The red fluorescent protein DsRed has been extensively engineered for use as an in vivo research tool. In fast maturing DsRed variants, the chromophore maturation half-time is ~40 min, compared to ~12 h for wild-type DsRed. Further, DsRed has been converted from a tetramer into a monomer, a task that entailed mutating approximately 20% of the amino acids. These engineered variants of DsRed have proven extremely valuable for biomedical research, but the structural basis for the improved characteristics has not been thoroughly investigated. Here we present a 1.7 Å crystal structure of the fast maturing tetrameric variant DsRed.T4. We also present a biochemical characterization and 1.6 Å crystal structure of the monomeric variant DsRed.M1, also known as DsRed-Monomer. Analysis of the crystal structures suggests that rearrangements of Ser69 and Glu215 contribute to fast maturation, and that positioning of the Lys70 side chain modulates fluorescence quantum yield. Despite the 45 mutations in DsRed.M1 relative to wild-type DsRed, there is a root-mean-square deviation of only 0.3 Å between the two structures. We propose that novel intramolecular interactions in DsRed.M1 partially compensate for the loss of intermolecular interactions found in the tetramer.

**Keywords:** DsRed/fast maturation/fluorescent protein/monomeric/structure

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**Introduction**

The red fluorescent protein DsRed (Matz et al., 1999) has become important both as a model for understanding fluorescent proteins and as a tool for biomedical research. DsRed and its engineered derivatives have found broad use in cell and molecular biology including as a marker for fluorescence microscopy, fluorescence correlation spectroscopy (FCS) and fluorescence activated cell sorting (FACS). These proteins can be expressed on their own or as fusions to heterologous partner proteins or organellar targeting sequences. The wavelengths of light used to excite DsRed are scattered less and cause less phototoxicity in cells than the wavelengths used for GFP. Further, there is little cellular autofluorescence in the region of the spectrum used to excite DsRed, resulting in high signal to noise ratios. Finally, DsRed can be used as a complementary marker to other fluorescent proteins such as GFP.

The DsRed chromophore is generated autocatalytically in the presence of molecular oxygen from the tripeptide Gln66-Tyr67-Gly68. The initial steps of DsRed chromophore formation are thought to resemble those in GFP, which has the chromophore tripeptide Ser65-Tyr66-Gly67 (Gross et al., 2000; Verkhusha et al., 2004). In GFP, protein folding is followed by nucleophilic attack of the Gly67 nitrogen on the Ser65 carbonyl carbon, resulting in backbone cyclization. This step is followed by dehydration of the Ser carbonyl oxygen and oxidation of the Cα–Cβ bond of Tyr67 (Tsien, 1998; Barondeau et al., 2003). The acquisition of green fluorescence proceeds relatively quickly in wild-type (WT) GFP (t_{1/2} ~1.4 h; Heim et al., 1994) and in the fast maturing variant termed EGFP (t_{1/2} ~0.3 h; Heim et al., 1995). The steps following cyclization have been shown to be rate-determining for GFP (Heim et al., 1994; Cubitt et al., 1995; Zhang et al., 2006). DsRed undergoes an additional oxidation that forms an acylamine with sp² geometry at Gln66 Cα, and this oxidation is coupled to an isomerization that forms a cis peptide bond between Phe65 and Gln66 (Gross et al., 2000; Wall et al., 2000; Tubbs et al., 2005). These additional maturation steps extend the pi-conjugated electron system of the chromophore, accounting for its red-shifted emission (Gross et al., 2000). In WT DsRed, acquisition of red fluorescence proceeds slowly (t_{1/2} ~12 h; Baird et al., 2000; Wiehler et al., 2001; Bevis and Glick, 2002) and remains incomplete even after prolonged incubation of the protein. The precise molecular mechanism of DsRed chromophore maturation and the identity of the rate-determining step(s) are not yet fully understood (Gross et al., 2000; Verkhusha et al., 2004).

Although the spectral properties of DsRed ideally complement those of GFP, WT DsRed has shortcomings as a research tool because it suffers from slow chromophore maturation, oligomerization into a stable tetramer and association of the tetramers into higher-order aggregates (Baird et al., 2000; Jakobs et al., 2000). Several groups have engineered useful DsRed variants (Terskikh et al., 2000; Bevis and Glick, 2002; Campbell et al., 2002; Yanushevich et al., 2002; Sörensen et al., 2003; Shaner et al., 2004). In our previous work, multiple rounds of random mutagenesis and screening yielded a set of fast maturing tetrameric variants with reduced higher order aggregation (Bevis and Glick, 2002). The most widely used of these variants are DsRed.T1, known commercially as DsRed-Express (Clontech), and the closely related DsRed.T4. The fast maturation of these variants is due primarily to the substitution of Asn42 with Gln. In WT DsRed, Asn42 faces the interior of the protein and lies adjacent to Gln66 of the chromophore. DsRed-Express and DsRed.T4 contain N42Q plus eight other mutations,
which collectively accelerate the maturation ~17-fold relative to WT DsRed (Bevis and Glick, 2002). However, the mechanism of this acceleration is unknown. Previous work based on comparative modeling of DsRed mutants suggested that increased space around the chromophore allows for faster and more complete maturation (Terskikh et al., 2001), but this simple principle does not explain how introducing a larger amino acid at position 42 accelerates maturation.

Subsequent directed evolution was used to generate monomeric DsRed variants. The first such variant was mRFP1, which was derived from DsRed-Express (Campbell et al., 2002). Later mRFP1 derivatives include mCherry as well as other ‘mFruits’ with different spectral properties (Shaner et al., 2004). In parallel, we developed a monomeric DsRed.T4 derivative called DsRed.M1, which is described here and is known commercially as DsRed-Monomer (Clontech). The creation of fluorescent monomeric variants required a large number of mutations. Many of these mutations altered the former tetramerization interfaces, but additional surface and interior substitutions were needed to stabilize the monomers. Unfortunately, the brightest of the available DsRed monomers are still only about half as bright as WT DsRed, due largely to reduced fluorescence quantum yield (QY) (Shaner et al., 2004).

Here we present the high-resolution crystal structure of the fast maturing tetramer DsRed.T4. Comparison with WT DsRed suggests that structural rearrangements centered on Glu42 and the chromophore-proximal residues Ser69 and Glu215 are responsible for the accelerated maturation. We also describe the crystal structure and spectral characteristics of the fast maturing monomer DsRed.M1. Compared to the WT protein, DsRed.M1 shows no gross structural changes, but has a slightly non-coplanar chromophore and a disordered conformation of the chromophore-proximal residue, Lys70. Both of these changes may contribute to the low QY. These data shed light on the molecular determinants of chromophore maturation and brightness, and will facilitate the engineering of further improved DsRed variants.

Materials and methods

Mutagenesis and screening

Screens to evaluate the brightness and color of mutagenized DsRed employed pQE60NA, a modified version of the pQE60 expression vector (Qiagen) in which the hexahistidine tag codons have been eliminated. Translation was initiated in pQE60NA at the DsRed start codon. Site-directed mutagenesis was performed using the QuikChange method (Strategene). For semi-random mutagenesis of particular codons, overlap extension PCR (Ho et al., 1989) was performed using partially degenerate codons at the targeted positions. Random mutagenesis of the entire DsRed gene was performed using error-prone PCR as previously described (Bevis and Glick, 2002). Colonies of Escherichia coli strain DH10B containing mutagenized DsRed were screened using the previously described slide projector assay (Cronin and Hampton, 1999; Bevis and Glick, 2002). Briefly, fluorescence excitation was achieved by placing a 520 ± 20 nm bandpass filter over the lens of a slide projector, and fluorescence emission was detected visually using laboratory goggles covered with a Kodak Wratten filter no. 22, which passes wavelengths >550 nm. Screening was performed in bacteria expressing the untagged proteins in order to avoid any artificial contribution of the tag to expression or oligomerization.

Spectral analysis

For protein purification from E.coli strain DH10B, DsRed variants were N-terminally hexahistidine tagged. These experiments employed the pQE31 expression vector together with the repressor plasmid pREP4 (Qiagen). Fifty-milliliter bacterial cultures in baffled flasks were grown to an OD600 of 0.5 in LB medium. Cells were induced with 1 mM isopropyl-beta-d-thiogalactopyranoside (IPTG). After 4 h of induction, protein synthesis was inhibited with 170 µg/ml chloramphenicol and 50 µg/ml tetracycline, and the cultures were allowed to continue shaking for an additional 12 h. Bacteria were lysed with B-PER II (Pierce), and protein was purified using Ni-NTA agarose beads as previously described (Bevis and Glick, 2002). Immediately following purification, protein was dialyzed into 50 mM Na+-phosphate, 50 mM NaCl, 1 mM EDTA, pH 7.4 and incubated for 5 days at 4°C to allow complete maturation. Fluorescence excitation and emission spectra were collected on a HORIBA Jobin Yvon Fluoromax-3 spectrophotometer, and absorbance measurements and spectra were collected on a Shimadzu UV-1650PC spectrophotometer. Protein concentrations were determined by amino acid analysis at the Molecular Structure Facility, University of California, Davis. QY values were determined as described (Lakowicz, 1999; Baird et al., 2000) using Rhodamine 101 in ethanol as a reference. For QY determination, the excitation wavelength was 510 nm and fluorescence emission was integrated from 525 to 750 nm.

Maturation kinetics

Escherichia coli DH10B/pREP4 cells were transformed with pQE31 derivatives containing DsRed variant genes. Three clones from each transformation were used to inoculate a 5-ml LB starter culture, which was grown to an OD600 of 0.5. Those starter cultures were each diluted 1:20 into 10 ml of fresh LB in a 50-ml baffled flask, and shaken at 275 rpm at 37°C. After 1 h, a pulse of gene expression was induced with 1 mM IPTG. A chase was initiated after 20 min by inhibiting protein synthesis with 170 µg/ml chloramphenicol and 50 µg/ml tetracycline. At designated timepoints, 200 µl aliquots were removed from the continuously shaking cultures, and red fluorescence (550 nm excitation, 585 nm emission) was immediately measured using a Tecan Safire² Microplate Reader.

Analytical ultracentrifugation

DsRed.M1 protein was purified as described above with the following modification. The protein was expressed using pQE31-TEV, a modified pQE31 vector in which a tobacco etch virus (TEV) protease cleavage site has been inserted between the hexahistidine tag and the polylinker. After purification on a Ni-NTA column, the hexahistidine tag was removed by cleavage with hexahistidine-tagged TEV protease (Kapust et al., 2001), and the released DsRed.M1 was collected as flow-through from a second Ni-NTA column. The purified protein was buffer exchanged into 50 mM Na+-phosphate, 50 mM NaCl, 1 mM EDTA, pH 7.4 and concentrated to 10 mg/ml with Amicon Ultra centrifugal
filter units (Millipore). Velocity ultracentrifugation and analysis were performed by B. Demeler at the Center for Analytical Ultracentrifugation of Molecular Assemblies at the University of Texas Health Science Center, San Antonio. Ultracentrifugation was performed with a Beckman Optima XLA analytical ultracentrifuge using Rayleigh interference optics. The sample was centrifuged using a 3 mm centerpiece at 20 °C and 42 000 rpm for 8.5 h, and data were analyzed using the UltraScan software package (Demeler, 2005).

Crystallization and X-ray diffraction data collection

DsRed.T4 was expressed from pQE31-TEV at 37 °C in 250 ml LB cultures of E.coli DH10B/pREP4. Two hours after inoculation with a 5-ml saturated overnight culture, the cells were induced with 0.1 mM IPTG, grown for an additional 4–6 h, and pelleted by centrifugation. Cell pellets were resuspended in lysis buffer (20 mM HEPES, pH 8.0, 500 mM NaCl, 1 mg/ml lysozyme, 20 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100), subjected to a single freeze-thaw cycle, and then sonicated. The soluble fraction was purified by Ni-NTA agarose column chromatography, and the hexahistidine tag was removed by TEV protease digestion as described above. The purified protein used for crystallization was buffer exchanged and concentrated to ~10 mg/ml in 10 mM HEPES, pH 8.0, 1 mM DTT and stored at −80 °C.

DsRed.M1 was expressed from pQE31-TEV at 30 °C in 1 l LB cultures of E.coli DH10B/pREP4. After inoculation with a 10-ml saturated overnight culture, the cells were induced with 1 mM IPTG and grown overnight at 30 °C. Cell pellets were resuspended in 50 mM HEPES pH 7.5, 300 mM NaCl, 20 mM imidazole and 5 mM β-mercaptoethanol, and disrupted by passage through a microfluidizer. The soluble fraction was purified by Ni-NTA chromatography and cleaved by TEV protease digestion as described above. EDTA was added to 2 mM, and the protein was buffer exchanged and concentrated to ~10 mg/ml in 5 mM HEPES, pH 8.0, 200 mM NaCl, 1 mM TCEP and stored at −80 °C.

Initial crystallization conditions were identified using Crystal Screen HT and Index HT screens (Hampton Research) at room temperature in 96-well, sitting drop vapor diffusion format. Optimized crystals were grown in 24-well, hanging drop format using equal volumes of protein and reservoir solution. Crystals of DsRed.T4 were grown from well solution containing 100 mM HEPES, pH 7.5, 200 mM MgCl₂, 22% polyacrylic acid. These crystals were mounted in nylon loops after briefly transferring them to cryoprotectant containing 100 mM HEPES, pH 7.5, 300 mM MgCl₂, 25% polyacrylic acid, 20% ethylene glycol. Diffraction data were collected at 100 K at APS beamline 17ID-B (Advanced Photon Source, Argonne, IL) on an ADSC Quantum 210 detector (Table I). DsRed.M1 crystals were grown from well solution containing 100 mM HEPES, pH 7.6, 25% PEG3350, 200 mM MgCl₂, 5 mM ZnSO₄. These crystals were mounted in nylon loops after briefly transferring them to cryoprotectant containing 100 mM HEPES, pH 8.0, 250 mM MgCl₂, 2 mM ZnSO₄, 25% PEG 3350, 20% ethylene glycol. Diffraction data were collected at 100 K at APS beamline 14BM-C on an ADSC Quantum 315 detector (Table I).

Table I. Data collection and refinement statistics

<table>
<thead>
<tr>
<th></th>
<th>DsRed.T4</th>
<th>DsRed.M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P1</td>
<td>P2222</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td>a = 71.3, b = 79.5, c = 83.0</td>
<td>a = 38.9, b = 62.2, c = 81.9</td>
</tr>
<tr>
<td>X-ray source</td>
<td>APS 17ID-B</td>
<td>APS 14BM-C</td>
</tr>
<tr>
<td>Resolution range</td>
<td>50–1.64 (1.70–1.64)</td>
<td>50–1.59 (1.65–1.59)</td>
</tr>
<tr>
<td>Total/unique observations</td>
<td>416 199/210 765</td>
<td>181 013/27 397</td>
</tr>
<tr>
<td>Completeness (%)&lt;br/&gt;µ (Å)</td>
<td>95.9 (87.9)</td>
<td>99.7 (99.6)</td>
</tr>
<tr>
<td>Rsym (%)</td>
<td>4.7 (13.8)</td>
<td>5.5 (39.7)</td>
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<tr>
<td>Rcryst (%)</td>
<td>16.2</td>
<td>17.6</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>19.7</td>
<td>19.7</td>
</tr>
<tr>
<td>Average B-factor (Å²)</td>
<td>9.7</td>
<td>15.8</td>
</tr>
<tr>
<td>RMS Bond lengths (Å)</td>
<td>0.015</td>
<td>0.014</td>
</tr>
<tr>
<td>RMS Bond angles (°)</td>
<td>1.7</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*Values in parentheses are for the high-resolution shell.

Crystallographic refinement

Diffraction data were processed with DENZO and SCALEPACK (Otwinowski, 1997). Structures were solved by molecular replacement with PHASER (Storoni et al., 2004), using as a search model wild-type DsRed (PDB code 1ZGO; Tubbs et al., 2005) with the chromophore and waters omitted. REFMAC5 (Murshudov et al., 1997) was used for crystallographic refinement (Table I), and COOT (Emsley and Cowtan, 2004) was used for manual model building into 2Fo–Fc and Fo–Fc maps. Chromophores were built into the model after two rounds of coordinate and isotropic B-factor refinement. Inspection of difference maps revealed the presence of predicted green and red forms of the chromophore in DsRed.T4 and DsRed.M1; in each case, these forms were modeled with equal occupancy. Chromophore restraints were generated using the PRODRG server (Schuttelkopf and van der Spoel, 2004), using as a search model wild-type DsRed with the chromophore and waters omitted. Structural superimpositions were done in COOT, PyMol was used to prepare all structure figures. Atomic coordinates and structure factors have been deposited with the Protein Data Bank (PDB; Berman et al., 2000) and given codes 2VAE for DsRed.T4 and 2VAD for DsRed.M1.

Results

The fast maturing tetramer DsRed.T4

Structural overview Data collection and refinement statistics for the DsRed.T4 crystal structure are shown in Table I. Upon superposition of alpha carbons from each of the eight monomers (residues 7–222) in the DsRed.T4 crystal structure with each of the four monomers in WT DsRed [PDB code (1ZGO; Tubbs et al., 2005)], an average root-mean-square deviation (RMSD) of 0.2 Å is observed. The electron density shows an approximately equal mixture of green
(trans-Phe65-Gln66 peptide bond with sp³-hybridized Gln66 Ca) and red (cis-Phe65-Gln66 peptide bond with sp²-hybridized Gln66 Ca) chromophores (Supplementary Material, Fig. S1), as observed in the high-resolution structure of WT DsRed (Tubbs et al., 2005). The largest difference in the conformation of the chromophore occurs at the side chain of Gln66, which shifts by ~0.8 Å relative to its position in the WT protein.

Of the nine substitutions in DsRed.T4 relative to WT DsRed, three (R2A, K5E and N6D), are located at the disordered N-terminus and are not visible in the electron density maps, while a fourth (T21S) is located at the surface, more than 15 Å from the chromophore. Two substitutions (H41T and A145P) are in the vicinity of the chromophore, but the side chains point toward the surface and the substitutions cause little structural change. The remaining three substitutions are internal. T217A is in the second shell of residues surrounding the chromophore, while N42Q and V44A point directly at the chromophore (Fig. 1). Our analysis therefore focused on how these three internal substitutions affect the chromophore and its local environment.

Chromophore environment The Asn-to-Gln substitution at position 42 greatly accelerates chromophore maturation (Bevis and Glick, 2002). In DsRed.T4, the N42Q substitution is partially accommodated by displacement of the backbone atoms of residues 41–43 by ~0.5 Å away from the chromophore chamber. This displacement eliminates a hydrogen bond between Gln66 and the backbone carbonyl of residue 42. An intriguing change is seen with Ser69, which exists in

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Fig. 1. The local chromophore environments of WT DsRed, DsRed.T4 and DsRed.M1. The chromophores are colored orange, teal and yellow, respectively. The figures are drawn in stereo and in approximately the same orientation. Polar interactions (<3.4 Å) are indicated with dashed grey lines. Waters are indicated as red spheres. Amino acid substitutions relative to WT DsRed are indicated with asterisks. Coordinates used for WT DsRed are from Tubbs et al. (2005) (PDB code 1ZGO).
WT DsRed as two rotamers. One rotamer places the Ser69 side chain proximal to the chromophore, where it forms a hydrogen bond with Asn42, while the other rotamer rotates the side chain ∼120° so that it is more distal to the chromophore and cannot hydrogen bond with Asn42 (Fig. 1). In DsRed.T4, the additional ∼0.8 Å protrusion of the longer Gln42 side chain restricts Ser69 to the distal rotamer, which now forms a hydrogen bond with Gln42 (Fig. 1). Another change is seen with Glu215. In WT DsRed, Asn42 hydrogen bonds with Glu215. In DsRed.T4, Gln42 interacts instead with the carbonyl oxygen of Ser69 and with three adjacent water molecules (Fig. 1), two of which are unique to DsRed.T4 (see below).

The Val-to-Ala substitution at position 44 eliminates two methyl groups that are adjacent to Gln66 and the Phe65-Gln66 cis/trans peptide bond. The side chains of the surrounding hydrophobic residues, including Phe14, Val16, Leu46 and Phe65, move only slightly into the resulting cavity. However, the Gln66 side chain moves ∼0.8 Å and partially fills the cavity (Fig. 1).

The Thr-to-Ala substitution at position 217 alters a residue that is one shell removed from a direct interaction with the phenolate moiety of the chromophore. Loss of the hydroxyl group at residue 217 disrupts two hydrogen bonds, one with the side chain of Ser197 and the other with the side chain of Lys70 (Fig. 1). Although neither Lys70 nor Ser197 undergoes a significant rearrangement in DsRed.T4, two water molecules fill the cavity created by the smaller Ala217 side chain. These two water molecules, along with a conserved water molecule adjacent to the imidazolinone ring of the chromophore, mediate a network of interactions involving Gln42, Ser69, Lys70, Tyr72 and Glu215 (Fig. 1).

All anthozoan fluorescent proteins contain a conserved residue corresponding to Glu215 (Glu222 in GFP). In DsRed.T4, the Glu215 χ3 torsion is rotated up to ∼60° from its WT position such that Glu215 now forms a 3.2 Å hydrogen bond to the N2 nitrogen of the chromophore imidazolone ring, while maintaining a hydrogen bond to Lys70 (Fig. 1). In this new conformation, the carboxylate of Glu215 also makes a close contact (3.1 Å) with Cβ of Gln66 (Fig. 1).

The substitutions in DsRed.T4 cause additional conformational shifts in the chromophore chamber. For example, the carbonyl of Tyr72 now adopts two conformations, one of which is part of the new network of water-mediated interactions surrounding the chromophore (Fig. 1). Similarly, the side chain of Leu199, which is located below Glu215, assumes at least two distinct conformations in DsRed.T4 (not shown). We interpret these data as indicating increased flexibility in the chromophore environment.

### Table II. Summary of spectral and biophysical properties

<table>
<thead>
<tr>
<th>Protein</th>
<th>Excitation maximum (nm)</th>
<th>Emission maximum (nm)</th>
<th>Extinction coefficient (M⁻¹ cm⁻¹)</th>
<th>Fluorescence yield</th>
<th>Brightness (mM⁻¹ cm⁻¹)</th>
<th>t¹/₂ for maturation at 37°C (h)</th>
<th>Oligomeric state</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT DsRed</td>
<td>559</td>
<td>584</td>
<td>46 400</td>
<td>0.71</td>
<td>33</td>
<td>∼12²</td>
<td>Tetramer</td>
</tr>
<tr>
<td>DsRed.T4</td>
<td>556</td>
<td>586</td>
<td>33 600</td>
<td>0.47</td>
<td>16</td>
<td>0.7</td>
<td>Tetramer</td>
</tr>
<tr>
<td>DsRed.M1</td>
<td>557</td>
<td>592</td>
<td>27 300</td>
<td>0.14</td>
<td>4</td>
<td>1.3</td>
<td>Monomer</td>
</tr>
</tbody>
</table>

*All reported values were measured for this study, except WT DsRed maturation, which is from Bevis and Glick (2002).
tetramer. Whereas tetrameric DsRed buries ~1300 and ~1000 Å² of surface area in the polar and hydrophobic interfaces, respectively, the largest contact region in the DsRed.M1 crystal lattice buries only ~450 Å² (Fig. 3A and B). These observations are consistent with the analytical ultracentrifugation measurements, and they further indicate that DsRed.M1 is fully monomeric.

Despite the multiple amino acid substitutions in DsRed.M1, its structure reveals no gross distortions of the GFP-like fold. Superposition of alpha carbons from DsRed.M1 (residues 7–222) onto each of the eight monomers in DsRed.T4 yields an average RMSD of 0.3 Å, with the largest conformational differences occurring in the loops.

Several surface mutations that disrupt intermolecular interactions in the tetramer form intramolecular interactions in the monomer. For example, Arg153 and the adjacent Lys158 mediate interactions in the polar and hydrophobic interfaces, respectively (Fig. 3C). In DsRed.T4, Arg153 is replaced with a Gln, thereby disrupting an intermolecular salt bridge with Glu100 and forming a new intramolecular hydrogen bond between Gln153 and Lys158 (Fig. 3C). Similarly, a Glu-to-Tyr substitution at position 26 disrupts an intermolecular hydrogen bond in the hydrophobic interface of the tetramer (Fig. 3D). In DsRed.M1, Tyr26 fills a void by packing against the surface and making a compensatory hydrogen bond with Glu28, in an identical manner to Tyr26 in the closely related chromoprotein Rtm5 (Prescott et al., 2003).

Chromophore environment

DsRed.M1 has six internal substitutions relative to DsRed.T4. Four of them, V71A, K83M, V175C and S179T, are second-shell substitutions, located approximately ~8 Å from the chromophore. The fifth substitution, K163H, alters a residue that directly contacts the chromophore. The final internal substitution, Y193H, is located ~13 Å from the chromophore. Together with His75 and two chloride ions, His193 chelates a buried zinc ion from the crystallization solution. Incubation of DsRed.M1 with concentrations of zinc comparable to those in the crystallization buffer did not alter the shape or amplitude of the absorbance and fluorescence spectra (data not shown).

As with WT DsRed and DsRed.T4, the DsRed.M1 electron density is best fit by an approximately equal mixture of green (sp³-hybridized Gln66 Cα and a trans-Phe65-Gln66 peptide bond) and red (sp²-hybridized Gln66 Cα and a cis peptide bond) chromophores (Supplementary Material, Fig. S1). The repositioning of Ser69 and Glu215 seen in DsRed.T4 is also observed in DsRed.M1 (Fig. 1).

The electron density for the DsRed.M1 chromophore clearly defines small conformational changes in the N2-Cα2-Cβ2-Cγ2 and Cα2-Cβ2-Cγ2-Cδ1 dihedrals, corresponding to approximately +10° and −11° deviations from coplanarity (Fig. 1 and Supplementary Material, Fig. S1). These deviations are more modest than those observed in the crystal structures of the mFruits (Shu et al., 2006), but small amounts of residual difference density below the plane of the phenolate are consistent with chromophore flexibility.
The most striking structural feature in the DsRed.M1 chromophore environment is a shift in the position of Lys70 from its usual location stacked above the chromophore phenolate. Although partially disordered in DsRed.M1, the electron density reveals a dramatic 2.4 Å shift in the position of the Lys70 N\textsuperscript{1} atom relative to its position in the tetrameric structures. As a result, Lys70 no longer interacts with Glu215 or Ser197, but instead forms a salt bridge with Glu148. In place of the Lys70 side chain, a new water molecule mediates a network of hydrogen bonds between Glu215, Ser197 and the repositioned Lys70 (Fig. 1 and Supplementary Material, Fig. S2). The lack of additional well-ordered water molecules surrounding Lys70, coupled with the relatively weak electron density of its side chain, suggests that Lys70 adopts multiple conformations and sweeps out a broad arc above the chromophore. Reorientation of Lys70 is likely influenced by the K83M substitution, as observed in a K83M mutant of DsRed and also in mFruits that replace Lys83 with a hydrophobic residue (Shu et al., 2006). Additionally, the V71A and S179T substitutions may further modulate the position of Lys70.

**Discussion**

**Fast maturation**

The molecular details of chromophore maturation are the subject of debate for both DsRed and GFP (Remington, 2006). For DsRed, it is generally accepted that the initial steps of backbone cyclization and oxidation of the Tyr67 Cα–Cβ bond parallel the same reactions in GFP. It is also generally accepted that a ~480 nm-absorbing green fluorescent species appears before the ~560 nm-absorbing red fluorescent species that represents mature DsRed (Baird et al., 2000; Terskikh et al., 2000; Wiehler et al., 2001), and that fully matured DsRed contains a mixture of red and green species (Gross et al., 2000; Yarbrough et al., 2001; Tubbs et al., 2005). One possible interpretation is that the green chromophore is a precursor to the red chromophore, and that a fraction of the green molecules become permanently trapped instead of completing their maturation. Recently, Verkhusha and colleagues proposed an alternative scheme in which formation of the red and green chromophores results from a branched rather than a linear maturation pathway.
(Verkhusha et al., 2004). In any case, the red chromophore differs from the green chromophore by an additional oxidation that generates an sp²-hybridized Gln66 Ca (acylimine) with planar geometry, and by trans–cis isomerization of the peptide bond between Phe65 and Gln66 (Wall et al., 2000; Yarbrough et al., 2001; Tubbs et al., 2005).

From a practical standpoint, the presence of the green chromophore is undesirable. Shaner et al. (2004) demonstrated that a Q66M substitution reduces the ~480 nm absorbing species in mCherry, and Tubbs et al. (2005) showed structurally that in WT DsRed, the Q66M substitution results in “all red” chromophores. However, the mechanism for the formation of green versus red chromophores remains a mystery. Compared to WT DsRed, the fast maturing variants contain a higher proportion of green chromophores, but the basis for this difference is not apparent in the structures. Further, high resolution structures of the Q66M mutant (Tubbs et al., 2005) and of mCherry (Shaner et al., 2004; Shu et al., 2006), do not highlight the mechanism by which this substitution shifts the chromophore populations to “all red”.

The accelerated maturation of DsRed-Express, DsRed.T4 and their derivatives is largely due to the N42Q substitution, with an additional boost from the T217A substitution (Bevis and Glick, 2002). Our structural studies provide insights into the possible mechanisms underlying fast maturation. We suggest that N42Q and T217A exert their effects by rearranging Ser69 and Glu215. In WT DsRed, the side chain of Ser69 adopts two conformations with approximately equal occupancy: one that points toward the chromophore (proximal), and another that is rotated ~120° away from the chromophore (distal) (Fig. 1) (Yarbrough et al., 2001; Tubbs et al., 2005). Early work suggested that the proximal conformation of Ser69 might be catalytic, functioning to abstract a proton from the Gln66 N-Ca bond during the final oxidation step (Yarbrough et al., 2001). However, later analysis of an S69A mutant suggested that Ser69 is not directly required for DsRed chromophore maturation (Tubbs et al., 2005). In support of that conclusion, we find that in DsRed.T4 and DsRed.M1, the longer Gin side chain at position 42 sterically occludes the proximal conformation of Ser69, thereby forcing Ser69 into the distal conformation and increasing space around Gln66 Ca (Fig. 1). This rearrangement could impact acylimine formation by two non-exclusive mechanisms. First, displacement of the Ser69 hydroxyl may increase the affinity of molecular oxygen for transient binding above Gln66 Ca, thereby increasing the rate of acylimine formation. Second, displacement of Ser69 may lower the energetic barrier to isomerization of the Phe65-Gln66 peptide bond.

Glu215 is highly conserved among fluorescent proteins and chromoproteins. It has been hypothesized to function as a catalytic base during the initial cyclization reaction in GFP (Sniegowski et al., 2005) and during green-to-red phototransformation of EosFP (Nienhaus et al., 2005). In DsRed.T4, the carboxylate group of Glu215 is rotated by ~60° relative to its position in WT DsRed, placing Glu215 within hydrogen bonding distance (3.2 Å) of the chromophore imidazolinone N2 and within ~3.1 Å of Gln66 Cβ (Fig. 1). A similar rearrangement of Glu215 is observed in the crystal structures of the mFruits (Shu et al., 2006) and of DsRed.M1. The proximity of the Glu215 carboxylate group to the imidazolinone N2 suggests that Glu215 is protonated and unable to act as a general base during acylimine formation. However, Glu215 might facilitate dehydration of the Gln66 carbonyl after the initial cyclization reaction. Dehydration has been proposed to be one of the rate-determining steps for GFP maturation (Zhang et al., 2006), and the same may be true for DsRed.

Quantum yield

The rigidity of the chromophore matrix correlates positively with QY for both proteinaceous and non-proteinaceous chromopores (Niwa et al., 1996; Kummer et al., 2002; Mauring et al., 2005; Fayed and Etaiw, 2006). For example, the crystal structure of the far red fluorescent protein HcRed, which has a very low QY (0.04), reveals a highly mobile chromophore (Gurskaya et al., 2001; Wilmann et al., 2005). The QY values for DsRed.T4 (0.47) and DsRed.M1 (0.14) are significantly lower than for WT DsRed (0.71). In DsRed.T4 and DsRed.M1, the increased space around the chromophore may decrease QY by increasing the probability of non-radiative energy loss from the excited state chromophore.

Coplanarity of the chromophore ring system has also been hypothesized to correlate positively with QY in fluorescent proteins. Thus, deviations from chromophore coplanarity are proposed to be responsible for the relatively low QY values for mCherry (0.22) and mStrawberry (0.31) (Shaner et al., 2004; Shu et al., 2006), and also for the chromoproteins KFP (Quillin et al., 2005) and RmTs5 (Prescott et al., 2003). Despite its modest QY (0.14), the structure of DsRed.M1 reveals only a minor distortion of the chromophore from coplanarity (Fig. 1). Further, non-coplanar chromophore geometry does not always correlate with a low QY. For example, mOrange has a significant tilt to the chromophore but a high QY (0.69; Shaner et al., 2004; Shu et al., 2006), and the recently described teal fluorescent protein, mTFP1, has non-coplanar chromophore geometry but a very high QY (0.85; Ai et al., 2006). We propose that additional factors may contribute to the low QY in DsRed.M1.

When comparing DsRed.M1 with the brighter tetrameric variants, the clearest structural difference near the chromophore is at Lys70, which in DsRed.M1 has moved away from its position above the chromophore (Fig. 1 and Supplementary Material, Fig. S2). This Lys70 repositioning is likely due to the K83M substitution, because a similar Lys70 shift is observed in DsRed-K83M and in the mFruits, all of which contain bulky hydrophobic residues at position 83 (Shu et al., 2006). What are the consequences of Lys70 repositioning? Relative to WT DsRed, the DsRed-K83M variant has substantially red-shifted fluorescence spectra (6 and 19 nm for the excitation and emission maxima, respectively), and these changes have been attributed to movement of Lys70 (Shu et al., 2006). However, the DsRed.M1 red shifts are much smaller (1 and 6 nm for the excitation and emission maxima, respectively, compared to DsRed.T4) because the Y193H substitution compensates for the K83M substitution (data not shown). Thus, Lys70 may not actually be a major determinant of fluorescence color. On the other hand, Lys70 positioning may be important for brightness because a reduced QY is seen in a number of DsRed variants with a displaced Lys70, including DsRed-K83M (0.44), mCherry (0.22) and DsRed.M1 (0.14) (Baird et al., 2000; Shaner et al., 2004; Shu et al., 2006; this work). We propose that stacking of Lys70 over the chromophore contributes to
the high QY in WT DsRed by stabilizing a rigid conformation of the chromophore.

Surface engineering of DsRed.M1

Most fluorescent proteins are oligomeric and in DsRed, the tetramerization interfaces bury ~2300 Å², nearly one quarter of the total surface area of the protein (Wall et al., 2000; Yarbrough et al., 2001). It is therefore not surprising that the first engineered monomeric variants of DsRed, including the precursors to mRFP1 (Campbell et al., 2002) and DsRed.M1, were largely non-fluorescent, and that multiple additional mutations were needed to recover significant red fluorescence. If tetramerization provides stability to DsRed, then disrupting the tetramer should decrease stability. To compensate for loss of the tetrameric interactions, we attempted to engineer novel intramolecular interactions. In support of this approach, polar intramolecular surface interactions are a feature of thermostable proteins, and engineering these types of interactions has been shown to increase protein stability (Kumar et al., 2000; Strickler et al., 2006). With DsRed.M1, our attempts to engineer novel intramolecular interactions seem to have been somewhat successful. For example, the E26Y substitution is observed to recapitulate a similar interaction in the close homologue Rtm5, by packing against the protein surface and forming a hydrogen bond with Glu28 (Fig. 3D). Similarly, novel intramolecular interactions seen in the crystal structure of DsRed.M1, such as Gln153–Lys158 (Fig. 3C) and Glu100–Thr174 (not shown), may contribute to rigidifying the monomer.

Conclusion

Analysis of the fast maturing DsRed.T4 suggests that maturation rate can be altered by minor conformational rearrangements surrounding the chromophore. In particular, Asn/Gln42, Ser69, and Glu215 are the best candidates for residues that influence maturation rate. Analysis of the monomeric DsRed.M1 suggests that fluorescence brightness is affected by chromophore mobility and the position of Lys70. This improves our understanding of the mechanisms that underlie chromophore maturation and fluorescence in DsRed and related proteins, and should facilitate efforts to engineer variants with improved photophysical properties.

Supplementary Material

Supplementary data are available at PEDS online.

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Conflict of Interest statement

DES, BB, and BSG have financial interest in DsRed.M1.

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