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Enhancing the biophysical properties of mRFP1 through incorporation of fluoroproline

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ABSTRACT

Here we enhanced the stability and biophysical properties of mRFP1 through a combination of canonical and non-canonical amino acid mutagenesis. The global replacement of proline residue with (2S, 4*R*)-4-fluoroproline [(4*R*)-FP] into mRFP1 led to soluble protein but lost its fluorescence, whereas (2S, 4*S*)-4-fluoroproline [(4*S*)-FP] incorporation resulted in insoluble protein. The bioinformatics analysis revealed that (4*R*)-FP incorporation at Pro63 caused fluorescence loss due to the steric hindrance of fluorine atom of (4*R*)-FP with the chromophore. Therefore, Pro63 residue was mutated with the smallest amino acid Ala to maintain non coplanar conformation of the chromophore and helps to retain its fluorescence with (4*R*)-FP incorporation. The incorporation of (4*R*)-FP into mRFP1-P63A showed about 2–3-fold enhancement in thermal and chemical stability. The rate of maturation is also greatly accelerated over the presence of (4*R*)-FP into mRFP1-P63A. Our study showed that a successful enhancement in the biophysical property of mRFP1-P63A[(4*R*)-FP] using non-canonical amino acid mutagenesis after mutating non-permissive site Pro63 into Ala.

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1. Introduction

In general, protein stability can be enhanced through canonical amino acid mutagenesis with either rational or irrational approaches. Even though, this methodology is successful for generating highly stable variants, it is limited due to 20 canonical amino acids [1,2]. Recently, alternative approaches were developed to enrich the stability and folding efficiency of proteins through incorporation of non-canonical amino acids (NCAA) [3,4]. Incorporation of NCAA is a fascinating tool in synthetic biology to create proteins with novel functionality [5,6]. Genetic code engineering (residue-specific or global incorporation), which reassigns the canonical amino acid of the sense codon with NCAA is the most frequently used methodology. In this method, NCAAs are isostructural to canonical amino acids and therefore recognized by the endogenous host cell machinery. The advantage of this methodology is acquiring the synergistic effect through multiple site incorporation of NCAA that can alter the biophysical properties of proteins [7–9]. For example, incorporation of methionine analogue, norleucine into P450 BM-3 Heme domain showed 2-fold enhanced peroxygenase activity [10]. On the other hand, L-proline (Pro) plays a critical role in the protein structure by forming *cis* and *trans* peptidyl-proline bond conformation. The pyrrolidine ring of Pro structure adopts two alternative conformations as the C^{γ}-exo and C^{γ}-endo puckering. The substitution of C^{γ}(C-4) fluorine atoms in (2*S*, 4*R*)-4-fluoroproline [(4*R*)-FP] and (2*S*, 4*S*)-4-fluoroproline [(4*S*)-FP] favors the C^{γ}-exo and C^{γ} endo puckering, respectively [11–13]. In the past few decades, the replacement of proline with fluoroproline (FP) was proven to tune or alter the stability, folding, and the biophysical properties of proteins such as collagen, barstar, elastin–mimetic polypeptide, FK506-Binding proteins, EGFP, small helical protein, DNA polymerase, Single chain Fv fragment and human ubiquitin by exploring the stereoelectronic effects [14–23].

Genetically encoded fluorescent proteins are extensively utilized in the field of targeted labeling and imaging of proteins, organelles, cell tissues, and whole organisms [24]. Among different fluorescent proteins, the red fluorescent protein is highly advantageous due to their longer wavelength which helps to penetrate deeper into the target and it exhibits less autofluorescence in the background during imaging [25]. The slow maturation and tendency to oligomerization is considered to be the main disadvantage of red fluorescent proteins [26]. To circumvent this problem, protein engineers used directed evolution method for generating monomeric form of variants with faster maturating property. Among them, mRFP1 is generated by introduction of 33 mutations into DSRed which showed ~10 times faster maturation speed [27].

Abbreviations: NCAA, non-canonical amino acid incorporation; FP, fluoroproline; (4*R*)-FP, (2S, 4*R*)-4-fluoroproline; (4*S*)-FP, (2*S*, 4*S*)-4-fluoroproline; MM, minimal media; GdmCl, guanidine hydrochloride.

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Here we tried to further enhance the stability and biophysical properties of mRFP1 by using FP incorporation.

2. Materials and methods

2.1. Expression and purification of Pro analogues incorporated into mRFP1 variants

The mRFP1 variants in pQE-80L was transformed into *Escherichia coli* BL21 (DE3)pLysS (KC1325) Pro auxotroph and expressed in minimal medium (MM) lacking Pro according to previously described protocols [8]. Briefly, the limiting concentration of Pro (0.05 mM) allowed the cells to attain an OD_{600} 0.6–0.8 and the target proteins were induced with 1 mM IPTG followed by simultaneous addition of Pro or (4*R*)- and (4*S*)-FP (0.5 mM) and allowed the expression for 7 h. The harvested cells were subjected to centrifugation and stored at −70 °C until further use. Briefly, collected cell pellet was suspended in lysis buffer (5 mM immidazole and 50 mM sodium-Phosphate buffer pH 7.5 containing protease inhibitor), followed by sonication and centrifuged at 16,000 rpm, 4 °C for 20 min. The supernatant was saved as a soluble protein fraction and the pellet was saved as insoluble protein fraction, and analyzed by SDS–PAGE (12% acrylamide gel). The remaining soluble protein fractions were purified by Ni–NTA column chromatography (GE Healthcare Bio-Sciences, Sweden) by standard protocol. Elution fractions were analyzed by SDS–PAGE, and those that were enriched in the desired mRFP1 variants were pooled and dialyzed against 1× phosphate buffered saline. The concentration of protein was quantified using Bradford assay. Fluorescence spectra of mRFP1 variants were recorded on a Perkin Elmer LS-55 fluorescence spectrometer equipped with digital software, Winlab.

2.2. Homology modeling

The protein sequence of mRFP1 was obtained from NCBI protein sequence databank (AAM54544). Sequence homology search was performed for the mRFP1 sequence against PDB database using BlastP. The potential template was retrieved from the protein data bank (PDB ID-2QLI) and multiple sequence alignment was carried out for the query and the template using ClustalW with default parameters. Three dimensional model was constructed based on the structural template and the sequence alignment using Modeler tool. The overhangs at the both ends were excised during the model construction. Generated models satisfy the spatial restraints on the distance and the dihedral angles. The stereo chemical quality of the model was validated using SAVES server. The resulting model structure was energy minimized and optimized using modeler. Then the structure was analyzed using pymol molecule viewer.

2.3. Circular dichroism

A far UV CD spectrum was recorded for the mRFP1, mRFP1-P63A and mRFP1-P63A[(4*R*)-FP] on a JASCO J-715 spectrometer. For the analysis, 250 μl of 3 μM protein was prepared in 1× PBS buffer (pH 7.5) and placed in a 0.2 cm cell, and the CD absorption spectrum was obtained at room temperature. Twenty scans were accumulated per spectrum and raw data were processed using Jasco software package. Finally, the obtained results were analyzed and graph was drawn by using Origin software.

2.4. Thermal and chemical stability of mRFP1 and its variants

To determine the half-life ($t_{1/2}$) of thermal stability for mRFP1, mRFP1-P63A and mRFP1-P63A[(4*R*)-FP], the protein (1 mg/ml) was incubated in 100 mM Phosphate buffer (pH 7.5) at 25, 40, 50, and 60 °C for 20 h and 10 μl was aliquot at pre-defined time and diluted to 10 μg/ml with 100 mM Phosphate buffer (pH 7.5). Then the fluorescence intensity of mRFP1was measured at 607 nm and mRFP1-P63A and mRFP1-P63A[(4*R*)-FP] were measured at 577 nm using PerkinElmer LS55 fluorescence spectrophotometer. To determine the $t_{1/2}$ of chemical stability for mRFP1, mRFP1-P63A and mRFP1-P63A[(4*R*)-FP], each protein (1 mg/ml) were incubated with either 5% SDS, 8 M urea, 6 M guanidine hydrochloride (GdmCl) in 100 mM Phosphate buffer (pH 7.5) and 10 μl was aliquot at pre-defined time and diluted to 10 μg/ml with 100 mM Phosphate buffer (pH 7.5). Then the fluorescence intensity of mRFP1was measured at 607 nm and mRFP1-P63A and mRFP1-P63A[(4*R*)-FP] were measured at 577 nm using PerkinElmer LS55 fluorescence spectrophotometer.

2.5. Maturation assay

For maturation assay in LB media, *E. coli* BL21 (DE3)pLysS (KC1325) containing mRFP1 and mRFP1-P63A were grown at 37 °C in 1 L LB media containing 10 mg/ml ampicillin. When the OD_{600} reached 0.6, the target protein was induced with 1 mM IPTG. After induction, cells were collected at different time intervals and were readily purified at 4 °C and the concentration of protein was quantified using Bradford assay [27]. The fluorescence intensity of mRFP1 variants were measured by Perkin Elmer LS-55 fluorescence spectrometer.

For maturation assay in MM medium, *E. coli* BL21 (DE3)pLysS (KC1325) Pro auxotroph containing mRFP1 variants were grown at 37 °C in 1 L MM medium containing 10 mg/ml ampicillin. Briefly, the limiting concentration of Pro (0.05 mM) allowed the cells to attain an OD_{600} 0.6–0.8 and the target proteins were induced with 1 mM IPTG followed by simultaneous addition of Pro or (4*R*)-FP analogues (0.5 mM). After induction, cells were collected at different time intervals and were rapidly purified at 4 °C and the concentration of protein was quantified using Bradford assay. The fluorescence intensity of mRFP1 variants were measured by Perkin Elmer LS-55 fluorescence spectrometer.

3. Results and discussion

3.1. Incorporation of Pro analogues into mRFP1

First, we tried to establish the incorporation condition of FP into mRFP1 with genetic code engineering method. *E. coli* BL21 (DE3)-pLysS (KC1325) Pro auxotroph harboring pQE80L-mRFP1 were grown in MM medium containing optimal limiting concentration of 0.05 mM Pro and allowed the production of cell mass up to an OD_{600} value of 0.8–1, within 6 h (Supplementary Fig. S1). Under this condition, the cultures were divided into four subsets, as follows: a medium supplemented with Pro (0.22 mM), (4*R*)-FP (0.5 mM), (4*S*)-FP (0.5 mM), and a control lacking Pro. Later, the protein expression was induced with 1 mM IPTG. The whole cell protein expression profile clearly showed that the mRFP1expression level was comparable with the protein expressed with (4*R*)-FP or (4*S*)-FP. The soluble and insoluble proteins were collected from each variant and the protein expression patterns were analyzed by SDS–PAGE (Fig. 1A). The incorporation of (4*S*)-FP into mRFP1 resulted in majority of the protein were found in insoluble form, which suggests that the protein as folded improperly. Conversely, the (4*R*)-FP into mRFP1 was favored to form soluble proteins as comparable with the mRFP1. Rather surprisingly, soluble protein with mRFP1[(4*R*)-FP] was lost its fluorescence, which indicated that the protein folded properly, but the Pro residue replaced with (4*R*)-FP have affected the formation of fluorophore or the (4*R*)-FP disrupt proper interaction between the formed fluorophore

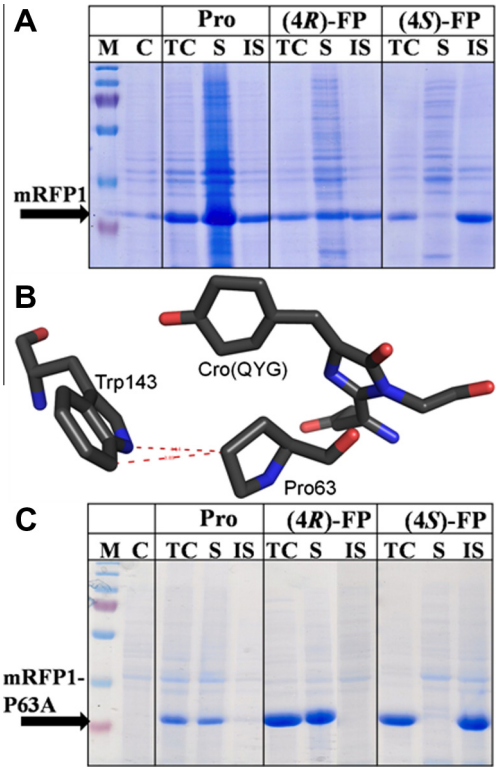

Fig. 1. (A) SDS–PAGE analysis of the expression of mRFP1 with the incorporation of proline analogues (4*S*)- and (4*R*)-FP; Lane 1; Marker, Lane 2; total cell extract of *E. coli* BL21 (DE3)pLysS (KC1325), Lane 3–5; mRFP1 expression with Pro, Lane6–8; mRFP1 expression with (4*R*)-FP, Lane 9–11; mRFP1 expression with (4*S*)-FP. M = Marker, C = Control (*E.coli* BL21 (DE3)pLysS (KC1325) carrying pQE80-mRFP1 without IPTG induction), T.C = Total cell extract, S = Soluble Protein, I.S = Insoluble protein. (B) Stick representation of the fluorophore moiety and its nearest residues Trp143 showing edge to edge interaction with Pro63. (C) SDS–PAGE analysis of the expression of mRFP1-P63A with the incorporation of proline analogues (4*S*)- and (4*R*)-FP; Lane 1; Marker, Lane 2; total cell extract of *E. coli* BL21 (DE3)pLysS (KC1325), Lane 3–5; mRFP1-P63A expression with Pro, Lane6–8; mRFP1-P63A expression with (4*R*)-FP, Lane 9–11; mRFP1-P63A expression with (4*S*)-FP. M = Marker, C = Control (*E.coli* BL21 (DE3)pLysS (KC1325) carrying pQE80-mRFP1-P63A without IPTG induction), T.C = Total cell extract, S = Soluble Protein, I.S = Insoluble protein.

and surrounding residues in mRFP1. Moreover, it is notable that when (4*S*)- and (4*R*)-FP were incorporated into EGFP, the (4*S*)-FP incorporation led to a soluble fluorescent protein, whereas the (4*R*)-FP incorporation into EGFP resulted in colorless, indicating deposition of unfolded non-fluorescent protein in inclusion bodies [19]. In mRFP1 case, (4*R*)-FP incorporation gave soluble protein but it lost its fluorescence. Even though EGFP and mRFP1 are structurally similar, this result is unexpected and need to be studied furthermore to understand and is currently under investigation.

3.2. Homology modeling to find non-permissive site for the fluorescent loss in mRFP1[(4R)-FP]

The residue-specific incorporation globally replaces either one of the natural amino acid residue with a NCAA into a target protein usually occurs at multiple sites. Therefore, incorporating NCAA into a non-permissive site or removing an essential natural amino acid often leads to a substantial loss of folded structure and activity in the target protein. To find out non-permissive site is a big challenge in protein engineering with residue-specific incorporation of NCAA. As mentioned above, the incorporation of (4*R*)-FP into mRFP1 resulted in soluble but lost its fluorescence. The replacement of Pro residue with (4*R*)-FP at specific residue (non-permissive site) may interrupt the formation of fluorophore or fluorophore interaction with the surrounding residue which is necessary for the fluorescence. If the non-permissive Pro residue of mRFP1 is replaced with other canonical amino acid, which mutant possibly give fluorescence protein with the (4*R*)-FP incorporation. To find out non-permissive Pro residue, we constructed a 3D model for the mRFP1 using the Modeller (Supplementary Fig. S2). The constructed model depicts that the mRFP1 contains 12 Pro residues, of which 7 residues are surface exposed and the remaining 5 residues are buried inside (Supplementary Table. S1). We believe that the formation of the new interactions with the neighboring amino acids by the (4*R*)-FP may result in the loss of fluorescence. It is reported that the fluorine atom in (4*R*)-FP able to establish new interactions with the atoms of the surrounding residues [22]. To investigate it, we structurally replaced the hydrogen atom of the proline with fluorine atoms using Discovery studio 2.1. Prior to the structural analysis, we have energy minimized the protein structure. Then the distance between the fluorine atom and the indolinone ring of tryptophan and the chromophore tyrosine is measured. Among them the Pro63 residue present near the fluorophore and forming hydrophobic interaction with p-hydroxy phenyl ring of fluorophore and its nearby Trp143 residue (Fig. 1B). There is steric hindrance between the fluorine atom of (4R)-FP at Pro63 and fluorophore which might be the reason for the loss of fluorescence.

To circumvent this problem non-permissive site Pro63 was subsequently mutated with canonical amino acid which might help in retaining the fluorescence and (4*R*)-FP accommodation. The Pro63 is highly conserved among all the red fluorescent protein [28], first we chose to replace with P63T (based on the sequence alignment with EGFP (PDB ID: 2Q6P). However, the expression of the mRFP1-P63T in LB medium resulted in insoluble form of protein, which might be due to steric hindrance by the T63 towards the fluorophore. The methyl side chain of the T63 resides very closely to the fluorophore, which might have affected the fluorophore formation or through the non coplanar conformation of the fluorophore (Supplementary Fig. S4). Subsequently, we mutated P63 residue with the smallest amino acid Ala to maintain non coplanar conformation of fluorophore (Supplementary Fig. S5). The mRFP1-P63A gave soluble protein but it alters the absorbance spectrum of the protein by ~28 nm (556 nm) compared to that of wild-type mRFP1 (584 nm) (Fig. 2A). Further, the mRFP1-P63A mutant showed a distinct blue-shift in its emission maximum (587 nm) of ~20 nm compared to that of wild-type mRFP1 (607 nm) (Fig. 2B). In earlier report that the chromophore p-hydroxy phenyl group of far red fluorescent protein mCherry was stabilized in the non-coplanar conformation by forming van der Waals interactions with the Pro63 and Trp143 [29]. Therefore, the fluorescence spectrum of mRFP1-P63A was altered when compared with mRFP1.

3.3. The thermal and chemical stability of mRFP1 and its variants

Next, we tried to incorporate (4*R*)-FP into mRFP1-P63A in which expected non-permissive Pro residue is replaced with Ala as described above. As expected, *E. coli* BL21 (DE3)pLysS (KC1325) Pro auxotroph expressing mRFP1-P63A[(4*R*)-FP] gave fluorescence (Fig. 1C). Subsequently, we purified the mRFP1-P63A and mRFP1-P63A[(4*R*)-FP] protein through Ni–NTA affinity column at 4 °C (Supplementary Fig. S6). The incorporation of mRFP1-P63A[(4R)-FP] (32 mg/l) showed high level of expression compared to that of mRFP1 (25 mg/l) and mRFP1-P63A (26 mg/l). The introduction of (4*R*)-FP into mRFP1-P63A showed similar kind of fluorescence pattern with mRFP1-P63A (Fig. 2A and B). Further the ESI-MS/MS analysis confirmed that residue-specific incorporation of (4*R*)-FP into mRFP1-P63A (Supplementary Table S2). CD spectroscopy analysis revealed the majority of sharp negative major deflection around 218–220 nm and minor deflection around 230 nm in

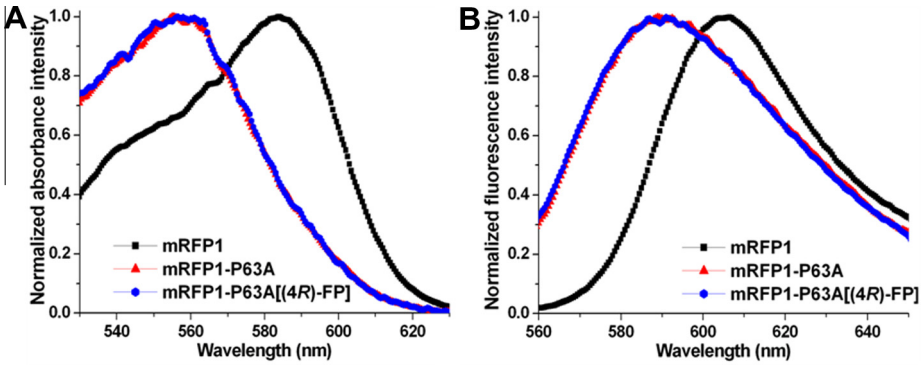

Fig. 2. Fluorescence spectra of mRFP1 and its variants excitation (A) and emission spectra (B).

mRFP1, mRFP1-P63A and mRFP1-P63A[(4*R*)-FP], respectively. These dichroic profiles confirmed that the overall secondary structural characteristics of the protein remained unaltered even after incorporation of (4*R*)-FP (Supplementary Fig. S7). Finally we found and demonstrate that Pro63 is non-permissive site for FP replacement by using structural analysis.

In the case of EGFP, incorporation of (4*S*)-FP showed faster refolding than parent EGFP [19]. Here, we also expected that (4*R*)-FP incorporation into mRFP1-P63A will enhance the stability of the protein even though the opposite chiral FP was incorporated into mRFP1-P63A. To substantiate the effect of (4*R*)-FP in the Pro sites of mRFP1-P63A, we analyzed the thermal stability at 25, 40, 50, and 60 °C by measuring the fluorescence intensity. The thermal residual activity of mRFP1-P63A[(4*R*)-FP] showed an increasing stability when compared to that of the mRFP1 and mRFP1-P63A, at 25, 40, 50, and 60 °C. The $t_{1/2}$ of mRFP1 at 25, 40, 50, and 60 °C were 5.6, 5.4, 5.01, and 3.01 h, respectively. The $t_{1/2}$ of mRFP1-P63A at 25, 40, 50, and 60 °C were 7.05, 6.48, 6.17, and 4.28 h, respectively. It is interesting that mRFP1-P63A showed higher thermal stability than wild-type mRFP1. For example, the $t_{1/2}$ of mRFP1-P63A (4.28 h) at 60 °C was about 1.4-fold higher than that of mRFP1(3.01 h). The $t_{1/2}$ of mRFP1-P63A[(4R)-FP] at 25, 40, 50, and 60 °C were 16.77, 15.65, 13.25, and 9.28 h, respectively (Fig. 3). The successful replacement of Pro residue with (4*R*)-FP into mRFP1-P63A, dramatically enhanced the thermal stability at all tested temperature. For example, the $t_{1/2}$ of mRFP1-P63A[(4*R*)-FP] (9.28 h) at 60 °C was about 2.2-fold higher than that of parent mRFP1-P63A (4.28 h). Moreover, the $t_{1/2}$ of mRFP1-P63A[(4R)-FP] (9.28 h) at 60 °C was about 3.1-fold higher than that of wild-type mRFP1 (3.01 h). These results clearly showed that the thermal stability of mRFP1 was enhanced by (4*R*)-FP incorporation.

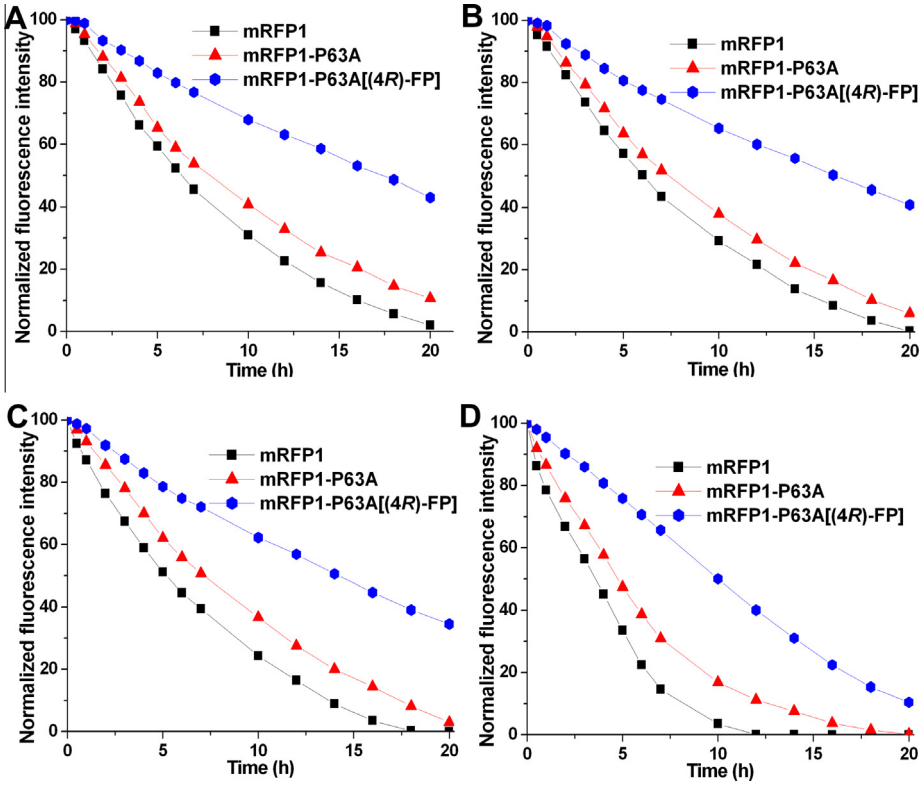

Fig. 3. Thermal stability of mRFP1 and its variants. Reaction conditions: each protein (1 mg/ml) was incubated in 100 mM Phosphate buffer (pH 7.5) at 25 (A), 40 (B), 50 (C) and 60 °C (D) for 20 h and 10 μl was aliquot at pre-defined time and diluted to 10 μg/ml with 100 mM Phosphate buffer (pH 7.5).

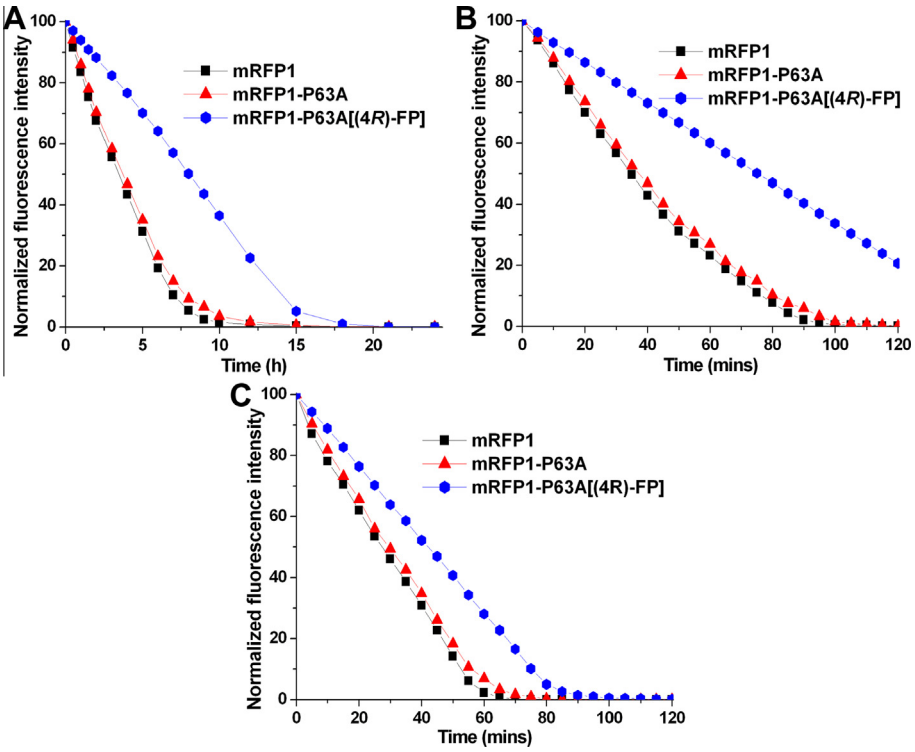

Fig. 4. Chemical stability of mRFP1 and its variants. Reaction conditions: each protein (1 mg/ml) were incubated with either 5% SDS (A) 8 M urea (B) 6 M guanidine hydrochloride (GdmCl) (C) in 100 mM Phosphate buffer (pH 7.5) and 10 μl was aliquot at pre-defined time and diluted to 10 μg/ml with 100 mM Phosphate buffer (pH 7.5).

Further, the chemical stability of mRFP1, mRFP1-P63A and mRFP1-P63A[(4*R*)-FP] were examined in the presence of 5% SDS in 1× PBS buffer (pH7.5). The $t_{1/2}$ of mRFP1 and mRFP1-P63A was about 3.5 h, whereas that of mRFP1-P63A[(4*R*)-FP] was about 8.0 h (Fig. 4A). The mRFP1-P63A[(4*R*)-FP] showed about 2.3-fold enhanced stability in the presence of 5% SDS. In addition, the stability of proteins was examined in the presence of denaturant such as Urea and GdmCl. The $t_{1/2}$ of mRFP1, mRFP1-P63A and mRFP1-P63A[(4*R*)-FP] in the presence of 8 M Urea were about 35, 35 and 75 min, respectively (Fig. 4B). The $t_{1/2}$ of mRFP1, mRFP1-P63A and mRFP1-P63A[(4*R*)-FP] in the presence of 6 M GdmCl were about 25, 25, and 40 min, respectively. The mRFP1-P63A[(4*R*)-FP] showed about 2.1 and 1.6-fold enhanced stability than parent mRFP1-P63A and wild-type mRFP1 with 8 M Urea and 6 M GdmCl, respectively (Fig. 4C). These results clearly showed that the chemical stability of mRPP1 was enhanced by (4*R*)-FP incorporation.

3.4. The maturation study of mRFP1 and its variants

The major drawback of DSRed is the slow maturation time, among its variants, mRFP1 showed faster maturation time. Here, we intend to analyze the maturation time of mRFP1-P63A and mRFP1-P63A[(4*R*)-FP]. We performed maturation assay at 37 °C in MM medium, the mRFP1-P63A[(4*R*)-FP] showed faster maturation time when compared to that of the mRFP1 and mRFP1-P63A. The maturation of mRFP1 and mRFP1-P63A in MM was ~6 h. However, the half-life of maturation of mRFP1-P63A[(4*R*)-FP] in MM was ~5 h (Supplementary Fig. S8). These results indicate that (4*R*)-FP incorporation helps for the faster folding of the protein. The mRFP1 and mRFP1-P63A showed similar pattern of maturation assay in the MM medium. In order to confirm it we performed the maturation assay in LB broth. In the case of LB broth, the pattern was similar and the half-life of mRFP1 and mRFP1-P63A was ~1 h (Supplementary Fig. S9). Overall, the replacement of Pro residue with (4*R*)-FP into mRFP1-P63A, showed enhanced thermal and chemical stability and subsequently, it showed that the rate of maturation is greatly accelerated over that of mRFP1 and mRFP1-P63A.

In summary, in order to enhance the stability of mRFP1, (4*S*)- and (4*R*)-FP were incorporated into mRFP1. The (4*S*)-FP incorporation led to insoluble protein, whereas (4*R*)-FP incorporation into mRFP1 resulted in soluble but lost its fluorescence. The bioinformatics analysis revealed that Pro63 residue is non-permissive site for the replacement with (4*R*)-FP, the steric hindrance between the fluorine atom of (4*R*)-FP at Pro63 and fluorophore was the reason for the loss of fluorescence. To overcome this problem, mRFP1-P63A mutant was generated and (4*R*)-FP incorporation into mRFP1-P63A give soluble protein with fluorescence. To the best of our knowledge, this is the first study find out the non-permissive site for the incorporation of NCAA which was successfully overcome by canonical amino acid mutagenesis. The successful design of a protein with a novel (4*R*)-FP into mRFP1-P63A showed enhanced thermal and chemical stability along with faster maturation of protein. Finally we enhanced the biophysical property of mRFP1-P63A[(4*R*)-FP] using non-canonical amino acid mutagenesis after mutating non-permissive site P63A.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.062.

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