Oligomerization of DsRed is required for the generation of a functional red fluorescent chromophore

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Abstract **The coral red fluorescent protein (DsRed) absorbs and emits light at much higher wavelengths than the structurally homologous green fluorescent protein, raising questions about the properties of its chromophore. We have analyzed the relationship between the aggregation state and fluorescence of native, 6-histidine-tagged, or maltose-binding protein-fused DsRed. In all cases, newly synthesized DsRed molecules were largely monomeric and devoid of covalently closed chromophores. Maturation in vitro induces the expression of red fluorescent chromophores but only in oligomeric forms of the protein, whereas monomers are essentially devoid of fluorescence. NaOH-denatured samples demonstrated a generalized breakdown of the DsRed oligomers to monomers, which refolded after neutralization into weakly green fluorescent and still monomeric species. Red fluorescent chromophores were regenerated only upon oligomerization. These findings demonstrate that 'red' chromophores form and are functional only as oligomers, and suggest that the smallest red fluorescent functional unit is a dimer. A comparison of alkali-, acid- and guanidinium-denatured DsRed indicates that stabilization of the DsRed chromophore by concerted steps of folding and oligomerization may play a critical role in its maturation process.** **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Green fluorescent protein; Red fluorescent protein; Chromophore; Protein folding; Fluorescent energy transfer

1. Introduction

A homolog of the green fluorescent protein (GFP) gene that encodes a red fluorescent protein (drFP583, DsRed) has been cloned from the *Discosoma* coral [1]. In comparison to GFP, DsRed has excitation and emission spectra shifted significantly to the red [1], and is thus of great interest for multicolor labeling or fluorescent energy transfer (FRET) detection in living cells. However, the application of DsRed has been disappointing because of the low levels of fluorescence it exhibits in vivo and its relatively long maturation time. DsRed can also prove toxic to expressing cells because of a strong tendency to aggregate [2–5]. The recently obtained mutants DsRed-2 [6,7] and DsRed.T [8]) have provided only a partial solution to these problems, inasmuch as these proteins demonstrate greater fluorescence and a reduced maturation time, yet exist predominantly in tetrameric forms.

These findings have raised questions as to the mechanism by which the red fluorescence of DsRed is generated. Recent articles have demonstrated that the DsRed chromophore is extended by a double bond, compared to GFP, and postulate that this modification contributes significantly to the observed hypsochromic effect [3–5]. In this work, we demonstrate that the cyclization–oxidation of the DsRed chromophore requires aggregation of the DsRed monomers, and we present evidence for a dimeric nature of the smallest fluorescent isoform of DsRed. These findings support a model according to which both folding and oligomerization (aggregation) of DsRed are required for stabilization of the chromophore, and thus play a critical role in the maturation of the fluorophore.

2. Materials and methods

2.1. DsRed protein expression and purification

The pDsRed-N1 expression vector (Clontech) was utilized to express the full-length DsRed cDNA [1] in *Escherichia coli*. The coding sequence of DsRed was also inserted into the pRSETa vector (Invitrogen), thereby adding six histidines to its amino-terminus (6His tag). The expression of native or 6His-tagged DsRed in *E. coli* BL21(DE3) cells was induced by 1 mM IPTG for various lengths of time (3–24 h). To purify DsRed-6His the clarified cell lysate was adsorbed on Ni-NTA agarose overnight at 4°C, and DsRed-6His was eluted with 250 mM imidazole. The eluted fractions were dialyzed against 100 mM Tris, pH 8.5, 100 mM NaCl overnight.

The pMalE-DsRed vector, expressing DsRed fused to maltose-binding protein (MBP-DsRed), was obtained through the courtesy of Dr. Jered Floyd (Massachusetts Institute of Technology). MBP-DsRed expression was induced in XL1 Blue with 1 mM IPTG at 37°C for 5–48 h. Bacteria were lysed by sonication in phosphate-buffered saline, 1 mM phenylmethylsulfonyl fluoride, and lysates were incubated for 1 h with amylose resin (New England BioLabs), washed with 20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 10 mM β-mercaptoethanol (β-ME), 1 mM ethylenediaminetetraacetic acid, and eluted in the same buffer supplemented with 10 mM maltose.

Expression vectors for GFP and enhanced GFP (EGFP) were obtained from Clontech or were generated as described [9–11]. GFP and EGFP were purified over Ni-NTA agarose columns as described above.

2.2. PAGE analysis

Coomassie blue staining of polyacrylamide gels and absorbance at 558 nm (ε_{558} = ~75 000 cm^{-1} M^{-1} [4,5]) were used to quantify DsRed

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Abbreviations: GFP, green fluorescent protein; DsRed, red fluorescent protein; MBP, maltose-binding protein

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in whole-cell lysates. Fluorescent bands in gels were detected under UV light. Low molecular weight (17-0446-01, Pharmacia Biotech) and prestained (SDS-7B, Sigma) markers were used as size standards. Fluoresceinated bovine serum albumin (BSA), IgGs or native GFP were used as fluorescent size standards. Denaturation and renaturation assays and in vitro maturation studies of DsRed and GFP were performed as described below.

2.3. Absorption and fluorescence spectroscopy

Light absorption and fluorescence measurements were obtained with a Uvikon 860 spectrophotometer and a CM1T11I Spex spectrofluorimeter (Spex Industries, Edison, NJ, USA), respectively.

3. Results

3.1. Expression of DsRed in E. coli

Preliminary analysis confirmed that the maturation of native and tagged DsRed to a functional chromophore is very slow compared to that of GFP [2–5] (Figs. 1–3 and data not shown). SDS–PAGE analysis showed the appearance of DsRed protein 2 h after induction, but fluorescence was first detected only after 4–8 h of further incubation. DsRed fluorescence became significant 18–22 h after induction (10–12 h for MBP-DsRed), and reached a plateau at 48 h after induction (30 h for MBP-DsRed). The recombinant DsRed, His-tagged DsRed and MBP-DsRed accumulated in bacteria to fairly high levels (up to 60% of the total protein). A significant fraction of the native DsRed (up to 50%) was found in the insoluble fraction after centrifugation of bacterial lysates. Conjugation of DsRed to MBP increased its solubility to 85–90% of the total.

3.2. Fluorescence properties of DsRed

Light absorption and fluorescence were assessed in purified fractions of DsRed-6His and MBP-DsRed and in pre-cleared bacterial lysates in the case of native DsRed. The absorption peaked at 558 nm with shoulders at 485 and 525 nm and the emission peaked at 583 nm, in agreement with previous findings [1]. No significant spectral differences were observed between DsRed, DsRed-6His, and MBP-DsRed (Fig. 3), indicating that the protein tags did not alter the absorption properties of the red chromophores.

3.3. Protein size estimate by SDS–PAGE

DsRed fluorescence persists upon exposure to SDS. Thus, purified DsRed and whole bacterial lysates could be analyzed by SDS–PAGE (Fig. 1A) with retention of fluorescence in the protein bands. Denatured samples of purified MBP-DsRed demonstrated a single major band with the expected molecular weight (69 kDa) and minor breakdown products. Unboiled samples demonstrated a weaker Coomassie blue staining at this position plus three bands at much higher mass positions (210 kDa, corresponding to MBP-DsRed tetramers, and greater). Notably, the higher oligomeric species exhibited bright red fluorescence, whereas no fluorescence was detectable for the MBP-DsRed monomers (Fig. 1B). Essentially

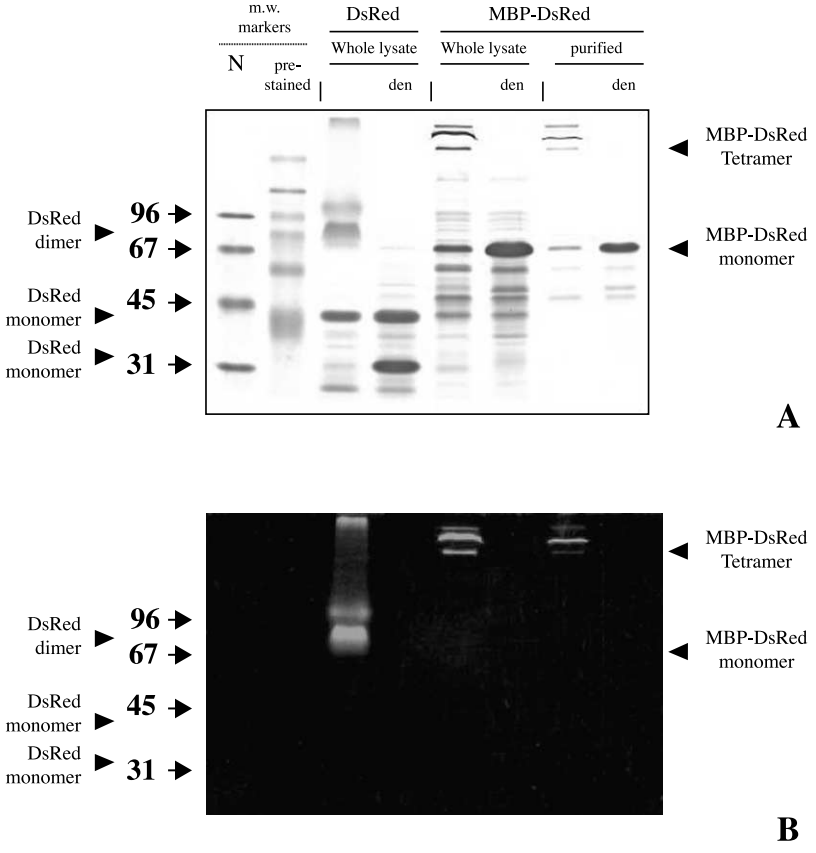


Fig. 1. SDS–PAGE analysis of native or denatured DsRed and MBP-DsRed. Purified MBP-DsRed and lysates of DsRed or MBP-DsRed expressing bacteria were analyzed at 48 h after induction of synthesis with IPTG. A: Coomassie blue staining. B: Fluorescence analysis. No fluorescence corresponding to the DsRed and MBP-DsRed monomers is detectable. Non-denatured samples demonstrate fluorescence in aggregated DsRed and MBP-DsRed. Corresponding denatured samples are indicated. Size markers: 94, 67, 43, 30, 20, 14 kDa.

identical results were obtained in whole-cell lysates expressing MBP-DsRed, thus excluding artefacts arising in the purification procedure.

Native DsRed monomers migrated as doublets at 30 and 40 kDa under denaturing conditions (Fig. 1, fourth lane). Comparison with the unboiled sample (preceding lane in Fig. 1A) and with GFP run under identical conditions indicated that the 40 kDa band arose from the renaturation of DsRed during electrophoresis, as happens with GFP ([12] and references therein). Non-denatured samples demonstrated several DsRed bands corresponding to dimers, tetramers and higher-order aggregates. Notably, no fluorescence was demonstrated by native or tagged DsRed monomers.

The electrophoretic data provide compelling evidence that red fluorescence is displayed only by DsRed oligomers. The aggregates of MBP-DsRed remained predominantly tetrameric or aggregated to higher-order structures. On the other hand, the lower resistance of native DsRed to SDS induced a significant breakdown of its tetrameric structure [3–5,13,14], and a release of dimers and monomers. Intriguingly, monomers lacked any detectable red fluorescence, with the smallest red fluorescent band appearing to correspond to DsRed dimers (Fig. 1B).

3.4. Spectroscopic and PAGE analysis of DsRed at different maturation stages

Purified DsRed-6His and MBP-DsRed or whole-cell lysates expressing native or MBP-tagged DsRed were analyzed by absorption fluorescence spectroscopy and SDS–PAGE at different induction times (Figs. 2 and 3). The results obtained with DsRed, DsRed-6His and MBP-DsRed matched closely (Figs. 2 and 3). All forms of DsRed appeared in significant quantities 5 h after induction (Fig. 2B), but demonstrated negligible red fluorescence (Figs. 2B and 3B, black trace) and absorbance at 558 nm (Fig. 3A, black trace). SDS–PAGE analysis demonstrated that most of the native DsRed was monomeric and lacked a functional chromophore. The formation of the red fluorescent species paralleled the appearance of aggregates and the disappearance of the monomers. MBP-DsRed demonstrated a stronger tendency to aggregate than DsRed and DsRed-6His, and detectable red fluorescence was visible in MBP-DsRed aggregates already at 5 h after induction, while MBP-DsRed monomers remained non-fluorescent (Fig. 2B). In summary, DsRed, DsRed-6His, and MBP-DsRed monomers lacked significant fluorescence at any maturation time point, whereas red fluorescent aggregates were easily visualized in progressively larger amounts over time.

Maturation was followed by measuring light absorbance and fluorescence at different times after IPTG induction as described [6,7]. We allowed DsRed, DsRed-6His and MBP-DsRed to mature at 4°C (Figs. 2 and 3A,B), at room temperature and at 37°C (Fig. 3A inset, C). Essentially identical results were obtained at the three temperatures, except for faster kinetics at higher temperatures (Fig. 3A versus inset). Soon after induction the protein extracted from bacteria exhibited absorption peaks at 480 and 558 nm. With time, the 558 nm peak increased much more dramatically than the 480 nm peak (Fig. 3A). Both peaks matched the corresponding fluorescence absorption peaks of DsRed [1]. Immediately after lysis the fluorescence emission of MBP-DsRed was largely green (Fig. 3B, black trace, C). The predominantly green state

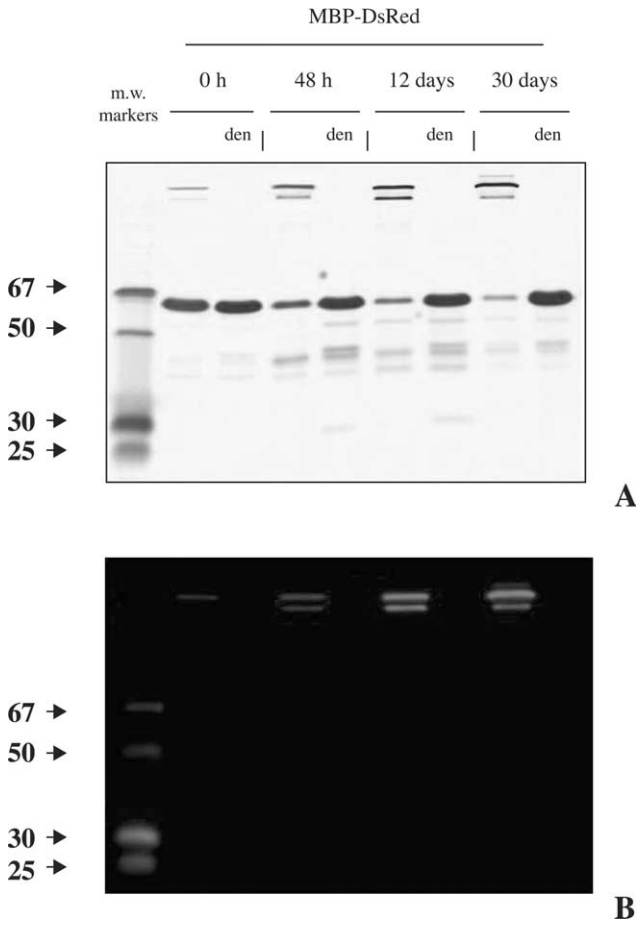
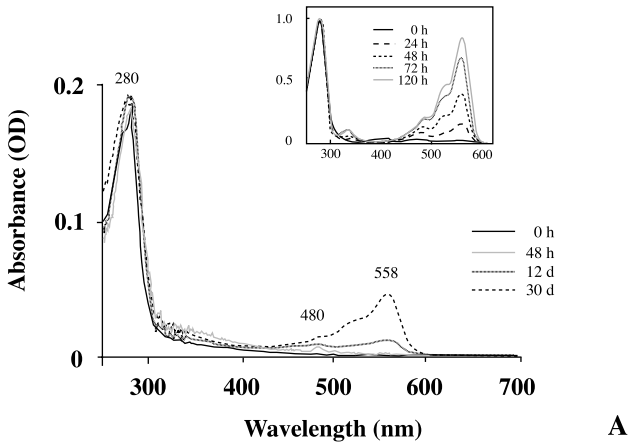


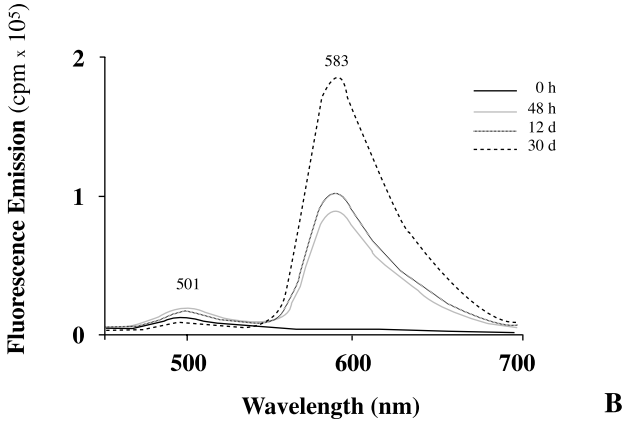
Fig. 2. SDS–PAGE analysis of MBP-DsRed at different maturation stages. MBP-DsRed was purified 5 h after IPTG induction in bacteria and allowed to mature for different lengths of time (0 h, 48 h, 12 days, 30 days) in vitro at 4°C. A: Coomassie blue staining. B: Fluorescence analysis. Non-denatured samples were run in the first lane for each time point of maturation. Corresponding denatured samples were run in each following lane. FITC–BSA (67 kDa), FITC–IgG (50 and 25 kDa) and GFP (30 kDa) were utilized as molecular weight markers.

of DsRed persisted and in fact increased over a considerable length of time (days at 4°C). Excitation at 558 nm and a concomitant red fluorescent emission were first detected at ~2–4 days after bacterial lysis, and increased steadily to much higher levels than the green emission (Fig. 3B, red trace, C). Maturation occurred in bacterial lysates and purified protein fractions with similarly slow kinetics, reaching plateau fluorescence levels after several days or weeks at 4°C, consistent with a spontaneous maturation and folding of DsRed. In all cases substantial red fluorescence was obtained, confirming the efficiency of our maturation procedures [6,7].

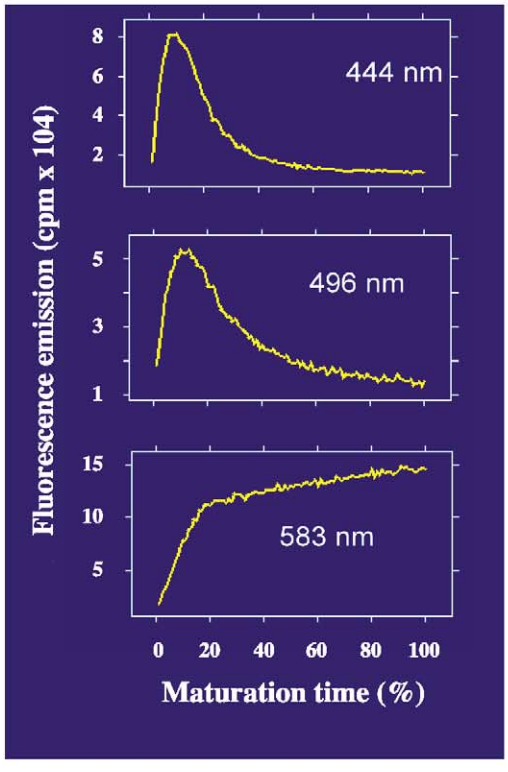
The crystal structure of DsRed demonstrates the presence of a mixed population of chromophores [13] that do ('red') or do not ('green') feature an extended double bond [3–5]. It was hypothesized that such mixed species generate red fluorescence by FRET. Thus, fragmenting the DsRed tetramers into functional monomeric chromophores was expected to generate a mixed population of 'red' and 'green' chromophores, with a corresponding reduction of the red emission and a strong increase in the green signal. To test this hypothesis, we performed solution spectroscopy experiments at low concentrations of DsRed (42 nM, 4.2 nM, and 0.42 nM) in

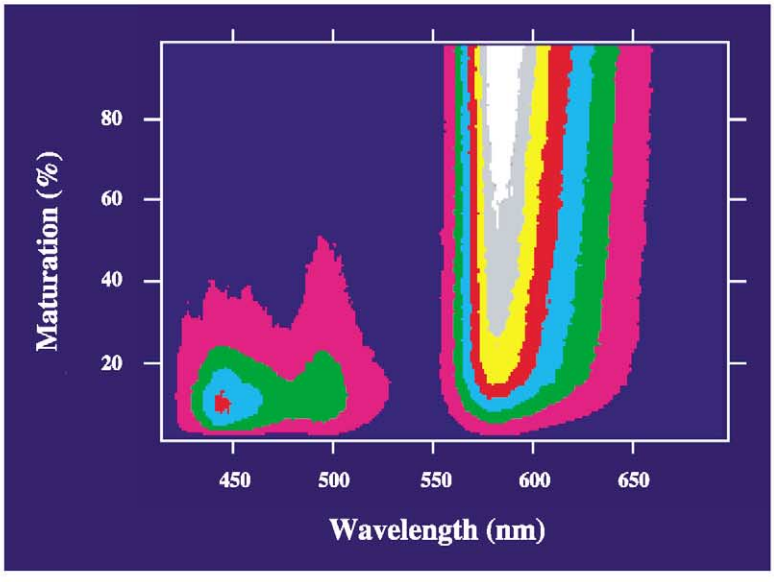

Absorbance (OD)
0.2
0.1
0
280
480
558
300
400
500
600
700
Wavelength (nm)
0 h
48 h
12 d
30 d
1.0
0.5
0
0 h
24 h
48 h
72 h
120 h
300
400
500
600

A


Fluorescence Emission (cpm x 10⁵)
2
1
0
583
501
500
600
700
Wavelength (nm)
0 h
48 h
12 d
30 d

B


Fluorescence emission (cpm x 104)
8
6
4
2
444 nm
5
3
1
496 nm
15
10
5
583 nm
0
20
40
60
80
100
Maturation time (%)


Maturation (%)
80
60
40
20
450
500
550
600
650
Wavelength (nm)

C

Fig. 3. Analysis of light absorption and fluorescence emission of DsRed at different stages of maturation. A: Light absorption of MBP-DsRed and DsRed-6His (inset) by spectrophotometric analysis. Absorbance was measured in OD units. MBP-DsRed samples were prepared as in Fig. 2 and analyzed immediately (solid black line) or after 48 h (solid gray line), 12 days (dotted) or 30 days (dashed) of maturation in vitro. Inset: DsRed-6His samples were analyzed immediately (solid black line) or after 24 h (dashed), 48 h (finely dashed), 72 h (dotted) or 120 h (solid gray line) of maturation in vitro at 37°C. B: Fluorescence emission of MBP-DsRed by spectrofluorimetric analysis. MBP-DsRed was excited at 558 nm. The fluorescence emission is given in cpm $\times 10^5$. MBP-DsRed fractions were the same as in A. C: Fluorescence emission of DsRed-6His over time. Maturation was at 37°C. Left: Time course analysis of the fluorescence emission at 444 nm, 496 nm and 583 nm; right: three-dimensional plot and pseudo-color representation of the relative amounts of fluorescence emission (cpm $\times 10^4$; 2–4: magenta; 4–6: green; 6–8: cyan; 8–10: red; 10–12: yellow; 12–14: gray; 14–16: white). Fluorescence scans were obtained every 15 min.
←

the hope of driving the monomer–oligomer equilibrium towards the monomeric state. However, extreme dilution failed to elicit appreciable spectral changes in DsRed. A high stability of the aggregated state of DsRed probably minimized the extent of dissociation of aggregates into monomers [3–5]. However, this result also suggests that the dissociated subunits of DsRed are inefficient chromophores, thereby confirming that aggregation of DsRed is required for an efficient generation of fluorescence.

3.5. Denaturation and renaturation of DsRed

Denaturation at pH extremes was used to establish the spectral characteristics and stability of the DsRed chromophore, as in [5]. The absorption spectrum of the red chromophore immediately after denaturation in 0.2 M NaOH demonstrated a single peak at 452 nm. A 385 nm peak was observed at pH 1.5. For comparison, the absorption peaks of wild-type and S65T-GFP were at 447 and 448 nm, respectively, in NaOH, and at 381 and 384 nm in HCl (Fig. 4). Thus, the absorption characteristics of acid- and alkali-denatured DsRed closely matched those of GFP. The NaOH-denatured DsRed was immediately neutralized to minimize chromophore degradation. Following pH neutralization, both GFP and DsRed recovered green fluorescence with similar kinetics (Table 1). Notably, DsRed demonstrated only a green emission (excitation 480 nm, emission 496 nm) (Fig. 5). The green fluorescence increased over time, indicating that the first product of the renaturation of DsRed was the green-emitting species. The red fluorescence also recovered, but extremely slowly (several days), suggesting that this was an event secondary to the reformation of the green chromophores. The renaturation of red fluorescent molecules reached plateau levels in about 10 days at room temperature or in 30 days at 4°C.

Electrophoretic analysis of the NaOH-denatured samples demonstrated a generalized breakdown of the DsRed oligomers into monomers (Fig. 6). As before, the recovery of red fluorescence correlated closely with the reformation of red oligomers. No red-emitting monomers were detected at any stage of the renaturation process.

The transition from 'green to red' has been proposed to rely on the oxidation of the green chromophore with the generation of a double bond that extends the chromophore to a 'redder' configuration [4,5]. Inasmuch as denaturation of DsRed leads to an immediate loss of fluorescence, an extreme sensitivity of the additional double bond to reduction was hypothesized [4,5]. We investigated the oxygen dependence of the renaturation of DsRed (with restoration of red fluorescence) in our procedures (Table 1). DsRed solutions were rapidly denatured in alkali, immediately neutralized into buffered solutions (extensively degassed to eliminate residual oxygen), and allowed to mature under nitrogen or upon re-exposure to atmospheric oxygen. H_2O_2 or β-ME was added to parallel samples to obtain markedly oxidizing or reducing environments, respectively. GFP was analyzed in parallel (Table 1). No significant differences in the recovery of red fluorescence in the presence or absence of molecular oxygen (Table 1) or H_2O_2 (not shown) were observed and β-ME did

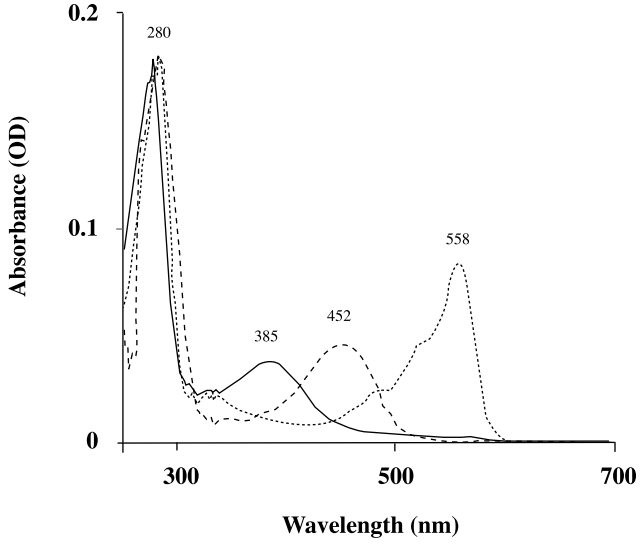


Fig. 4. Absorbance of denatured MBP-DsRed. The spectra of native (finely dashed), acid-denatured (solid black line) and alkali-denatured (dashed) MBP-DsRed were measured. Untagged DsRed demonstrated essentially identical spectra (not shown). Absorbance is in OD units.

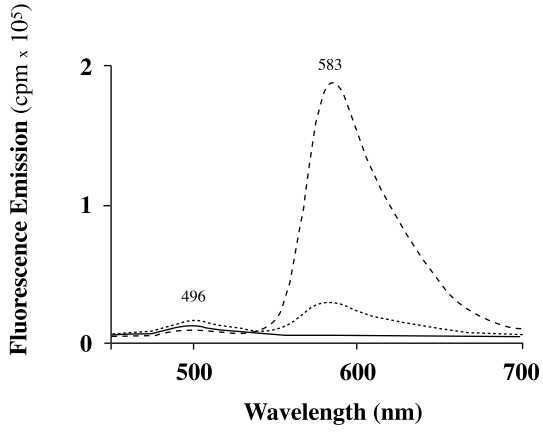


Fig. 5. Fluorescence analysis of denatured MBP-DsRed renatured in vitro. Alkali-denatured MBP-DsRed was neutralized and renatured at 4°C. The fluorescence emission of MBP-DsRed immediately after pH neutralization (solid black line) and after 25 days of renaturation (finely dashed) is shown, with the spectrum of the native protein for comparison (dashed). The fluorescence emission is given in units of cpm $\times 10^5$.

Table 1
Oxidation requirements for the green-to-red transition during the renaturation of DsRed

	Fluorescence emission[a]						
	GFP	DsRed					
		$+O_2$		$-O_2$		$-O_2$+β-ME	
		red	green	red	green	red	green
Native	100	100	0.8				
Denatured	0.3	0	1.5				
Renatured							
0 h	39	0.1	2.5	0.1	2.5	0.1	2.5
48 h	90	5	2.9	5.7	3.4	8.9	3.1
192 h	>90	8.7	3	9.5	3.4	10	2.4

[a]Fluorescence emission (510 nm for GFP and 583 nm for DsRed) at different time points after neutralization of NaOH-denatured proteins. Results are expressed as percentages of the native state. $+O_2$: exposed to air; $-O_2$: under nitrogen; +β-ME: in the presence of 1 mM β-ME.

not detectably diminish the rate of fluorescence recovery of DsRed. Thus, the redox potential of the chromophore environment is unlikely to be a (or the) significant factor in the formation of the DsRed chromophore. On the other hand, the strong correlation between the aggregation of DsRed and red fluorescence emission suggests that regeneration of the DsRed chromophore was favored energetically if and only when DsRed refolded and aggregated.

4. Discussion

The fluorescent protein DsRed has aroused considerable interest because of its potential use in FRET and in multicolor labeling of living cells. However, its tendency to oligomerize and its long maturation time have posed strong obstacles in many applications. Several mutants of DsRed have been obtained that emit green or orange fluorescence [3,15]. Improved DsRed-2 [6,7] and DsRed.T [8] mutants have been obtained that maintain the spectral characteristics of DsRed, but mature more quickly. However, they still form tetramers. Our findings may rationalize these difficulties, in that they demonstrate that the DsRed monomers do not emit red fluorescence, in contrast to all the aggregated states. Interestingly, the smallest red fluorescent aggregate appears to be a dimer by PAGE analysis. The size of functional DsRed molecules was also investigated by size exclusion chromatography, using EGFP as an accurate monomeric size standard. A consistent finding was the absence of fluorescence in the monomeric fraction of DsRed. On the other hand, the major peak of the elution curve of functional DsRed corresponded to a species with twice the mass of EGFP, confirming that the DsRed chromophore is oligomeric (unpublished). Although further work is required to conclusively determine if functional subdomains exist within the fundamental tetrameric structure of DsRed [3–5,14], the dimeric nature of at least one of the DsRed mutants obtained [16] is in accordance with this possibility.

A considerable fraction of newly synthesized DsRed molecules did not contain a cyclized chromophore, even at optimal induction times, in contrast to the GFP chromophore that cyclizes very shortly after induction (15–30 min [9,11,17,18]). Cyclization of the DsRed chromophore proceeded in parallel to the formation of aggregates, suggesting that the reaction is facilitated by or even requires concomitant aggregation, possibly for stabilization of the molecule. The MBP moiety of the MBP-DsRed fusion protein induced a stronger aggregation of DsRed monomers and a faster appearance of fluorescence, compared with untagged DsRed. The finding is consistent with the observation that tags can lead to better folding of fluorescent proteins [11], and further supports the notion of a stabilizing influence of aggregation. A further key finding of this work is that maturation in vitro generates a predominantly green state of DsRed and that this species is relatively stable, persisting for days. The green fluorescence increased significantly during the early and intermediate stages of maturation, indicating that new green chromophores form spontaneously, even at later times. However, the red fluorescence of DsRed subsequently increased much more dramatically than the green. This result and the progressive decrease of the green fluorescence at later maturation stages indicate that green green chromophores are likely to be intermediates in the generation pathway of red molecules.

NaOH-denatured samples demonstrated a generalized breakdown of the DsRed oligomers into monomers. In striking consistency with the maturation data, renaturation after alkali denaturation was characterized by rapid refolding into an inefficient green chromophore. PAGE analysis of the renatured samples demonstrated recovery of red fluorescence by

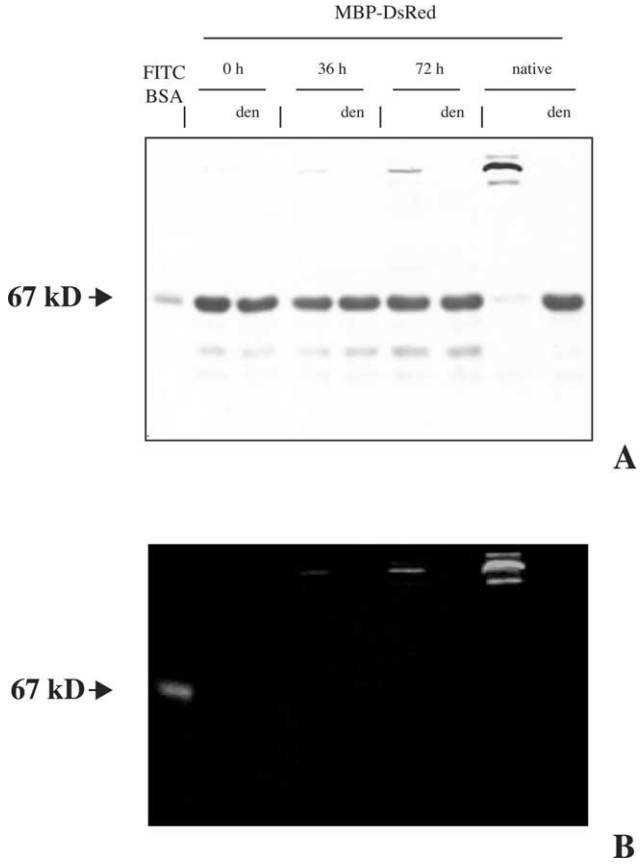


Fig. 6. SDS–PAGE analysis of denatured MBP-DsRed renatured in vitro. A: Coomassie blue staining. B: Fluorescence analysis. MBP-DsRed samples prepared as in Fig. 5 were analyzed by SDS–PAGE. Undenatured samples were run in the first lane for each time point of maturation in vitro. Corresponding denatured samples were run in each following lane. FITC–BSA was used as a size marker (67 kDa).

DsRed oligomers, whereas no red-emitting monomers were detected at any stage of the renaturation process. More generally, red fluorescent DsRed monomers were not observed under any of the conditions examined, in contrast to the intense red emission of all the oligomeric forms.

The autocatalytic formation of an acylimine in the DsRed chromophore appears to be responsible for the shift toward longer wavelength emission compared to GFP, as it causes a wider π electron sharing over an enlarged chromophore [3–5,14]. However, GFP and DsRed demonstrate similar light absorption spectra upon denaturation in alkali, acid or guanidine [4,5]. A profound sensitivity of the acylimine structure to reducing conditions was proposed to account for this phenomenon, as it would rapidly lead to a GFP-like chromophore upon denaturation [3–5]. Our findings, i.e. the lack of maturation and/or spectral change under widely differing redox conditions and the strong correlation between the aggregation of DsRed and its red fluorescence, suggest that the acylimine bond is stabilized by the folding and aggregation of DsRed, rather than by factors dependent on the redox conditions.

DsRed tetramers demonstrate roughly equivalent amounts of chromophores containing or lacking the double-bond-extended structure [13]. Thus, dissociation of DsRed into a population of monomeric, functional molecules was expected to generate mixed spectra of green and red species. However, extreme dilution experiments failed to show any spectral change in DsRed. Although the pronounced stability of the aggregated state of DsRed might serve to limit the degree of dissociation, the complete absence of spectral perturbations was unexpected, leading to the conclusion that dissociated DsRed monomers are inefficient emitters. The relatively low fluorescence of the green species has been attributed to quenching accompanying resonance fluorescence energy transfer [3,19], but may be explained by its inherently low fluorescence efficiency (extinction coefficient × quantum yield [1]).

This conclusion and the dimeric nature of the smallest functional chromophore of DsRed support a model according to which the red chromophore of DsRed originates from the folding and aggregation of (at least) two DsRed monomers. We postulate that the oligomerization of DsRed is required to stabilize the folding of the molecule and the configuration of the chromophore, particularly the acylimine double bond. However, other factors might also play a role. A network of hydrogen-bonded water molecules is trapped between two adjacent DsRed monomers in the crystal structure [14], connecting the two chromophores. As this structure is likely to influence the charge distribution of both chromophores, it might serve to generate a more red shifted configuration [20]. This might also facilitate the deprotonation of the DsRed chromophore or even induce additional/variant protonation events [21–23]). The spatial arrangement of four chromophores in close proximity also suggests the possibility of collective effects and mutual interactions, as demonstrated by Cotlet et al. [24], or a molecular exciton model of excitation transfer that has been postulated for this system [19]. While the exact details of the interaction mechanisms remain to be elucidated, it is possible to speculate that interactions between (at least) two monomers may be essential for the red fluorescence of DsRed.

5. Note added in proof

After the acceptance of this manuscript the generation of a monomeric DsRed through a stepwise evolution was described [25]. The first step was the production of a DsRed dimer, in consistency with the model presented in this article. To generate a monomeric red fluorescent protein 33 mutations had to be inserted, indicating that the structure of DsRed had to be profoundly altered to eliminate oligomerization while maintaining a red fluorecence capability.

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