Accelerated Publications

High Stability of *Discosoma* DsRed As Compared to *Aequorea* EGFP[†]

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ABSTRACT: Comparative analysis of conformational stabilities was performed for two widely used genetic reporters, EGFP and DsRed, proteins exhibiting similar β-can folds, but possessing different oligomeric organization and chromophore structures. Two factors affecting protein stability *in vitro*, such as elevated temperatures and a chaotropic agent guanidine hydrochloride, were studied. *In vivo* tolerance of the fluorescence proteins to proteasomal-based degradation was studied in insect and mammalian cells, and in *Xenopus* embryos. The apparent rate constants of thermal and GdmCl-induced denaturation were several orders of magnitude lower for DsRed than for EGFP. DsRed lifetimes severalfold longer than those of EGFP were observed in cultured cells and in embryos. The remarkable fluorescence stability of DsRed under the all conditions that have been studied is attributed to a significant extent to its tetrameric organization. Therefore, DsRed can be used as a genetic reporter and advanced population marker with a significantly extended intracellular lifespan.

Green fluorescent protein (GFP)[1] from the jellyfish *Aequorea victoria* is a widely used reporter in studies of gene expression, protein dynamics, and localization (*1*). Crystallographic structures of wild-type GFP and its enhanced mutants (ECFP, EGFP, and EYFP) have revealed that the GFP tertiary structure resembles a β-can (*2*, *3*). GFP fluorescence was shown to be rather stable under a variety of conditions, including treatment with detergents and pH (*4*), proteases (*5*), and temperature (*6*).

The recent discovery of a red GFP-like protein DsRed (*7*) from corallimorph *Discosoma* sp. and development of its improved mutants, DsRed-Timer (*8*), DsRed2 (*9*), and fast-maturating DsRed-Express (*10*), have significantly increased the range of FP applications, including multicolor protein tagging (*11*), intracellular reporting (*12*), and resonance energy transfer (*13*). Although the DsRed monomer folds, like GFP, into a β-can (*15*), both sedimentation (*16*, *17*) and crystallographic studies (*14*, *15*) have revealed that DsRed forms a tight tetramer with a nanomolar association constant (*17*). The tetramerization is probably one of the causes of the stability of DsRed being higher than that of GFP under mildly acid and alkaline conditions (*16*, *17*). To date, ∼30 distinct GFP-like proteins have been cloned (*18*). As for DsRed, many of them have obligate tetrameric structures (*9*), but the biochemical properties and role of their oligomerization for functioning are not yet known.

Fluorescence acquisition and stability of FPs under various physical and chemical conditions are important for biotechnological use of these reporters. Some *in vitro* properties of *Renilla* GFP (*19*) and DsRed are known (*13*, *16*, *17*), although systematic studies and comparison with *Aequorea* GFP mutants have not been performed. The fluorescence changes and lifetimes of FPs should also be taken into account to quantify the spatial and temporal events inside cells and model organisms. So far, only the intracellular lifetime of EGFP has been determined, and several its destabilized variants have been constructed (*20*, *21*).

To expand potential applications of DsRed as a complement to the EGFP reporter with a distinct color, we have

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[1] Abbreviations: GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; DsRed, red fluorescent protein; FP, fluorescent protein; GdmCl, guanidine hydrochloride.

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Table 1: Rate Parameters for the Kinetics of EGFP and DsRed Fluorescence Decreases

	k_{fT} (s^{-1})			k_{fG} (s^{-1})			S2, $t_{50\%}$ (days)	
protein	37 °C	55 °C	80 °C	4.0 M GdmCl	5.6 M GdmCl	6.2 M GdmCl	without Inh	with Inh
EGFP	8.25×10^{-8}	4.82×10^{-6}	2.12×10^{-4}	1.49×10^{-3}	3.36×10^{-3}	1.29×10^{-2}	3.8	1.0
DsRed	3.64×10^{-7}	6.39×10^{-7}	2.06×10^{-5}	not determined	1.62×10^{-8}	4.73×10^{-7}	8.1	4.6

employed two destabilizing factors *in vitro*, temperature and GdmCl, and have quantified the fluorescence changes. Their *in vivo* stabilities have been compared as this an important factor for the genetically encoded FP reporters.

MATERIALS AND METHODS

Plasmid Construction. The plasmids encoding EGFP and DsRed1 with polyhistidine tags were constructed as described previously (*12*) and were transfected into *Escherichia coli* BL21(DE3) (Invitrogen). For expression in *Drosophila* S2 cells, the PCR-amplified cDNAs of DsRed2 (from pDsRed2-N1, Clontech) and EGFP were inserted into *Kpn*I−*Bam*HI sites of the pRmHa-3 vector (*23*), carrying metallothionein promoter, which resulted in pRmHa-3-DsRed2 and pRmHa-3-EGFP plasmids, respectively. For *Xenopus* studies, EGFP and DsRed1 cDNAs were subcloned into the p35T vector which resulted in p35T-EGFP and p35T-DsRed1 plasmids (*7*).

Recombinant Proteins and Their Analysis. The FP expression in *E. coli* was induced by 1 mM IPTG (Nacalai tesque) over the course of 24 h at 37 °C, and proteins were purified with Ni−NTA agarose (Qiagen). The samples were at least 95% pure according to SDS−PAGE. Protein concentrations were determined with the Bio-Rad protein assay kit, and adjusted to 0.05 mg/mL in 50 mM Tris-HCl buffer (pH 8.0). For thermal inactivation, samples were incubated at elevated temperatures; aliquots were removed at intervals, and diluted 10-fold in the same buffer at 23 °C. For denaturation in GdmCl (Nacalai tesque), its concentrations were determined by refraction indexes using an Abbe refractometer (LOMO).

Fluorescence Spectroscopy. EGFP and DsRed1 were excited at 480 and 555 nm, and emission was detected at 510 and 585 nm. The fluorescence spectrophotometers described in ref *24* and model F-2500 (Hitachi) were used for steady-state spectroscopic analysis. Measurements were performed with samples adjusted to 23 °C.

Cell Culture. S2 cells were cultured at 25 °C in Schneider's medium (GibcoBRL) with 10% FCS (GibcoBRL) and cotransfected with pRmHa-3-DsRed2, pRmHa-3-EGFP, and pPC4 (*25*) (carrying α-amanitin resistant gene) plasmids in a 10:10:1 ratio as described previously (*25*, *26*). Stable S2 transfectants were selected by 5 μg/mL α-amanitin (Nacalai tesque) for 2 weeks. Human HEK293 cells (ATCC) were maintained in DMEM medium (GibcoBRL) with 10% FBS (Sigma) at 37 °C. The cells were transiently cotransfected with pDsRed1-N1 and pEGFP-N1 plasmids (Clontech) using FuGENE-6 (Roche).

To quantify EGFP and DsRed at different time points, 10^7 S2 cells were washed with D-PBS buffer (GibcoBRL), lysed by sonication, and centrifuged at 12000*g*. The fluorescence intensity of the supernatant was normalized per total protein concentration. Inhibitors of transcription and translation, actinomycin D (Sigma) and cycloheximide (Sigma), were used for cell treatment at 1 and 100 μg/mL, respectively. The viability of cells was determined to be more than 90% by the blue fluorescence of suspended aliquots stained with DAPI (excitation at 359 nm and emission at 461 nm) (Molecular Probes), and confirmed by Trypan Blue exclusion.

Xenopus Experiments. p35T-EGFP and p35T-DsRed1 plasmids were digested with *Eco*RI, and synthetic mRNAs were transcribed using the SP6 Message Machine kit (Ambion). The mRNAs were purified with the RNeasy column kit (Qiagen) and microinjected into embryos (1 ng per blastomere). To observe the expression of FPs, the cultured cells or *Xenopus* embryos were photographed using an MZIII fluorescent microscope (Leica). EGFP and DsRed1 fluorescence was acquired by standard FITC (excitation at 490/20 nm and emission at 530/30 nm) and TRITC (excitation at 550/20 nm and emission at 580/30 nm) filter sets.

Data Analysis. For quantification of kinetics of FP unfolding, a two-state model was applied:

$$\mathrm{N} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \mathrm{A}$$

where N and A represent the native and affected state of the FP and k_f and k_r are the forward and reverse rate constants, respectively. In cases of thermal and GdmCl unfolding, the forward rate constants (k_{fT} and k_{fG}, respectively) were calculated to be equal to a relative initial rate of fluorescence decrease:

$$k_f = -\frac{1}{I_0}\frac{dI}{dt}\bigg|_{t=0}$$

where I_0 is the intensity at time zero and the initial slope is determined from the tangent. The time for a half-maximal fluorescence decrease ($t_{50\%}$) in S2 cells was determined assuming $k_r = 0$. The maximal fluorescence intensity of each FP was taken to be 100%. Calculations and fitting were performed with Origin (Microcal).

RESULTS

Conformational Stability of EGFP and DsRed in Vitro

Kinetics of Thermal Denaturation. Elevated temperatures in the range of 37−80 °C caused rather slow changes in the fluorescence intensities of FPs. Furthermore, DsRed lost its fluorescence significantly more slowly than EGFP did (Figure 1A). Incubation of both proteins at lower temperatures (room temperature and colder) did not result in intensity changes within 1 month. The time necessary to cause a similar extent of fluorescence decrease was usually ~1 order of magnitude longer for DsRed than for EGFP (Figure 1A). This is reflected well in the determined unfolding rate constants, k_{fT} at 55 and 80 °C (Table 1), although at 37 °C the difference in k_{fT} for DsRed and EGFP was smaller (4.4 times).

Kinetics of GdmCl-Induced Unfolding. The difference in the stability of FPs was even more striking in the case of

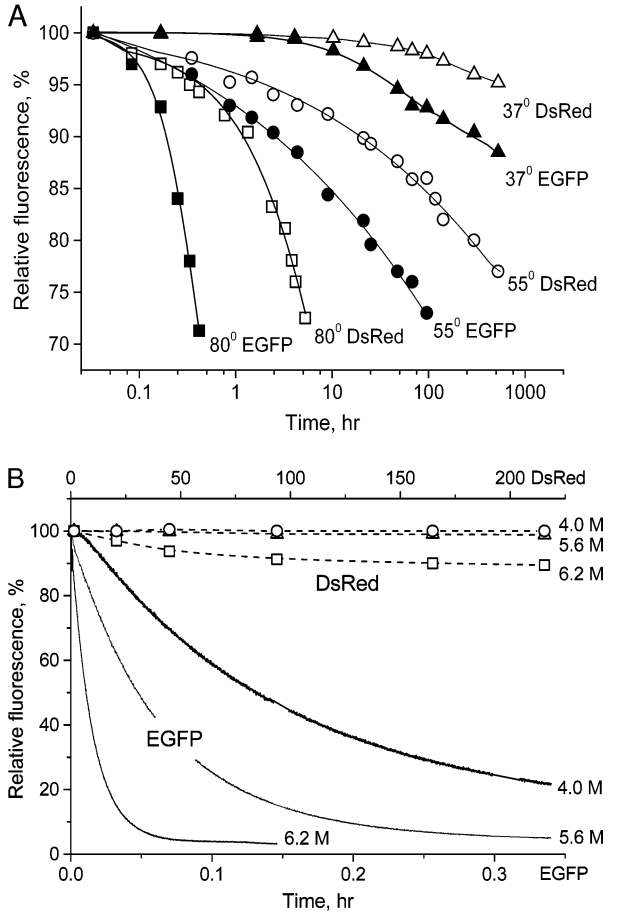

FIGURE 1: Behavior of EGFP and DsRed at elevated temperatures and in GdmCl. (A) Kinetics of fluorescence changes for EGFP (filled symbols) and DsRed (empty symbols) for selected temperatures are shown: 80 (squares), 55 (circles), and 37 °C (triangles). (B) Fluorescence changes accompanying protein denaturation in GdmCl for EGFP (—) and DsRed (- - -) at chaotropic agent concentrations of 4.0, 5.6, and 6.2 M.

GdmCl treatment at 23 °C. Figure 1B shows that EGFP notably unfolds in the concentrated GdmCl solution, whereas incubation of DsRed at concentrations of <5.6 M does not cause any fluorescence changes (at least within the time interval presented herein). Kinetically, the unfolding of EGFP is a uniexponential process (*22*) with the half-time decreasing with the increase in denaturant concentration (11.2, 4.96, and 1.29 min for 4.0, 5.6, and 6.2 M GdmCl, respectively). On the other hand, DsRed lost ~11% of its fluorescence intensity after incubation for more that 200 h in the presence of 6.2 M GdmCl. The estimated unfolding rate constants (k_{fG} for 5.6 and 6.2 M GdmCl) were 5 orders of magnitude lower than that of EGFP (Table 1). At the same time, the increase in temperature up to 80 °C during incubation in 6.2 M GdmCl caused the complete loss of DsRed fluorescence (not shown).

Quasi-Equilibrium Unfolding Induced by GdmCl. Unfolding curves in panels A and B of Figure 2 reflect the GdmCl-induced changes in fluorescence intensity measured for a given protein after incubation for the desired amount of time in the presence of the desired GdmCl concentration. These data have been used to plot the kinetics of the approaching of unfolding equilibrium (Figure 2C) as time courses of corresponding $C_{1/2}$ values (half-transition concentrations of

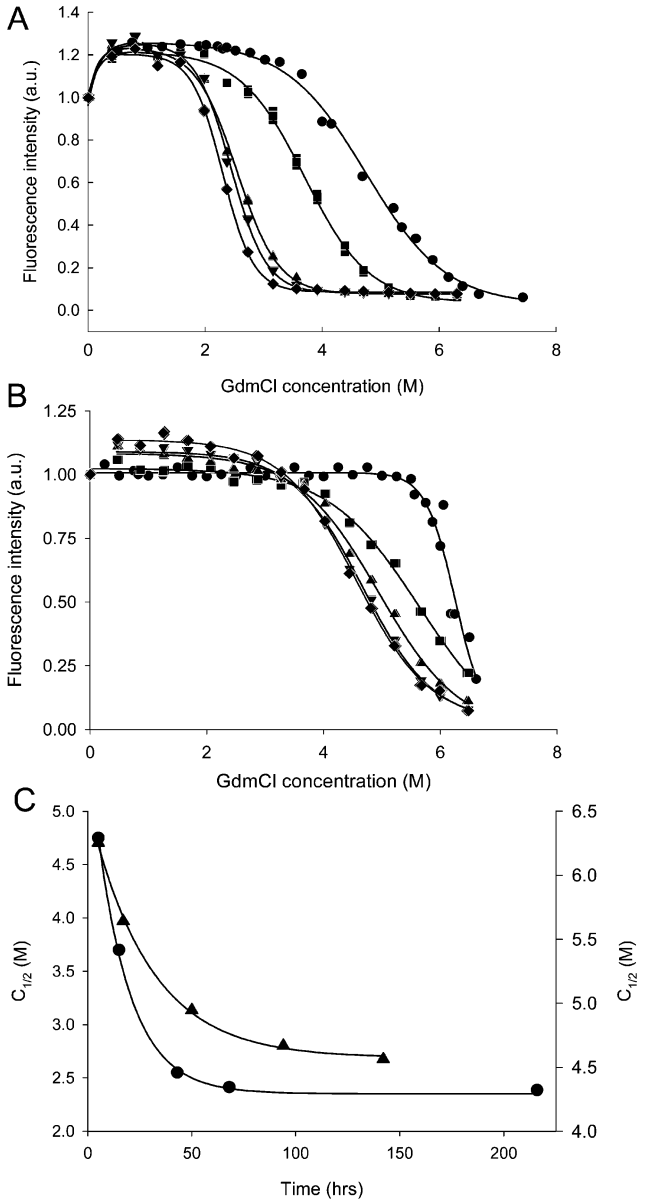

FIGURE 2: Quasi-equilibrium unfolding of EGFP and DsRed induced by GdmCl. (A) Measurements for EGFP were performed after incubation for 5 (●), 15 (■), 43 (▲), 67 (▼), and 216 h (◆) in the presence of the desired GdmCl concentration. (B) Measurements for DsRed were taken after incubation for 5 (●), 17 (■), 50 (▲), 94 (▼), and 142 h (◆) in the presence of the desired GdmCl concentration. (C) Kinetics of the approaching of unfolding equilibrium determined as $C_{1/2}$ vs incubation time dependences for EGFP (●, left axis of the ordinate) and DsRed (▲, right axis of the ordinate). $C_{1/2}$ values have been estimated from the sigmoidal fits of the corresponding data sets in panels A and B.

GdmCl). This analysis gave rate constants of $(9.59 \pm 0.76) \times 10^{-6}$ and $(17.73 \pm 0.22) \times 10^{-6}$ s^{-1} for DsRed and EGFP, respectively. Overall, data presented in Figure 2 confirm our conclusion that DsRed is considerably more stable toward GdmCl-induced unfolding than EGFP. This is manifested by its almost 2-fold higher equilibrium $C_{1/2}$ value (4.6 vs 2.4 M GdmCl), and by the 2-fold increase in the time required to reach the unfolding equilibrium.

In Vivo Stability of Fluorescent Proteins

FPs in Cell Cultures. To maintain the stable coexpression of EGFP and DsRed, we used *Drosophila* S2 cells. This was

done because these cells exhibit the post-translational modification and proteasomal-based protein degradation systems very similar to mammalian ones. They grow in suspension at 25 °C, therefore reducing the nonspecific effect of temperature on FPs. They are widely used for large-scale protein production because of the high viability and tolerance to Cu^{2+} used for regulation of metallothionein promoters (*23*). For more adequate comparison with EGFP, we used the DsRed2 mutant, which exhibits slightly faster maturation (*8*) but significantly lower cytotoxicity than DsRed1 (*9*). At the same time, this variant preserves DsRed tetrameric organization and chromophore structure (*9*). To complete the fluorescence acquisition, induction with 1 mM $CuSO_4$ for 2 h was followed by a 24 h culture. Cleared lysate of S2 aliquots was used to follow EGFP and DsRed fluorescence over time. Figure 3A shows that DsRed fluoresces longer than EGFP. The determined $t_{50\%}$ values were 3.8 and 8.1 days for EGFP and DsRed, respectively (Table 1).

To test whether the higher viability of DsRed fluorescence in the cells was due to the increased efficiency of transcription and translation or the extended life span of the folded polypeptide, we treated the cells with the inhibitors of transcription and translation, actinomycin D and cycloheximide. Both inhibitors appeared to shorten the lifespan of DsRed and EGFP fluorescence, although to different extents (data not shown). To elucidate the input of the proteasomal degradation, both inhibitors have been applied simultaneously. This resulted in an additive effect on the decrease in fluorescence (Figure 3A). The estimated half-times were ~1.0 and ~4.6 days for EGFP and DsRed, respectively (Table 1), reflecting the higher resistance of DsRed to proteasome action. $t_{50\%}$ ratios for DsRed and EGFP in the absence and presence of inhibitors were 2.1 and 4.6 times, respectively.

To exclude cell specificity as an explanation for the extended DsRed2 stability in S2 cells, and to rule out the possibility that the DsRed2 mutant behaves in a manner different from that of DsRed1, the tolerance of EGFP and DsRed1 in human HEK293 cells was studied. The increased viability of DsRed1 fluorescence was also observed in these cells transiently cotransfected with EGFP and DsRed1, both without and with inhibitors added (data not shown).

FP in Xenopus Embryos. To elucidate this effect in living organisms, we have induced expression of EGFP and DsRed1 in *Xenopus* by co-injection of their mRNAs into eight-cell embryos. The green and red fluorescence was easily observed in developing tadpoles (Figure 3B). Although the red fluorescence has only appeared on the third day because of the slower maturation of DsRed1 (*12*), it later persisted in descendants of the injected blastomeres for more than 1 month, whereas the EGFP fluorescence disappeared almost completely on the 21st day. No visible toxic effects were detected during the experiment. From the exposure times (0.055, 1.30, 3.02, and 3.32 s for 1, 3, 17, and 21 days, respectively) and brightness of EGFP and DsRed images (Figure 3B), one can conclude that the behavior of the FPs in *Xenopus* embryos was rather similar to that observed in insect S2 and human HEK293 cells without inhibitors.

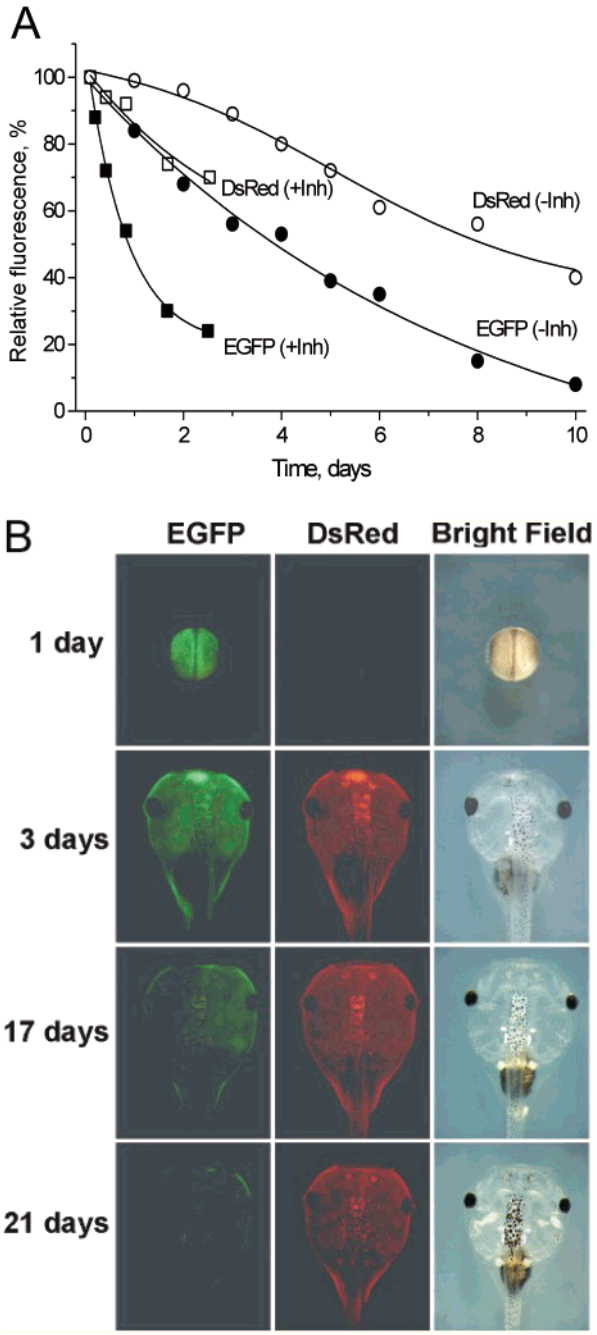

FIGURE 3: (A) Fluorescence stability of EGFP (filled symbols) and DsRed (empty symbols) after termination of induction of the FP expression in *Drosophila* S2 cells with (squares, +Inh) or without (circles, −Inh) inhibitors of transcription and translation. (B) Extent of EGFP (left column) and DsRed (central column) fluorescence on the indicated days after co-injection of the mRNAs into *Xenopus* embryos. The respective bright fields are shown in the right column.

DISCUSSION

Results of unfolding studies (both kinetic and equilibrium) reflect the tightness of the packing of protein core and hydrophobic contacts. In contrast to the pressure-induced transfer of solvent molecules into the protein interior, thermal and chemical-induced unfolding results in the exposure of internal nonpolar residues to the solvent (*29*). We show here that both FPs that have been studied possess unusually slow kinetics of the approaching of the unfolding equilibrium (Figure 2). In fact, it took 3 and 6 days for EGFP and DsRed, respectively, to reach the quasi-equilibrium unfolding conditions, where DsRed was essentially more stable than EGFP. Furthermore, DsRed was shown to unfold considerably slower than EGFP at both high temperatures and high GdmCl

concentrations (see Figures 1 and 2 and Table 1). The high degree of homology in packing of internal residues in EGFP and DsRed cannot explain the outstanding DsRed stability. Furthermore, we assume that the increased DsRed stability compared to that of EGFP cannot be explained by the simple increase in size of DsRed due to its tetramerization. In other words, we do not think that the mechanical increase in size is solely responsible for the strengthening of cooperative interactions within the individual protein globule that would better resist unfolding. On the contrary, additional structure-stabilizing mechanisms need to be assumed to explain the data. These assumptions are confirmed by the analysis of the DsRed crystal structure. In fact, each monomer of DsRed buries 10.4 and 13.6 nm^2 in the interfaces due to tetramer formation (*14*, *15*). In particular, the former has a highly hydrophobic organization involving 20 residues with compact packing. The tight packing of C-termini in hydrophobic pockets of the neighboring β-barrels further stabilizes the DsRed tetramer. This specific packing may explain the high stability of the DsRed tetramer and the impossibility of its dissociation on monomers by pH (*13*, *16*), detergents, and reducing agents (*13*). GFP-like proteins from other *Anthozoa* organisms with obligate dimeric organization exhibited greater resistance to unfolding also. *Renilla muelleri* GFP had a melting temperature higher than that of EGFP (*30*). *Renilla reniformis* GFP lost its fluorescence in detergents significantly more slowly than *Aequorea* GFP (*19*). Interestingly, the characteristic family fold of fluorescent proteins, β-can, is not more compact than most other folds in terms of residue packing within the globule, as it has cavities and even several water molecules buried within the protein core (*14*, *15*). Thus, it cannot be considered a major factor determining the extremely slow unfolding kinetics of FPs. We assume that the mutual interactions between the chromophore and the reminder structure of FPs contribute to the extended lifetime of fluorescence of these proteins under the highly denaturing conditions. This question definitely requires further analysis.

Cellular proteins differ widely in their stabilities. Their turnover is caused by signals that induce protein degradation. These signals consist of protein modifications, such as phosphorylation (*31*), or protein−protein interaction (*32*). Prior to degradation, most proteins need the 76-residue ubiquitin to be attached mainly to the Lys residues, a process catalyzed by several modification enzymes of the E2 family (*33*). Only ubiquitinated proteins are recognized by the 26S proteosome, a 2000 kDa multiprotein complex, which is responsible for the ATP-dependent turnover of up to 90% of the cellular contents (*34*). A few, mainly short-lived proteins, which contain specific "degradation sequences" such as PEST and CDB (*20*, *21*), do not require attachment of the ubiquitin for degradation. Because no degradation sequences, phosphorylation sites, or interaction with endogeneous proteins was reported for GFPs and DsRed (*1*, *7*), we suppose that ubiquitination takes place during *in vivo* turnover. Although the single-DsRed polypeptide has a slightly larger amount of lysines than EGFP does, in our *in vivo* experiments the DsRed tetramer exhibited significantly higher stability (Figure 3). One possible explanation is that oligomerization hinders some of the Lys residues that are critical for ubiquitination. Indeed, in the crystal structure of tetrameric DsRed, lysines 121, 123, 158, 163, 178, and 198 are located inside of interfaces between the monomers (*15*). Another possibility is that distinct members of E2-ubiquitin conjugating enzymes with different activities are responsible for the ubiquitination of EGFP and DsRed polypeptides. It has been noted that the lifetime of green monomeric unmaturated species of DsRed before their tetramerization, which is essential for the development of the red fluorescence (*35*), is even slightly shorter than the lifetime of EGFP in mammalian cells (Y. Miwa, personal communication). To further study this phenomenon, it would be interesting to determine intracellular lifetimes of the recently developed monomeric DsRed mutant, mRFP1 (*36*), and its variants mutated at lysine residues.

The outstanding stability of DsRed as compared to that of EGFPs will allow its use *in vivo* as an advanced genetic reporter in biotechnological applications. Also, in transgenic organisms, many strains exhibit changes in their expression patterns during development. EGFP and lacZ, commonly used as reporters, do not allow a determination of whether the cells have stopped expression or just died during the course of metamorphosis. Reporters with long intracellular lifespans like DsRed would aid in addressing this problem. Recently, we have shown the different latencies in fluorescence acquisition by compounds coexpressed under the same promoter EGFP and DsRed provided information about the time after the onset of promoter activation in *Drosophila* tissues (*12*). The higher *in vivo* stability of DsRed will also provide a fluorescence timing of promoter inhibition or inactivation. If coexpression of EGFP and DsRed is suppressed shortly after the generation of certain types of cells, the fluorescence hue would reflect the order of their formation. Single-protein fluorescent timers such as DsRed-Timer (*8*) and wild-type *Anthozoa* FP-timers (*18*), which emit a green signal when they are immature, are also useful for formation-order studies. However, their green fluorescence at the constitutive expression state makes them less suitable for double labeling with GFP-based reporters. DsRed and its improved version, DsRed2 (*9*), can be used as fluorescence timers during development and as a counter stainer of EGFP in the mature state. These features are very useful in model genetic organisms, where introduction of several reporters into one animal is achieved by simply crossing transformant strains.

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