 350 nm was divided by the intensity at 330 nm to assess the relative tryptophan environment in WT, reduced WT, and DM samples.

3. Results

3.1. Overall Structure and Hydrophobic Core-Mediated Stabilization of the Human PGFS C44S/C47S Double Mutant (DM)

To gain insight into the function of human PGFS, we determined the crystal structure of the human PGFS double mutant (DM) at a resolution of 2.3 Å. Despite extensive efforts, the crystallization of PGFS wild-type (WT) proved to be extremely challenging. While PGFS WT structure was resolved at 3.38 Å resolution, the limited resolution and crystal quality precluded its use as a reliable model for structural comparison (Figure S1, Table S1). PGFS DM comprises seven α -helices and seven β -strands (Figure 2A). Five of these β -strands form an antiparallel β -sheet, creating a hydrophobic core surrounded by seven α -helices arranged in a sandwich-like manner (Figure 2A,B). These structural features support the classification of PGFS as a thioredoxin-like protein. Structural similarity searches using the DALI server [28] also showed that PGFS is structurally similar to the thioredoxin or peroxiredoxin family proteins, and two bacterioferritin comigratory protein-related proteins in the peroxiredoxin family are most structurally similar to PGFS with the highest Z scores of 17.3 and 15.3 (Table S2) [30].

The stability of Helix 3 (H3) in PGFS is mainly sustained by interactions within a hydrophobic core involving β -strands 3 and 6 ($\beta 3$ and $\beta 6$), loops adjacent to the CXXC motif, and helix 4 (H4) (Figure 2C). Structurally, both ends of H3 are stabilized by interactions with Leu68 in $\beta 4$. The N-terminus of H3, where the active site is located, is stabilized through hydrophobic effects centered around Leu39 (Figure 2C). Specifically, Phe42 and Phe81 form a π - π interaction at a distance of 3.7 Å, and Leu91 engages in CH- π interactions with Phe42 and Phe81 at distances of 4.4 Å and 3.8 Å, respectively (Figure 2C). In addition, Phe42, Phe81, and Leu91 interact with Leu39 at distances of 4.4 Å, 3.8 Å, and 4.5 Å, respectively, and Ile50 from H3 interacts with Leu39 at a distance of 4.6 Å, further stabilizing the N-terminal region of H3 (Figure 2C). Notably, Val45 and Val46 residues in the CXXC motif appear distant from the hydrophobic core and thus do not noticeably influence the stability of the active site (Figure 2C). The C-terminal end of H3 is stabilized through hydrophobic effects involving Val35, Leu54, Leu68, Leu91, and Leu148, which are all within a 5 Å range. Hydrophobic effects maintained by Leu91 with Leu68 and Val35 at distances of 4.6 Å and 4.9 Å, respectively, result in a distinct helix kink within H3 at 128.4° (Figure 2C). On the opposite side relative to the β -sheet, Val36 from β -strand 3 serves as a central residue mediating hydrophobic effects. Surrounding residues, including Leu25, Trp29, Val69, Val71, Leu99, and Leu149, cluster within 4–5 Å, reinforcing this hydrophobic

core (Figure 2D). Leu25 in H2 is located at distances of 4.9 Å, 4.7 Å, and 3.9 Å from Val 36, Val69, and Leu149 in the central β -sheet, respectively (Figure 2D). Leu99 in H5 is located 3.9 Å from Leu25, while Leu25, Val36, and Val69 are also located each other within a distance of 4.7 Å (Figure 2D). The unique tryptophan located in hydrophobic environment in PGFS is Trp29, which is also located near Leu25 and Leu149 at distances of 3.6 and 4.6 Å, respectively (Figure 2D).

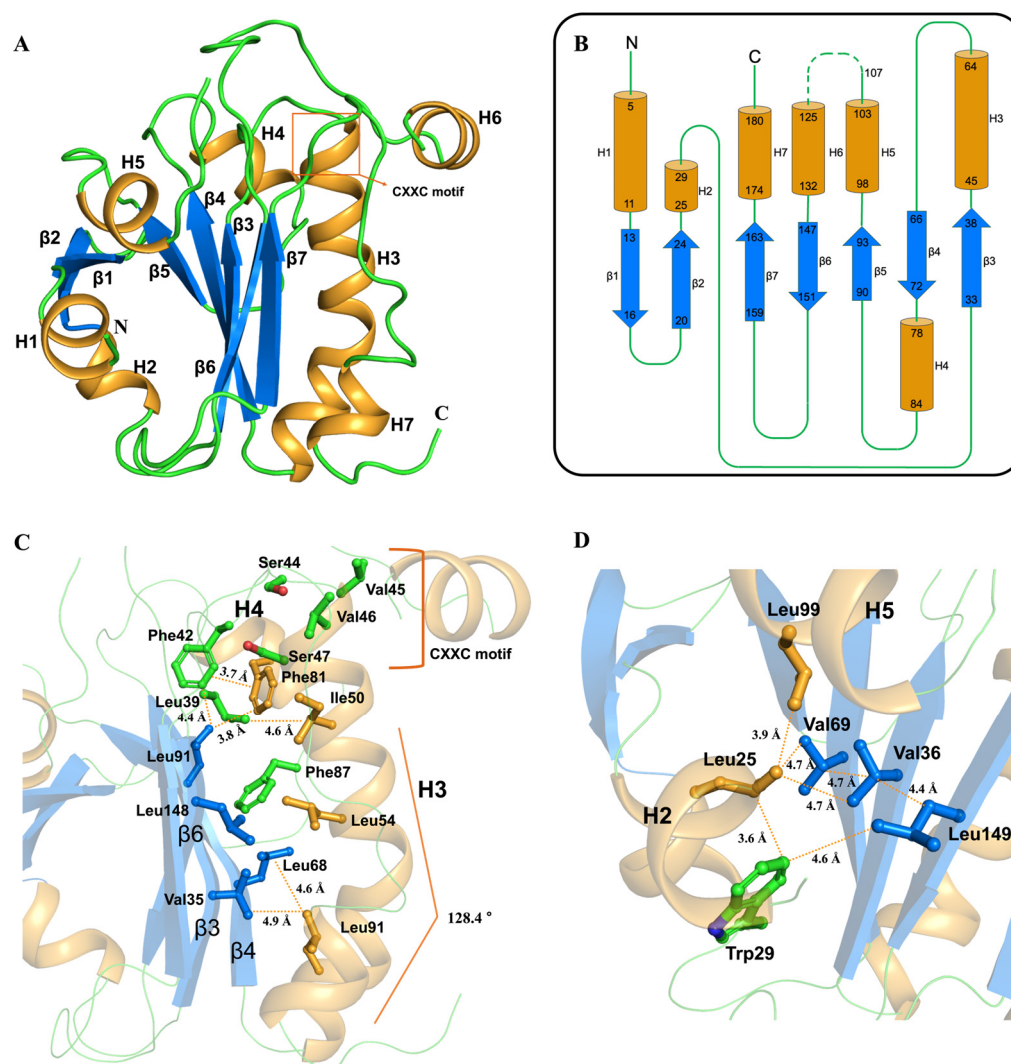


Figure 2. Overall structure of PGFS C44S/C47S double mutant (DM) with topology diagram and detailed features of the hydrophobic core surrounding the active site. (A) Overall structure of PGFS. PGFS is shown in a cartoon with marine β -sheets, light orange α -helices, and green loops. (B) Topology diagram of PGFS highlighting its characteristic sandwich-like fold. The color of secondary structures is applied as in A. The secondary structure analysis results were adopted from the ESPript 3.0 web-server [31]. (C) Structural view of the hydrophobic core that stabilizes helix H3, which harbors the CXXC motif. (D) Residues contributing to the hydrophobic core centered on the β -sheet that is opposite to H3 are shown in stick and colored by the secondary-structure element to which they belong.

3.2. Symmetric Homodimeric Assembly of Human PGFS DM and the Key Role of the C-Terminal Region of Helix 3

Human PGFS was determined to exist as a homodimer based on size-exclusion chromatography (SEC) analysis. The theoretical molecular weight of PGFS is 21.22 kDa and both PGFS WT and DM were eluted at the same volume corresponding to 46.55 kDa

(Figures 3A and S2). Structurally, H3 plays a critical role in dimer formation. X-ray crystallography revealed the presence of monomeric PGFS within each asymmetric unit. The kinked C-terminal region of H3 mediates dimerization through direct interactions between adjacent asymmetric units, as confirmed by the electron density maps illustrating the dimerization interface (Figure S3). These interactions occur symmetrically around the central axis between the monomers, demonstrating point symmetry (Figure 3B).

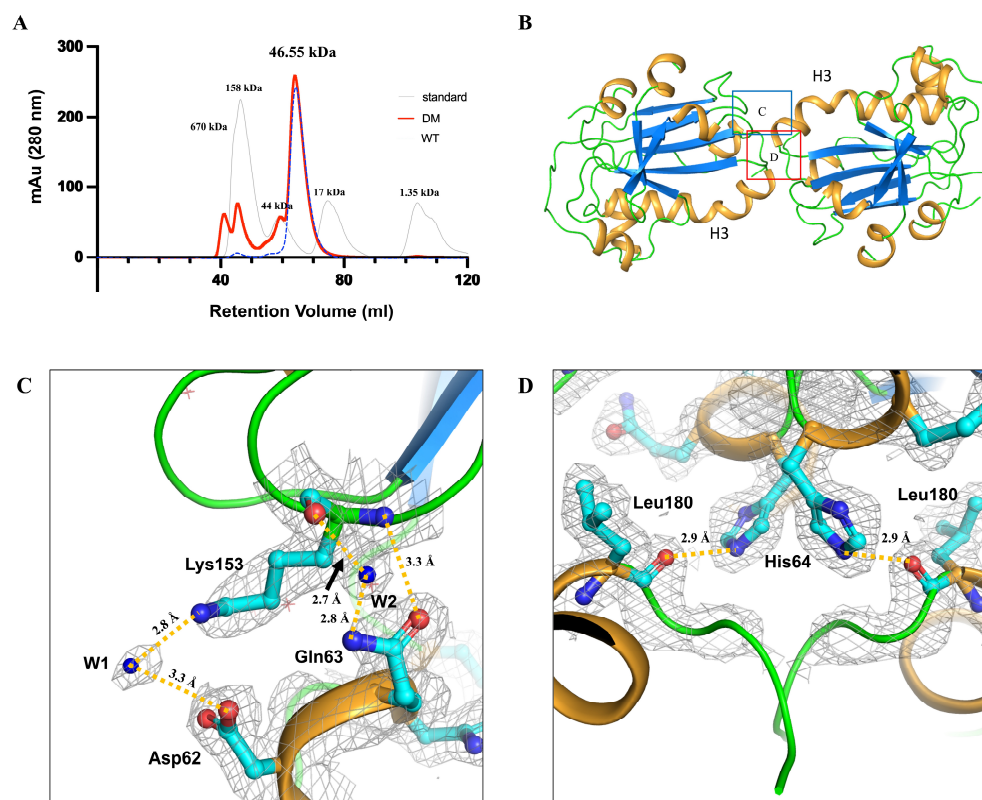


Figure 3. Dimerization of human PGFS DM and structural features of the dimeric interface. **(A)** Size-exclusion chromatography (SEC) profile of human PGFS C44S/C47S DM, wild-type (WT), and standard. The SEC profiles of PGFS DM and PGFS WT, and standard are represented by red line, blue dashed lines, and light gray line, respectively. Bio-Rad gel filtration standard (catalog number 1511901) was used to generate calibration curve containing bovine thyroglobulin (670 kDa), bovine γ -globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B12 (1.35 kDa) **(B)** Dimeric structure of human PGFS. PGFS is shown in a cartoon with marine β -sheets, light orange α -helices, and green loops. The twofold symmetry is centered at the kinked helix H3. The regions corresponding to **(C,D)** are highlighted in **(B)** with a blue box and a red box, respectively. **(C,D)** Residues forming the dimeric interface and their intermolecular interactions are shown. Interface-forming residues are depicted as cyan sticks, while water molecules contributing to the interface are labeled as W1 and W2. Intermolecular interactions are indicated by dark yellow dashed lines.

The residues involved in the dimer interface are Asp62, Gln63, and His64 located on the C-terminus of H3, and Lys153 and Leu180 located on helix 7 (Figure 3C,D). The β -carboxyl group of Asp62 forms a water-mediated salt bridge with Lys153 via a single water molecule (W1) positioned at approximately 3 Å (Figure 3C). Additionally, another water molecule (W2) mediates hydrogen bonding between the backbone carbonyl of Lys153 and the side-chain amine group of Gln63 at distances of 2.7 Å and 2.8 Å, respectively (Figure 3C). Furthermore, the side-chain carboxyl group of Gln63 forms a direct hydrogen bond with the backbone amide of Lys153 at approximately 3.3 Å (Figure 3C). These interactions symmetrically stabilize both ends of the dimer interface. Moreover, the imidazole ring of

His64 forms a hydrogen bond with the backbone carbonyl group of Leu180 at 2.9 Å, and the interaction also symmetrically is located at the core of the interface (Figure 3D).

3.3. Structural and Functional Insights into the CXXC Motif Including Conservation, Active-Site Electrostatic Environment, Substrate Accessibility, and Loop Disorder in Human PGFS

In PGFS, Cys44, Val45, Val46, and Cys47 constitute the conserved CXXC motifs. PSI-BLAST (v2.15.0) analysis of 500 sequences retrieved from BLAST results revealed that the XX dipeptide in the CXXC motif is highly variable across species [32], frequently differing from the two Val residues found in PGFS. Specifically, the first position of the CXXC motif predominantly appeared as Gln (32.5%), followed by Val (26.7%) (Figure S4).

Interestingly, when we mutated residues Cys44/Cys47 of PGFS to Ser44/Ser47, designed to mimic the reduced form of the active-site CVVC residues, the SVVS region exhibited a partially positive charge in their vicinity (Figure 4A). Electron density maps confirmed the stable localization of Arg40 and Arg41, explaining the positive charge observed in this region (Figure 4B). Ser47 formed a bifurcated hydrogen bond with the backbone of Arg40 at distances of 3.5 Å and 3.0 Å, which contributed to stabilizing the position of Arg40 (Figure 4B). Furthermore, docking analyses demonstrated that the peroxide moiety of PGH2 could approach sufficiently close to Ser44 (which mimics the reduced catalytic Cys44) to enable catalytic reduction (Figure 4C). Arg41, located 3.1 Å from the peroxide, likely plays a significant role in facilitating peroxide approach. Ser44 is located at a distance of 3.3 Å from the peroxide moiety of PGH2, which seems to be optimal for facilitating the reduction reaction between thiolate of Cys44 and the peroxide of PGH2 (Figure 4C).

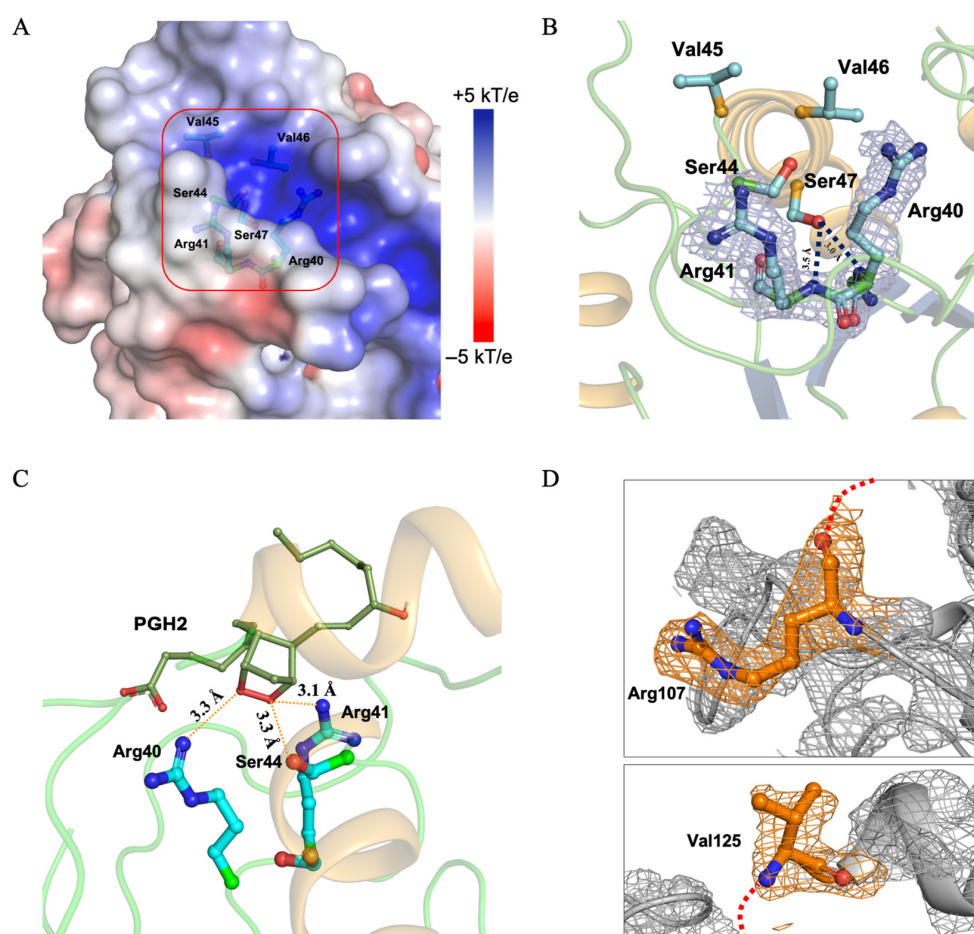


Figure 4. Structural features of the PGFS DM active site and surrounding residues (A) Electrostatic surface potential around the active site CXXC motif. Electrostatic surface potentials calculated using

APBS are shown, with colors ranging from +5 kT/e (blue) to −5 kT/e (red). The CXXC motif is highlighted by a red box. (B) Arg40 and Arg41 at the active site. Arg40 and Arg41 are shown with the 2Fo–Fc electron density map contoured at 1.0 σ (light gray). Hydrogen bonds between the backbone atoms of the arginines and the functional group of Ser47 are indicated by black dashed lines. (C) Docking model of PGFS with PGH2 generated using AutoDock Vina. PGH2 is depicted as a green line, and the active-site residues, Arg40, Arg41, and Ser44, are shown as cyan sticks. (D) Disordered region defined by the terminal residues Arg107 and Val125. Electron density maps of Arg107 and Val125, which mark the beginning and end of the disordered region, are shown contoured at 1.0 σ for the 2Fo–Fc map (light gray). The missing residues between Arg107 + 1 and Val125 − 1 are indicated by red dashed lines.

The electron densities for residues Tyr108–Asp124 were completely absent and predicted to pass near the CXXC motif (Figure S5A). The region connecting helices 5 and 6 was expected to traverse near the active site (Figure 4D). Arg107 and Val125 exhibited clear and distinct electron densities with sharply defined boundaries. Given the absence of nearby interacting asymmetric units, this observation strongly suggests that the Tyr108–Asp124 residues constitute an intrinsically disordered region (Figure S5B).

3.4. Conserved Residues and Their Role in the Localization of the Proline-Containing Loop

PGFS structure exhibited markedly different positioning of the P-1 residue compared with other thioredoxin-like superfamily proteins. In human PGFS, cis-proline corresponds to Pro167, and P-1 corresponds to Ser166. Structural analysis of PGFS DM revealed that the distance between Ser166 and the CXXC motif is 10.7–11.6 Å, which seems too long for direct functional interaction (Figure 5A). Notably, electron density maps revealed that Pro167, which corresponds to cis-proline in other homologous proteins, adopts a trans-proline conformation in human PGFS DM (Figure 5B). To date, this has only been reported for the Eps1p protein disulfide isomerase [33].

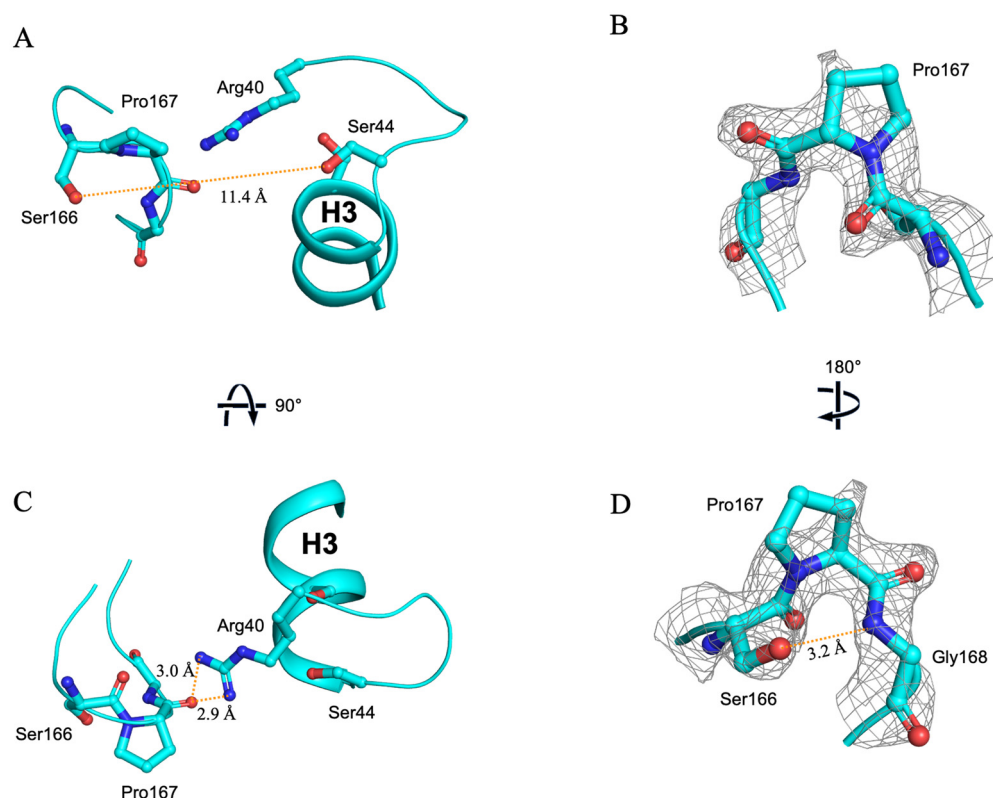


Figure 5. Proline-containing loop and the trans-Pro167 conformation. (A) The distance between the CXXC motif at the N-terminus of helix H3 and the proline-1 residue (Ser166) in the PGFS double mutant

(DM) is shown in cyan, highlighting the spatial relationship between the active-site motif and this residue. (B) The electron-density map for Pro167 is displayed, with carbon atoms colored cyan, oxygen atoms red, and nitrogen atoms blue, confirming the placement of the side chain within the structural model. (C) The localization of the Arg40 side chain is illustrated by its interaction with the backbone of Pro167, emphasizing how this contact contributes to the positioning of Arg40 near the active site. (D) The side chain of Ser166 forms interactions with the backbone between Pro167 and Gly168. The electron-density map surrounding Pro167 further indicates that this residue adopts a trans conformation, which is critical for maintaining the structural integrity of the local loop.

In the active site, the functional group of Arg40 is stabilized by the trans-proline Pro167. The guanidinium group of Arg40 forms hydrogen bonds with the backbone carbonyl group of Pro167 at distances of 2.9 Å and 3.0 Å (Figure 5C). Additionally, Ser166 forms a hydrogen bond with the backbone amide group of Gly168 at 3.2 Å (Figure 5D). The trans conformation of Pro167 likely positions the functional group of Ser166 in a manner consistent with this structure. The hydroxyl group of Ser166 formed a hydrogen bond with Gly168, enabling the backbone carbonyl group of Gly168 to interact with Arg40. BLAST analysis confirmed that Pro167, its surrounding residues, and Arg40, Arg41 at the active site are strictly conserved among most human PGFS-like proteins (Figure S3).

3.5. Prediction of Flexibility Based on Molecular Dynamics (MD) Simulation and B-Factor Comparison, and Structural Comparison with the AlphaFold Model

In the case of our experimentally determined PGFS DM structure, the active site does not form a well-defined substrate-binding pocket, so the structural stability of helix H3 may play a critical role in modulating PGFS enzymatic activity. To investigate the structural dynamics and flexibility of helix H3 and the adjacent disordered segments, a comparative analysis of crystallographic B-factors from the PGFS DM structures was conducted along with an MD simulation. Residues Leu39, Phe42, Leu54, Leu68, Phe81, Leu91, and Leu148 form a hydrophobic core that surrounds and stabilizes helix H3 (Figure 2C). These residues consistently exhibited low backbone RMSF values (~ 0.5 Å) and stable normalized B-factors in MD simulations, supporting their cooperative roles in maintaining the structural integrity of helix H3. The opposing hydrophobic core formed by Leu25, Trp29, Val36, Val71, Leu99, and Leu149 remained stable (Figures 6A and 7B). Notably, Leu25 and Trp29 exhibited relatively higher normalized B-factors (above zero) than the other residues (Figure 6A). The PGFS DM structure exhibited partial disorder in the residues Tyr108–Asp124. Normalized B-factor analysis for the PGFS DM structure was performed to verify this disorder structurally. In the PGFS DM, the Tyr108–Asp124 region was unresolved, and the adjacent residues exhibited similarly high normalized B-factors, supporting the instability of this region (Figure 6A). And the flexible region spanning residues Lys105 to Lys130 exhibited elevated RMSF values (about 1–2 Å) compared to other regions (Figure 6B), indicating increased flexibility. Analyses of residue-wise protein secondary structure over the 200 ns simulations revealed that the Tyr108–Asp124 region does not form α -helices or β -sheet and instead remains as an unstable loop or an intrinsically flexible/disordered-like region (Figure S6). To compare PGFS with structurally related proteins, an AlphaFold (AF) model was generated from AlphaFold 3 using the human PGFS amino acid sequence to identify structural differences [34]. The AF model exhibited a predicted aligned error score of 0.88, indicating the high confidence and quality in the prediction (Figure S7). When compared with the AF model, the DM structure showed strong similarity, with an RMSD value of 0.420 Å (Figure 6C). Although Tyr108–Asp124 residues were not observed in the crystal structure, the AF model predicted this region to have a stable structure with high confidence (Figures 6C and S7). In the AF model, helix 6 (H6) was predicted to be positioned approximately 2.3 Å further outward than the experimentally determined structure, and Pro167 was predicted to be positioned approximately 2.0 Å closer to the CXXC motif (Figure 6D).

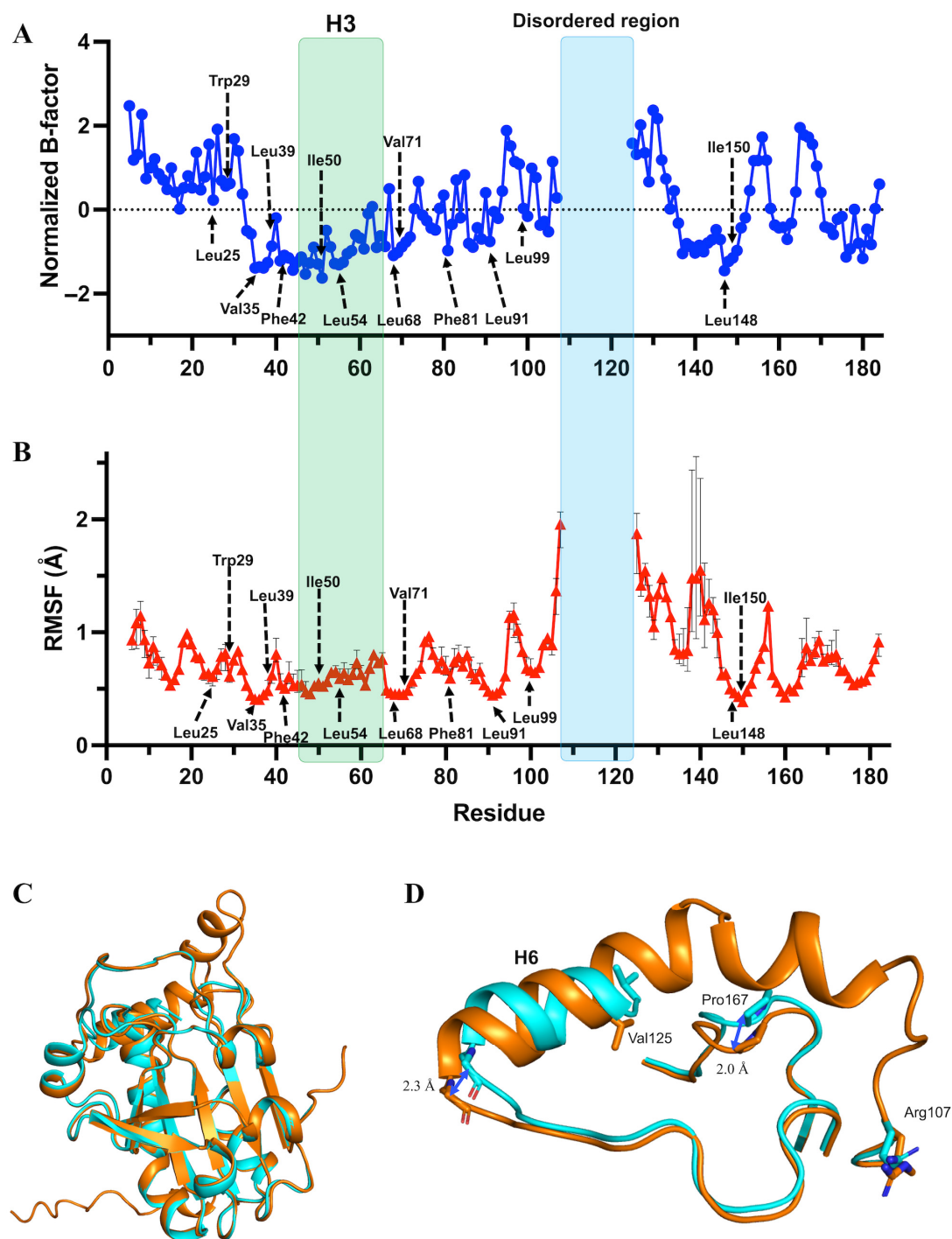


Figure 6. Comparison of residue-wise normalized B-factors and MD-derived RMSF values, including structural comparison between PGFS DM and the AlphaFold model. (A) Residue-wise normalized B-factors from the crystal structure, colored in blue. (B) Root-mean-square fluctuation (RMSF) values from molecular dynamics (MD) simulations of the PGFS DM structure, shown in red with error bars. Helix H3 is highlighted in light green, and the disordered region is shaded in sky blue. (C) Structural alignment of PGFS DM (cyan) with the AlphaFold (AF) model (orange). (D) Close-up view of regions showing structural differences between PGFS DM and the AF model.

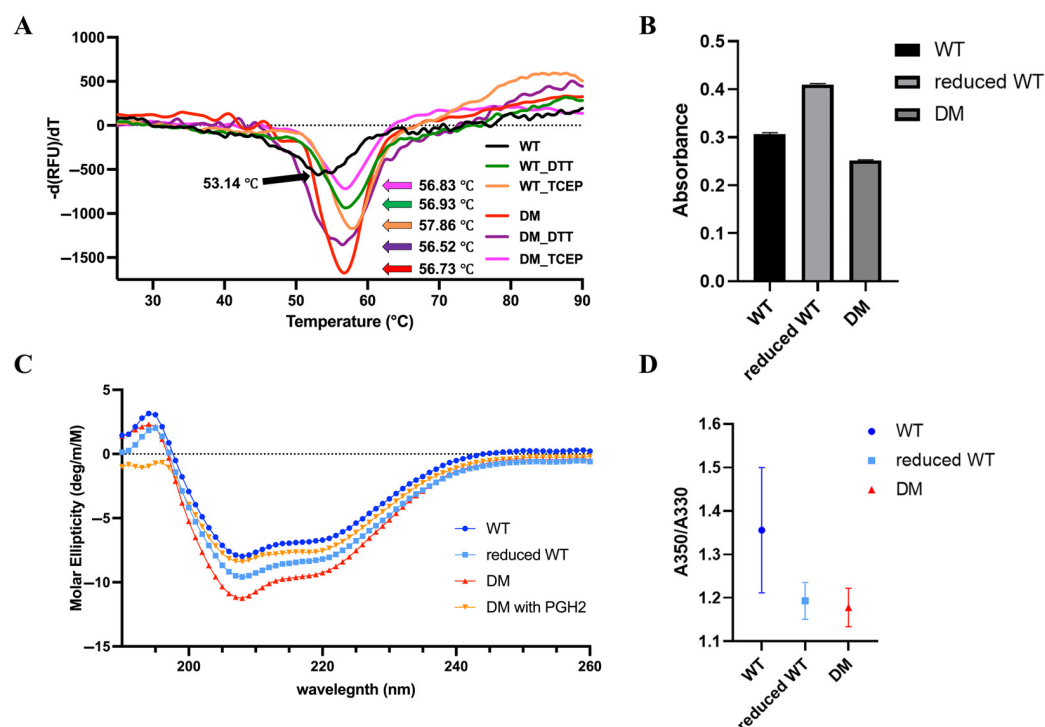


Figure 7. Spectroscopic analyses and in vitro assays for demonstrating biophysical characteristics of human PGFS. **(A)** Differential scanning fluorimetry (DSF) analysis of PGFS WT (black) and reduced PGFS WT treated with DTT (green), and PGFS WT treated with TCEP (light orange). PGFS DM is shown in red, while DM treated with DTT and TCEP are shown in purple and magenta, respectively. **(B)** Quantification of free cysteine residues in PGFS WT, reduced PGFS WT, and PGFS DM using Ellman's assay. **(C)** Circular dichroism (CD) spectroscopy of PGFS WT, reduced PGFS WT, PGFS DM, and PGFS DM with PGH2. PGFS WT is represented by blue circles, reduced PGFS WT by light blue squares, PGFS DM by red triangles, and PGFS DM with PGH2 by orange inverted triangles. **(D)** Intrinsic tryptophan fluorescence spectroscopy for PGFS WT, reduced PGFS WT, and PGFS DM. PGFS WT is represented by blue circles, reduced PGFS WT by light blue squares, and PGFS DM by red triangles, with error bars shown in the corresponding colors.

3.6. Activation-Dependent Stabilization of PGFS and Increase in Structurally Disordered Region upon Substrate Binding

To elucidate molecular determinants of PGFS stability in response to the activation by the breakage of a disulfide bond in the CXXC motif and subsequent formation of sulfhydryl groups of Cys, we implemented biophysical techniques such as differential scanning fluorimetry (DSF), circular dichroism (CD) spectroscopy, and intrinsic tryptophan fluorescence spectroscopy. Thermal unfolding profiles obtained from DSF revealed that PGFS WT exhibited a melting temperature (T_m) of 53 °C in the absence of reducing agents. The PGFS DM crystal structure confirmed the absence of any additional disulfide bonds. Upon reduction of disulfide bonds using DTT and TCEP, the T_m of PGFS WT increased to 57–58 °C, which implicates the involvement of redox-sensitive structural elements in the thermal stability regulation (Figure 7A). The T_m of the PGFS DM was similar to the T_m of reduced PGFS WT, with values in the range of 56–57 °C. Consistent with this observation, additional treatment of PGFS DM with reducing agents DTT and TCEP did not elicit any noticeable change in thermal stability, and the T_m values remained unchanged relative to the untreated PGFS DM (Figure 7A). PGFS contains six cysteine residues, including two in the conserved CXXC motif. The number of free thiol groups was quantified using Ellman's assay to assess whether the observed T_m shift could be attributed to disulfide bond disruption. The reduced PGFS WT exhibited a markedly elevated level of absorbance,

corresponding to six free cysteines. In contrast, PGFS WT and PGFS DM exhibited similar absorbance levels, corresponding to 4.5 and 4 cysteines, respectively (Figure 7B). These findings show that the CXXC-linked disulfide bond is a key determinant of the PGFS stability shift observed upon redox manipulation.

In addition, the contribution of the secondary structure to the thermal stability enhancement was assessed via far-UV CD spectroscopy. PGFS WT exhibited the smallest proportion of disordered content (9.7%). In contrast, the reduced PGFS WT and PGFS DM exhibited elevated disordered fractions (13.0% and 14.7%, respectively) despite their higher T_m values than that of PGFS WT (Table 2). Notably, when PGH2, an endogenous ligand of PGFS, was introduced into PGFS DM, the disorder content increased further to 16.7%, accompanied by a distinct negative ellipticity at 195–198 nm (Table 2; Figure 7C). To further elucidate whether thermal stabilization is correlated with the reorganization of the hydrophobic core of PGFS, the intrinsic fluorescence emission of tryptophan residues was measured. PGFS contains two tryptophan residues, solvent-exposed Trp49 and hydrophobic core-located Trp29 (Figure 2D). When Trp is buried in the hydrophobic core, the emission intensity at 330 nm increases. When Trp is exposed to the solvent, the emission intensity at 350 nm increases [35]. The reduced PGFS WT and PGFS DM exhibited consistent 350/330 nm emission ratios of approximately 1.2, indicating a stable core environment. In contrast, PGFS WT exhibited a higher 350/330 nm emission ratio of 1.36, with a greater deviation, implying partial destabilization or solvent exposure of the core Trp residues (Figure 7D).

Table 2. Comparison of secondary structure fractions of PGFS based on CD spectroscopy results.

	α -Helix (%)	β -Sheet (%)	Disordered (%)
PGFS WT	45.3%	45.0%	9.7%
Reduced PGFS WT	43.7%	43.2%	13.0%
PGFS DM	43.0%	42.3%	14.7%
PGFS DM with PGH2	41.7%	41.6%	16.7%

4. Discussion

In this study, we determined the PGFS DM structure at 2.3 Å resolution, revealing unique structural and biophysical features linked to its activity. PGFS adopts a Trx fold with a CXXC motif, but shows distinct differences from other thioredoxin-like proteins. The oligomeric state of Trxs is generally not well understood; however, our biophysical and structural data reveal that PGFS exists as a homodimer. Specifically, the C-terminus of H3, containing the CXXC motif, is essential for the dimeric interface. Furthermore, we observed that the position of the cis-proline-containing loop other Trx-like proteins differs from the corresponding loops found in PGFS. Notably, Pro167 adopts a trans conformation, and the P-1 residue Ser166 lies far from the CXXC motif, indicating minimal direct effect. Instead, Ser166 indirectly modulates the active site by stabilizing Arg40 through its interaction with the trans-proline, thereby influencing enzymatic activity. Strictly conserved Arg40 and Arg41 are positioned near the CXXC motif and stabilize its functional groups, generating a partially positive active site. This electrostatic environment likely facilitates the recruitment of the PGH2 endoperoxide moiety, explaining how PGFS selectively recognizes this short-lived substrate. Consistent with the proposed binding pose, MM/GBSA analysis following secondary energy minimization indicated favorable enthalpic contributions, primarily arising from van der Waals and electrostatic interactions (Table S2). We also attempted short MD simulations to assess pose stability. However, ligand dissociation was observed even within short timescales, likely reflecting the transient and shallow nature of the

substrate recognition site rather than limitations of the simulation setup. Considering the short half-life of PGH2, efficient formation of PGF2 α is expected to rely on rapid and transient substrate recognition rather than stable binding. Thus, the observed dissociation is consistent with the physiological role and inherent catalytic properties of the enzyme. Comparison of the PGFS DM structure with the AF model showed high overall similarity, except for the experimentally unresolved Tyr108–Asp124 region. Although AlphaFold predicted this region with high structural confidence, MD simulations, normalized B-factor analyses, and CD spectroscopy results suggest that this region exhibits high structural flexibility. Likewise, the region around the CXXC motif lacks a stably formed helix in PGFS DM structure and instead retains intrinsic flexibility, as inferred from elevated B-factors in nearby resolved regions and MD-derived fluctuations. Interestingly, reduction of the CXXC disulfide bond increased thermal stability but also expanded disordered regions, indicating that stabilization results from hydrophobic core integrity rather than increased secondary structure. This activation-dependent disorder contrasts with AF predictions and may play a key role in substrate recognition.

In vitro assays indicated that reduction of the CXXC motif enhances the hydrophobic core integrity of PGFS and contributes to its thermal stabilization. However, this stabilization is not accompanied by increased secondary structure elements but rather by a counterintuitive increase in disordered contents. This elevated disorder implies that PGFS may not possess a well-defined substrate-binding site. Instead, ligand interactions likely occurred through the flexible and disordered regions of PGFS, which undergo dynamic changes around the active site to accommodate these interactions.

The limitation of this study is the absence of data on the PGFS-PGH2 complex structure. In vitro assay data, including binding affinity measurements, have already been reported for mouse PGFS [1]. In the case of human PGFS, attempts to soak PGH2 into preformed crystals for a short duration resulted in crystal cracking and prevented structural determination. Furthermore, alternative approaches, such as co-crystallization, were not feasible due to the short half-life of PGH2 [36]. Determination of human PGFS structures in complex with more stable substrate analogs could help the elucidation of the detailed mechanism of substrate binding and catalysis. Nevertheless, we provided critical insights into the environment surrounding the active site, the factors influencing stability of the protein, and the impact of flexible regions on substrate binding. Substrate recognition typically stabilizes the protein's binding site structurally and decreases its flexibility. In contrast, we observed that residues around the binding site of PGFS become more flexible upon substrate recognition. Importantly, our elucidation of the human PGFS structure identifies this enzyme, which directly produces the clinically useful PGF2 α , as a compelling and previously underexplored druggable target. Structural insights into human PGFS suggest that small-molecule modulators targeting the active site, hydrophobic core, or allosteric regions could be engineered to modulate PGF2 α production, offering potential therapeutic strategies for PGF2 α -related disorders.

5. Conclusions

Our structural and biochemical analyses provide direct insight into the molecular basis of human PGFS function. The resolved active-form mimicking PGFS structure highlights the critical role of the CXXC motif and its surrounding charged environment in facilitating the recognition of the substrate PGH2. Notably, unlike other thioredoxin family proteins, PGFS lacks a stably formed helix around the CXXC motif and instead exhibits intrinsic flexibility in this region supported by structural and biophysical evidence. Together, these findings provide a structural framework for understanding PGFS-mediated prostaglandin biosynthesis and offers a foundation for future modulation of prostaglandin signaling.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biom16020262/s1>, Figure S1: Structural comparison between PGFS wild-type (WT) and C44S/C47S mutant (DM), Figure S2: Standard calibration curve of a Superdex 75 pg column generated using Bio-Rad gel filtration standards (catalog number 1511901), Figure S3: Dimeric interaction of human PGFS, Figure S4: PSI-BLAST results based on the human PGFS amino acid sequence, Figure S5: Location of the disordered region and its surrounding structure, Figure S6: Protein secondary structure analysis from Molecular Dynamics simulations, Figure S7: Overall structures of the AlphaFold model; Table S1: High Confidence DALI Homologs ($Z \geq 10$), Table S2: MM/GBSA analysis of docked PGH2 to PGFS DM.

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Abbreviations

The following abbreviations are used in this manuscript:

PGFS	Prostaglandin/prostamide F synthase
PGF2 α	Prostaglandin F ₂ α
PGH2	Prostaglandin H ₂
Trx	Thioredoxin
DSF	Differential scanning fluorimetry
CD	Circular dichroism spectroscopy
DTT	Dithiothreitol
TCEP	Tris(2-carboxyethyl) phosphine
RMSF	Root mean square fluctuation

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