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RESEARCH PAPER

Evaluating the Role of Puckering and Fluorine Atom in Stability and Folding of Fluoroproline Containing Proteins

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Abstract In the past decade, numerous studies have been reported that the residue specific incorporation of fluorine containing analogs into protein can enhance the stability of protein. On the other hand, the incorporation of fluoroproline can enhance both stability and refolding rate of recombinant proteins. The objective of this study was to determine the reason behind the enhanced stability and refolding rate of protein by comparing GFP variants containing fluoroproline or hydroxyproline. The fluorine atom of 4-fluoroproline played a significant role in enhancing stability, and C$^{\gamma}$-*endo* puckering property of (4*S*)-4-fluoroproline and (4*S*)-4-hydroxyproline plays a key role in enhancing protein refolding rate.

Keywords: enzyme stabilization, fluorination, fluoroproline, protein folding, unnatural amino acid

1. Introduction

Unnatural amino acid (UAA) based protein engineering has been one of the most important advances in biotechnology in the last few decades [1,2]. Rapid progress in this field has enabled the development of novel proteins exhibiting additional functional properties such as cross-linking nature, biophysical probes, metal ion binding, expanded substrate specificity, improved stability, activity, and so on [1,2]. Among UAA incorporation techniques, residue specific incorporation is a facile and economical method that provides synergistic effects for globally substituted UAA to the protein through addition of functional moiety [3]. An increasing number of studies are available for residue specific incorporation of UAA that utilizes endogenous tRNA synthetase to incorporate isostructural analogs of natural amino acids [3]. Among a wide range of UAAs, fluoro amino acids are considered as the most important unnatural amino acids that can increase stability and folding of proteins [4-7]. Earlier, it has been reported that fluorine atom enables the stability of protein by forming highly polarized bond with carbon atom compared to carbon-carbon bond. On the other hand, the fluorine atom is extremely electronegative and hydrophobic in nature which enables it to form hydrogen bond and hydrophobic interactions with nearby atoms, thus improving the stability of proteins [8,9]. Furthermore, many efforts have been dedicated to explore the role of functional moieties of UAA responsible for the highly valuable functional properties.

Among the 20 natural amino acids, proline (Pro) is a unique amino acid that plays an important role in protein structural conformation due to its five-membered pyrrolidine ring [8,9]. As a breaker of β-sheet and α-helical structures, natural Pro residue is mainly found in turns and links. It is rarely involved in substrate binding or active site [8-10]. Proline can form either a C$^{\gamma}$-*exo* ring pucker (in which C$^{\gamma}$ is puckered toward the C$^{\alpha}$ protron) or a C$^{\gamma}$-*endo* ring pucker (in which C$^{\gamma}$ is puckered toward the carbonyl group). Indeed, stereo electronic effects of ring substituents at C4 atom of proline favors C$^{\gamma}$-*exo* pucker and the *trans* conformation for (2*S*, 4*R*)-4-fluoroproline [(4*R*)-FP] and

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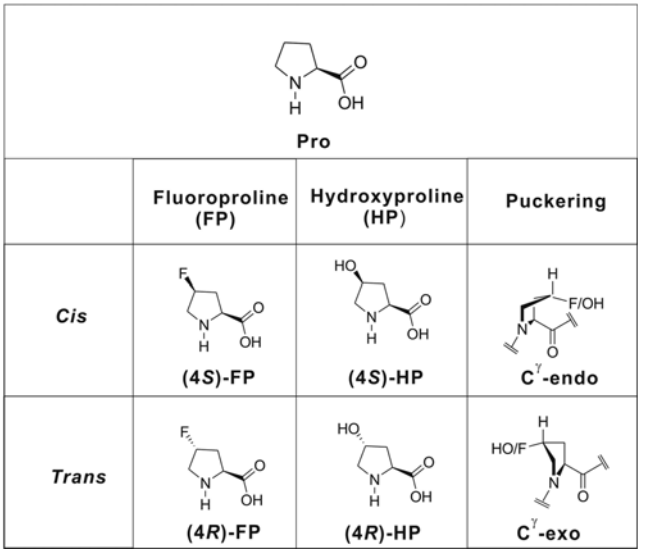

Fig. 1. Chemical structure and ring conformation of proline and its fluoro and hydroxy analogs.

(2*S*, 4*R*)-4-hydroxyproline [(4*R*)-HP], while its epimers promotes the *cis* conformation and C$^{\gamma}$-*endo* puckering for (2*S*, 4*S*)-4-fluoroproline [(4*S*)-FP] and (2*S*, 4*S*)-4-hydroxyproline [(4*S*)-HP] as illustrated in Fig. 1 [8-13]. Due to ring puckering nature, the preorganizing effect of protein backbone conformation of proline analogs might modulate protein stability [14,15]. It has been reported that the biased ring pucker can selectively stabilize or destabilize proteins [16].

Interestingly, EGFP with (4*R*)-FP or (4*S*)-FP has shown expression level comparable to that of wild type protein. However, EGFP incorporated with (4*R*)-FP has formed an irreversible and unfolded inclusion body [8]. Compared to the puckering of pyrrolidine rings, *cis*/*trans* proline affects the folding and stability of (4*R*)-FP or (4*S*)-FP incorporated EGFP in a lesser extent. In contrast, (4*S*)-FP incorporated monomeric red fluorescent protein can form inclusion body while (4*S*)-FP incorporated variant shows soluble protein and recovers its fluorescence after single amino acid mutation [17]. Apart from protein engineering, fluoroproline incorporation has been attempted in enzyme engineering for enzymes such as lipase, and DNA polymerase [18-20]. In the case of DNA polymerase, about 32 sites have been globally substituted with high efficiency without any detrimental effects on its structure or solubility [18]. Therefore, global replacement of fluoroproline at multiple sites might enable structural stability without losing native activity of proteins [18]. On the other hand, fluoroproline incorporation can increase stability of some proteins while it shows detrimental effects on others [17,20]. And recently, Dietz et al. used the bacteriophage T4 fibritin C-terminal domain (27 amino acid residues, called *foldon*) containing fluorinated, hydroxylated, and methylated prolines to investigate puckering effects on folding and stability [9]. In this context, the functional enhancement of protein through UAA incorporation can vary for different proteins. The reliability of enabling specific functional properties of proteins through UAA incorporation is lacking. To explore and discriminate the puckering effect and fluorine atom in terms of protein stability, we utilized *in vivo* incorporation of stereo isomers of fluoro and hydroxyl proline to enable similar puckering effect with different atoms at C$^{\gamma}$ (C-4) of proline. To derive conclusion, we carried out a comparative study to investigate the role of functional moiety and puckering nature in two different GFP variants by incorporating four different proline analogs in its enantiomeric pure and racemic forms.

2. Materials and Methods

2.1. Materials

PCR reagents, T4 DNA ligase, and restriction endonucleases were purchased from Promega (Madison, WI, USA). Isopropyl-D-thiogalactopyranoside (IPTG) was purchased from sigma chemicals (St. Louis, MO, USA). Host bacterium *Escherichia coli* (*E. coli*) strain XL1-blue (Stratagene, San Diego, CA, USA) was used for plasmid DNA preparation in this study. Proline auxotroph (BL21 (DE3) pLysS (KC1325)) for proline isostructural analog incorporation was purchased from NBRP at NIG (Japan). *E. coli* cells with plasmids were grown aerobically in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Michigan, USA) or on LB agar plate supplemented with appropriate antibiotics for the selection of transformants. *E. coli* BL21 (DE3)pLysS (KC1325) Pro auxotroph was kindly provided by Prof. Laszlo N. Csonka. Natural amino acids, urea, and SDS were purchased from Sigma (St. Louis, MO, USA). Isostructural analogs of proline residues such as (4*R*)-FP, (4*S*)-FP, (4*R*)-HP, and (4*S*)-HP were purchased from Bachem (Bubendorf, Switzerland). Plasmid pQE-80L and nickelnitrilotriacetic acid (Ni-NTA) affinity column were purchased from Qiagen (Valencia, CA, USA).

2.2. Cloning

DNA manipulations were performed according to the procedures described previously [17]. PCR reaction (50 μL) was carried out with 10 pM of each primer, 50 ng of template DNA, 1X *Taq* DNA polymerase buffer, 1U of Taq DNA polymerase (Promega, Madison, WI, USA), 0.2 mM of each deoxyribonucleotide triphosphates, and 1.5 mM $MgCl_2$. DNA amplification was performed in a DNA thermal cycler (Master Gradient thermal cycler, Eppendorf, Hamburg, Germany) using the following program: initial

denaturation at 94ºC for 1 min; 30 cycles of chain reaction at 94ºC for 1 min, at 60ºC for 1 min, and at 72ºC for 0.5 min; and a final extension step at 72ºC for 10 min. Plasmids pQE80-GFPHS and pQE80L-GFP3.1 were constructed using pET28a-GFPHS and pET28a-GFP3.1 as template, respectively. pET28a-GFPHS and pET28a-GFP3.1 were obtained from Prof. Sungu-lee, Pusan National University [22]. Amplified genes were digested with *Bam*H1 and *Hind*III restriction enzymes and sub cloned into pQE80L. The constructed pQE80-GFP was transformed into *E. coli* BL21 (DE3)pLysS (KC1325) Pro auxotroph to produce recombinant proteins with natural and unnatural amino acids. All constructs were sequenced and confirmed before expressing target proteins.

2.3. Expression and purification of proline analog incorporated GFP 3.1 and GFPHS

Plasmid pQE80L-GFP3.1 and pQE80L-GFPHS were transformed into *E. coli* BL21 (DE3) pLysS (KC1325) Pro auxotroph, respectively. Furthermore, these proteins were expressed in minimal medium (MM) lacking Pro according to previously described protocols [17]. Briefly, limited concentration of Pro (0.05 mM) was used to culture cells to reach OD_{600} of 0.6 ~ 0.8. These cells were washed three times with autoclaved saline. After centrifugation, cell pellets were resuspended in fresh minimal media lacking Pro. Target proteins were induced with 1 mM IPTG followed by simultaneous addition of Pro, (4*R*)-FP, (4*S*)-FP, (4*SR*)-FP, (4*R*)-HP, (4*S*)-HP, or (4*SR*)-HP (0.5 mM) to allow protein expression for 6 h. After centrifugation, cells were harvested and stored at -80°C until further use. Briefly, collected cell pellet was suspended in lysis buffer (5 mM imidazole and 50 mM sodium phosphate buffer pH 8.0 containing protease inhibitor) followed by sonication and centrifuged at 16,000 rpm and 4°C for 20 min. The supernatant was saved as soluble protein fraction while the pellet was saved as insoluble protein fraction. Both fractions were subjected to SDS-PAGE (12% acrylamide gel). Remaining soluble protein fractions were purified by Ni-NTA column chromatography (GE Healthcare Bio-Sciences, Sweden) using standard protocol. Elution fractions were analyzed by SDS-PAGE. Fractions containing desired GFP variants were pooled and dialyzed against 1X phosphate buffered saline. The concentration of protein was quantified using Bradford assay. Fluorescence spectra of GFP variants were recorded on a Perkin Elmer LS-55 fluorescence spectrometer equipped with digital software Winlab.

2.4. Sequence alignment and homology modelling studies

Multiple sequence alignment for GFP3.1, GFPHS, and EGFP was performed using online tool Mulalign. Proline residues were compared and analyzed using PDB structure (GFP-2B3P, GFP-Fluoro proline-2Q6P). To analyze surrounding amino acids near proline residues of GFP3.1 and GFPHS, GFPHS structure was modelled using homology modeller. Using Blastp search tool, a structural template (PDB Id-2B3P) was identified against protein databank. Using super folded GFP as structural template and sequence alignment for our target protein-GFPHS, a three-dimensional structure was constructed. By comparing structures of GFP3.1 and GFPHS, probable proline residues that might have steric hindrance with surrounding residue were predicted and analyzed using visualizing software Pymol.

2.5. Circular dichroic profile of GFPHS and its UAA variants

A far UV CD spectrum was recorded for GFPHS and its UAA variants on a JASCO J-715 spectrometer. For this analysis, 250 μL of 3 μM protein was prepared in 1× PBS buffer (pH 7.5) and placed in a 0.2 cm cell. The CD absorption spectrum was obtained at room temperature. Five scans were accumulated per spectrum. Raw data were processed using Jasco software package. Finally, obtained results were analyzed and graph was drawn using Origin software.

2.6. Thermal and chemical stability of GFPHS and its UAA variants

To investigate the thermal stability of protein, 100 μg/mL of each protein sample was incubated at 60ºC for 20 h. The zero-hour sample was taken after incubating the sample at 60ºC for 10 min in order to minimize error of fluctuation. To determine the half-life ($t_{1/2}$) of GFPHS and its UAA variants based on fluorescence intensity, samples were collected and fluorescence intensities were measured at different time points. After incubating protein samples at room temperature for 10 min, fluorescence intensities were measured using PerkinElmer LS55 fluorescence spectrophotometer. To determine the $t_{1/2}$ of chemical stability for GFPHS and its UAA variants, each protein (1 mg/mL) was incubated with either 8 M urea or 6 M guanidine hydrochloride (GdmCl) in 100 mM Phosphate buffer (pH 7.5). Then 10 μL aliquot was collected at pre-defined time points and diluted to 10 μg/mL with 100 mM Phosphate buffer (pH 7.5). Fluorescence intensity of GFPHS was then measured using PerkinElmer LS55 fluorescence spectrophotometer.

2.7. Differential scanning fluorimetry (DSC)

Differential scans were performed using a high-sensitivity DSC (Nano-DSC, TA Instruments, Delaware, USA). Sample and the reference cells were filled with 30 μL of protein (0.5 mg/mL) and 10 μL SYPRO orange dye (1:1,000 times

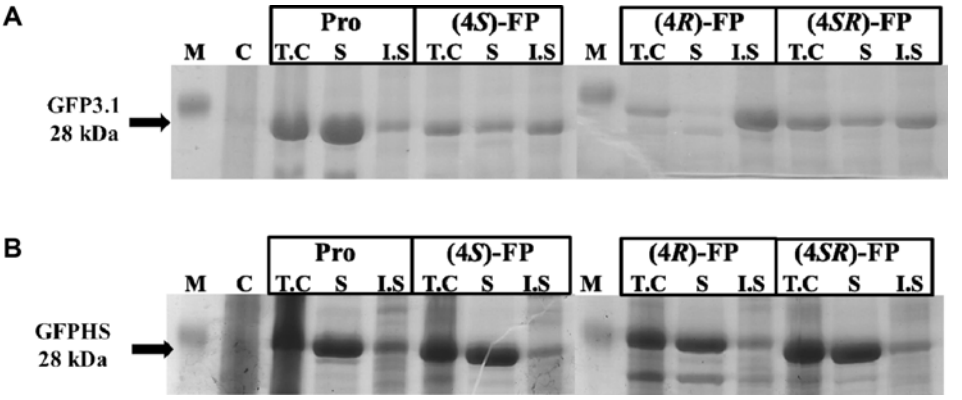

Fig. 2. Protein expression profiles of GFP3.1, GFPHS, and it UAA containing variants GFPHS (4*S*)-FP, GFPHS (4*R*)-FP, and GFPHS (4*SR*)-FP. (A) SDS-PAGE of total cell (TC), soluble (S), and insoluble fraction (IS) of GFPHS, GFPHS (4*S*)-FP, GFPHS (4*R*)-FP, and GFPHS (4*SR*)-FP. (B). SDS-PAGE of total cell (TC), soluble (S), and insoluble fraction (IS) of GFP3.1 and its UAA variants.

dilution) in 10 mM Tris-HCl buffer (pH 8.0). Calorimetric measurements were conducted in a microtitre plate at a scan rate of 1°C/min. Buffer tracings were obtained under the same conditions and subtracted from sample curves. Observed thermograms were baseline corrected. Normalized data were analyzed using NanoAnalyze software.

2.8. Refolding kinetics of GFPHS and its UAA variants

Denaturation of all purified samples of GFPHS and its UAA variants (30 μM each) was performed in 1× PBS buffer containing 8 M urea and 5 mM DTT at 95°C for 5 min. Urea denatured samples were renatured at room temperature by diluting them 100-fold with 1× PBS buffer and 5 mM DTT without urea. Protein refolding was monitored for 30 min by fluorescence recovery at excitation/emission wavelengths of 501/513 nm, respectively, using the option 'Timedrive' of the Perkin-Elmer spectrometer (LS50B) with an interval of 3 sec and a slit of 5 nm. Raw data were imported into Origin 6.1 (OriginLab Corporation, Northampton, MA, USA) and normalized before plotting. Data were fitted with Sigma Plot (Systat Software Inc., San Jose, CA, USA) using equations described elsewhere [22].

3. Result and Discussion

3.1. Incorporation of proline analogs into fluorescent proteins

In the past decade, numerous studies have reported that fluorine atom containing analogs can enhance the stability of target proteins compared to other analogs. On the other hand, the incorporation of fluoroproline can enhance both stability and refolding property of recombinant proteins (summarized in Supplementary Material Table S1). It has been reported that conformation and electronic effects of the side chain of proline and its analogs have considerable influence on the three-dimensional structures of proteins, thereby enhancing the stability and refolding nature of proteins [8,9]. However, none of these studies clearly distinguished these enhanced properties based on the role of puckering or through fluorine atom. Therefore, a comprehensive study and understanding between the role of puckering and side chain atom of proline analogs might make it possible to rationally incorporate protein analogs by their specific functional property. To compare and investigate the role of puckering and fluorine atom in protein stability, we incorporated enantiomerically pure and racemic mixture of hydroxyl and fluorine atom containing proline analogs into GFPHS in this study.

As an initial attempt, we incorporated fluoroproline in its racemic and enantiomerically pure form [(4*S*)-FP, (4*R*)-FP, and (4*SR*)-FP)] into GFP3.1 variant containing 10 proline residues. As shown in Fig. 2A, there was no detectable expression of GFP in the control experiment to depict the efficiency of UAA incorporation. It was evident that the incorporation of (4*S*)-FP or (4*SR*)-FP to GFP3.1 variant was successful with formation of soluble protein whereas the incorporation of (4*R*)-FP resulted in formation of insoluble protein (Fig. 2A). Although the incorporation of (4*S*)-FP was successful, to accomplish the main objective (evaluating the role of puckering and functional moiety in stability), a highly stable variant was required. A novel stable GFP variant has been engineered from GFP3.1 with the incorporation of unnatural amino acid [22]. We accomplished the incorporation of fluoroproline into highly stable GFP variant in a stereo isomeric and racemic form. As expected, the incorporation of the stereo isomeric and racemic form of hydroxyl and fluoroproline enabled the formation of soluble proteins (Fig. 2B, Supplementary material S1 and S2). Furthermore, GFPHS incorporated

with stereo isomeric and racemic form of hydroxyl and fluoroproline was purified on a Ni-NTA affinity column at 4ºC. Protein expression levels of unnatural amino acid (4*S*)-FP (30 mg/L), (4*R*)-FP (23 mg/L), (4*SR*)-FP (27 mg/L), (4*S*)-HP (28 mg/L), (4*R*)-HP (21 mg/L), and (4*SR*)-HP (24 mg/L) were found to be similar to that of proline (28 mg/L). Refolding and stability assays were then performed with soluble GFPHSs containing proline analog.

3.2. Structural analysis for successful incorporation of proline analogs in high stable GFP

In residue specific incorporation of unnatural amino acids, several factors may affect the formation of soluble protein after global replacement of corresponding amino acid. We modeled and compared three dimensional structures of GFP3.1 and GFPHS. Both these variants contained 10 proline residues with difference in 21 amino acids between their sequences (Fig. S3, Supplementary Material). Although GFP3.1 and GFPHS variants exhibited 10 proline residues, replacement of (4*R*)-fluoroproline enabled the formation of insoluble protein in GFP3.1 whereas GFPHS was a soluble protein. Similar formation of insoluble protein was observed in the earlier attempt after incorporation of (4*R*)-FP into EGFP [8]. The sequence of GFP3.1 protein was different from that of EGFP by 4 amino acids. They shared 10 proline residues. Based on sequence analysis, it is clear that the change in amino acid sequence (18 amino acids) might play a role in structural perturbation which is unfavorable for (4*R*)-FP incorporation. Sequence change might have affected the surrounding environment of 10 proline residues, thereby enabling successful incorporation of (4*S*)-FP into GFPHS.

Based on the models of GFPHS and GFP3.1, surrounding amino acids near the 4 proline residues (Pro13, Pro58, Pro192, and Pro192) varied among them. Proline residues and amino acid differences between GFP3.1 and GFPHS around 4 Å of corresponding proline residues are shown in Supplementary Material Table S2. Residues at 58 and 192 have steric clash with surrounding residues as shown in Fig. 3. Difference is observed in the loop region of GFP3.1. Residues 188-195 are modified in GFPHS. Pro192 might have hindered the surrounding residue after UAA incorporation, especially Leu 194 (Figs. 3 and S4, Supplementary Material). Replacing Pro58 with (4*R*)-FP might have been sterically hindered by Leu207 which is replaced with Ile in GFPHS, thereby reducing steric clash by taking different conformation (Fig. S5, Supplementary Material). It is evident that stereo-selective preference of each residue during global replacement of unnatural amino acid is important for the formation of soluble and active protein which is mainly dependent on surrounding residues.

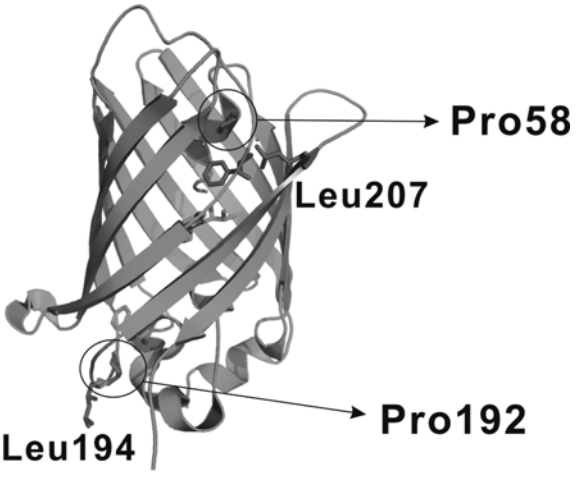

Fig. 3. Proline residues and its surrounding amino acids with steric clash during (4*R*)-FP incorporation in GFP3.1 predicted through *in silico* analysis.

3.3. Spectral and circular dichroic profile of GFPHS and it UAA variants

Fluorescence emission and excitation scan of GFPHS and UAA variants [GFPHS (4*S*)-FP, GFPHS (4*R*)-FP, and GFPHS (4SR)-FP] showed similar excitation maximum (Fig. S6, Supplementary Material). In contrast, GFPHS (4*S*)-FP and GFPHS (4*SR*)-FP showed distinct fluorescent red shift of 6 nm (519 nm) while wild type GFPHS, GFPHS (4R)-FP, and GFPHS (4R)-HP showed similar emission maxima at 512 nm (Fig. S7, Supplementary Material). To evaluate structural conformation of GFPHS and its UAA variants, we also performed circular dichroism (CD) spectroscopy analysis in the far UV range. Generally, the unique β-can structure of GFP has 11 β-pleated sheets. Therefore, the majority of sharp negative deflection can be observed. We observed the same spectrums during CD spectroscopy analysis at around 215 ~ 216 nm (Fig. S8, Supplementary Material). These dichroic profiles confirmed that GFPHS and its UAA variants had the same overall secondary structural characteristics.

3.4. Evaluating the role of puckering and fluorine atom in thermal and chemical stability

Thermal stability of GFPHS and its UAA variants at 60°C was examined in a time drive temperature controller. Half-lives of GFPHS, GFPHS (4*S*)-FP, GFPHS (4*R*)-FP, GFPHS (4*SR*)-FP, GFPHS (*4S*)-HP, GFPHS (*4R*)-HP, and GFPHS (4*SR*)-HP at 60°C were found to be 2.5, 6.1, 4.6, 5.4, 2.6, 1.5, and 2.4 h, respectively (Figs. 4 and S9, Supplementary Material). In the case of racemic form incorporated GFP variants, stability and refolding results showed the second-best values (next to the best variants). Interestingly, GFPHS (*4S*)-FP (half-life of 6.1 h), GFPHS (4*R*)-FP (half-life of 4.6 h), and GFPHS (4*SR*)-FP (half-life of 5.4 h)

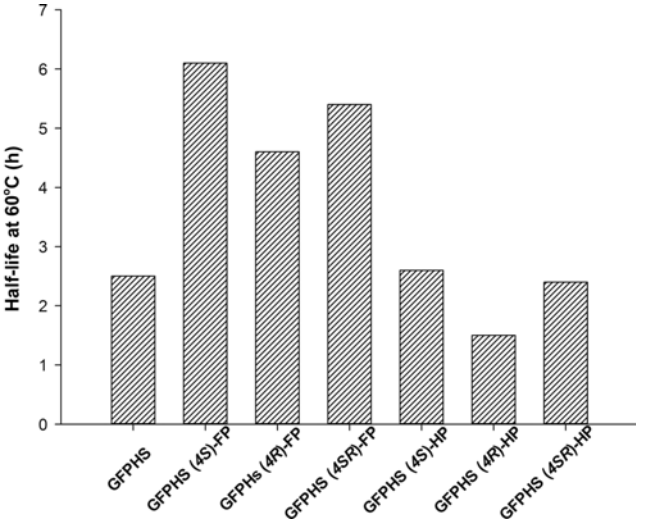

Fig. 4. Thermal stabilities of GFPHS and fluoroproline and hydroxyproline containing GFPHS.

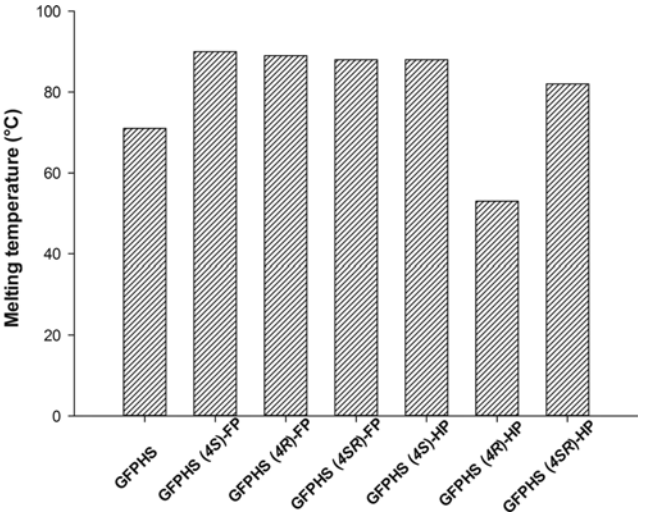

Fig. 5. Melting temperatures of GFPHS and its UAA containing variants GFPHS (4*S*)-FP, GFPHS (4*R*)-FP, GFPHS (4*SR*)-FP, GFPHS (4*R*)-HP, GFPHS (4*S*)-HP, and GFPHS (4*SR*)-HP.

were highly thermal stable compared to GFPHS (half-life of 2.5 h). Conversely, GFPHS (4*S*)-HP (half-life of 2.6 h) and GFPHS (4*SR*)-HP (half-life of 2.4 h) did not have much difference in thermal stability compared to that of GFPHS (half-life of 2.5 h). However, GFPHS (4*R*)-HP (half-life of 1.5 h) showed less thermal stability compared to its parent GFPHS (half-life of 2.5 h). Based on these results, it is clear that the fluorine atom substitution can enhance approximately two to three folds of increase in half-life of the protein compared to other C4 ring substituents. Furthermore, GFPHS (4*S*)-FP (half-life of 6.1 h) and GFPHS (4*S*)-HP (half-life of 2.6 h) showed minimal improvement in thermal stability compared to opposite chiral GFPHS (4*R*)-FP (half-life of 4.6 h) and GFPHS (4*R*)-HP (half-life of 1.5 h), respectively (Fig. 4). This shows that fluorine atom plays a main role in improving protein thermal stability while puckering plays a minimal role.

Melting temperature (Tm) of GFPHS and its UAA variants were examined through differential scanning fluorimetry. Wild-type GFPHS showed Tm value of ~71°C whereas GFPHS (4*R*)-HP showed lower Tm value of 53°C. GFPHS (4*S*)-FP, GFPHS (4*S*)-HP, GFPHS (4*R*)-FP, GFPHS (4*SR*)-FP, and GFPHS (4*SR*)-HP showed higher Tm values (at ~90, 88, 89, 88, and 82°C, respectively). GFPHS (4*S*)-FP, GFPHS (4*R*)-FP, and GFPHS (4*SR*)-FP showed higher Tm values than GFPHS (4*S*)-HP, GFPHS (4*R*)-HP, and GFPHS (4*SR*)-HP, respectively (Fig. 5). Overall, the replacement of Pro residue in GFPHS with either (4*S*)-FP, (4*R*)-FP, or (4*SR*)-FP enhanced the thermal stability and chemical stability of GFPHS compared to the replacement with (4*S*)-HP, (4*R*)-HP, or (4*SR*)-HP. Therefore, the fluorine atom played a major role in the stability while *endo* puckering showed little enhancement in the stability compared to *exo* puckering.

In similar fashion, chemical stability analysis results also showed that the fluorine atom was involved in the stability of GFPHS. As shown in Fig. S10, GFPHS (4*S*)-FP incorporated with (4*S*)-FP showed an increase in stability in 8M urea while GFPHS (4*SR*)-FP and GFPHS (4*R*)-FP showed moderate increase in chemical stability. This indicated that the fluorine atom was a major factor in protein stability compared to the puckering effect of proline. The stability of other UAA variants was comparable to that of parent GFPHS. The $t_{1/2}$ values of GFPHS treated with 8 M urea and its variants GFPHS (4*S*)-FP, GFPHS (4*R*)-FP, GFPHS (4*SR*)-FP, GFPHS (4*S*)-HP, GFPHS (4*R*)-HP, and GFPHS (4*SR*)-HP were about 21, 46, 27, 34, 23, 18, and 21 min, respectively. GFPHS (4S)-FP showed about 2.1-fold of enhanced stability compared to wild-type GFPHS in the presence of 8M urea. Stabilities of these proteins were also examined in the presence of 6 M GdmCl. The $t_{1/2}$ values of GFPHS, GFPHS (4S)-FP, GFPHS (4R)-FP, GFPHS (4SR)-FP, GFPHS (4S)-HP, GFPHS (4R)-HP, and GFPHS (4SR)-HP were about 23, 35, 28, 31, 25, 19, and 21 min, respectively (Fig. S9, Supplementary Material). GFPHS (4S)-FP showed about 1.5-fold enhanced stability compared to wild-type GFPHS in the presence of 6M GdmCl (Fig. S11, Supplementary Material). These results suggest that the stability pattern of these proteins in the presence of 6 M GdmCl was similar to that treated with 8 M urea. These results clearly showed that the chemical stability of GFPHS could be enhanced by (4*S*)-FP incorporation.

3.5. Refolding kinetics of GFPHS and its UAA variants

Earlier, we found that the incorporation of (4*S*)-FP into

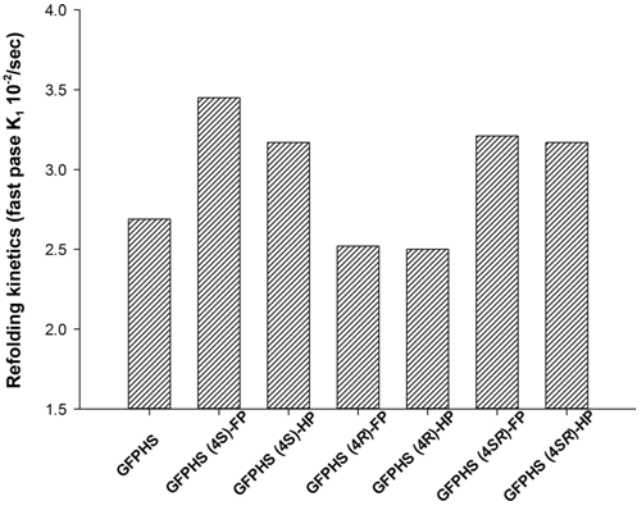

Fig. 6. Refolding rates of GFPHS and its UAA containing variants GFPHS (4*S*)-FP, GFPHS (4*R*)-FP, GFPHS (4*SR*)-FP, GFPHS (4*R*)-HP, GFPHS (4*S*)-HP, and GFPHS (4*SR*)-HP.

EGFP showed 2.1-fold faster refolding kinetics than its parent EGFP. Here, we also analyzed the folding properties of GFPHS and its UAA variants by unfolding these proteins after boiling them at 95°C in the presence of 8 M urea for 5 min followed by refolding at room temperature after 100-fold of dilution with PBS. Refolding kinetics were further monitored fluorometrically over a time period of at least 30 min and the refolding efficiency was assessed after 24 h incubation under non-denaturing condition. Compared to their initial fluorescence, GFPHS, GFPHS (4*S*)-FP, GFPHS (4*R*)-FP, GFPHS (4*SR*)-FP, GFPHS (4*S*)-HP, GFPHS (4*R*)-HP, and GFPHS (4*SR*)-HP recovered their fluorescence at about 91, 95, 84, 87, 91, 82, and 88%, respectively. Refolding kinetics of GFPHS, GFPHS (4*S*)-FP, GFPHS (4*R*)-FP, GFPHS (4*SR*)-FP, GFPHS (4*S*)-HP, GFPHS (4*R*)-HP, and GFPHS (4*SR*)-HP (Fig. 6) showed an initial fast phase of refolding with rate constants of 2.69×10^{-2}, 3.45×10^{-2}, 2.52×10^{-2}, 3.21×10^{-2}, 3.17×10^{-2}, 2.50×10^{-2}, and 3.11×10^{-2}/sec, respectively, followed by a slower refolding phase with rate constants of 0.39×10^{-2}, 0.43×10^{-2}, 0.34×10^{-2}, 0.39×10^{-2}, 0.40×10^{-2}, 0.32×10^{-2}, and 0.38×10^{-2}/sec, respectively. Based on these results, the *endo* puckering property of proline analogs plays a major role in enhancing protein refolding property whereas fluorine atom and hydroxyl group do not have any significant role in protein refolding.

4. Conclusion

In summary, by incorporating enantiomerically pure and racemic mixture into two different GFP variants, successful incorporation of specific isomer of proline analogs into target proteins was demonstrated. We also showed the role of puckering and fluorine atom in the stability of protein by incorporating *cis* and *trans* form of hydroxy and fluoro proline into GFP3.1 and its stable variant (GFPHS). Based on results of this study, it is clear that fluorine atom is the main reason behind the increase of protein stability. It increased the melting temperature of GFPHS variants by 18 ~ 20°C compared to parent GFPHS. In addition, the *endo* puckering property of (4*S*) form of proline enabled a slight increase in stability compared to its opposite isomer. On the other hand, GFPHS (4*S*)-FP and GFPHS (4*SR*)-FP exhibited superior refolding properties when compared to parent GFPHS. Their rate of refolding in the fast phase was 1.5-fold faster than that of parent GFPHS. Furthermore, fluorine atom enabled slight increase in refolding compared to hydroxyl atom. Finally, fluoroproline was found to be a versatile UAA, especially (4*S*)-FP. It enhanced both the stability and refolding property of GFPHS. The fluorine atom enhanced the stability while *endo* puckering enhanced the refolding property of the protein. In addition, the location of proline residue in target protein such as active site and sterically hindering surrounding residues must be considered during global incorporation of UAA.

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