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(54) **MONOMERIC RED FLUORESCENT PROTEINS**

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See application file for complete search history.

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(57) **ABSTRACT**

Disclosed are sequences encoding monomeric variants of DsRed fluorescent proteins and methods of use.

4 Claims, 2 Drawing Sheets

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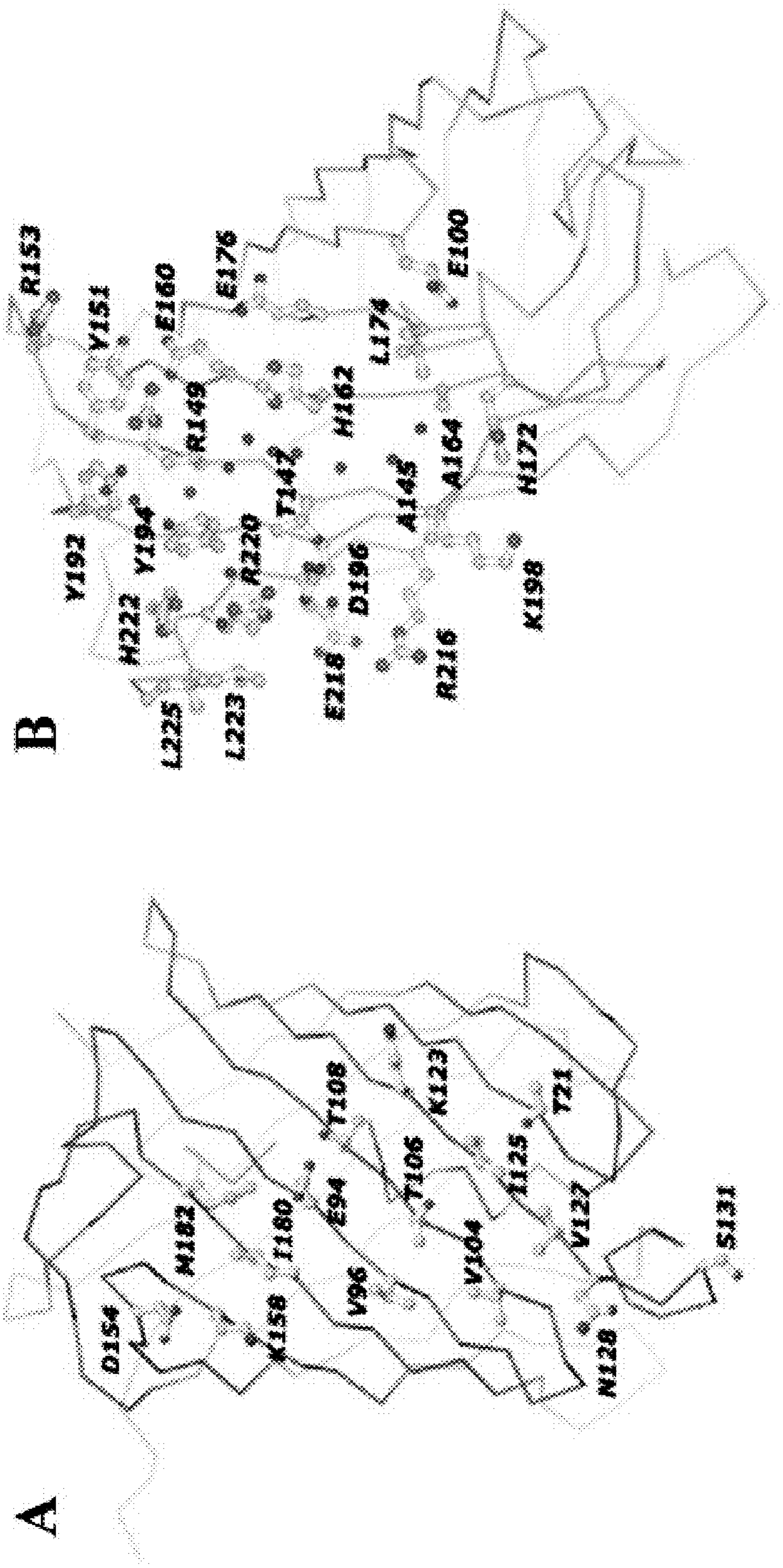
A
D154
K158
M182
I180
V96
E94
T108
T106
V104
K123
I125
T21
V127
N128
S131
B
R153
Y151
E160
E176
E100
L174
R149
H162
Y192
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L225
L223
E218
R216

FIG. 1

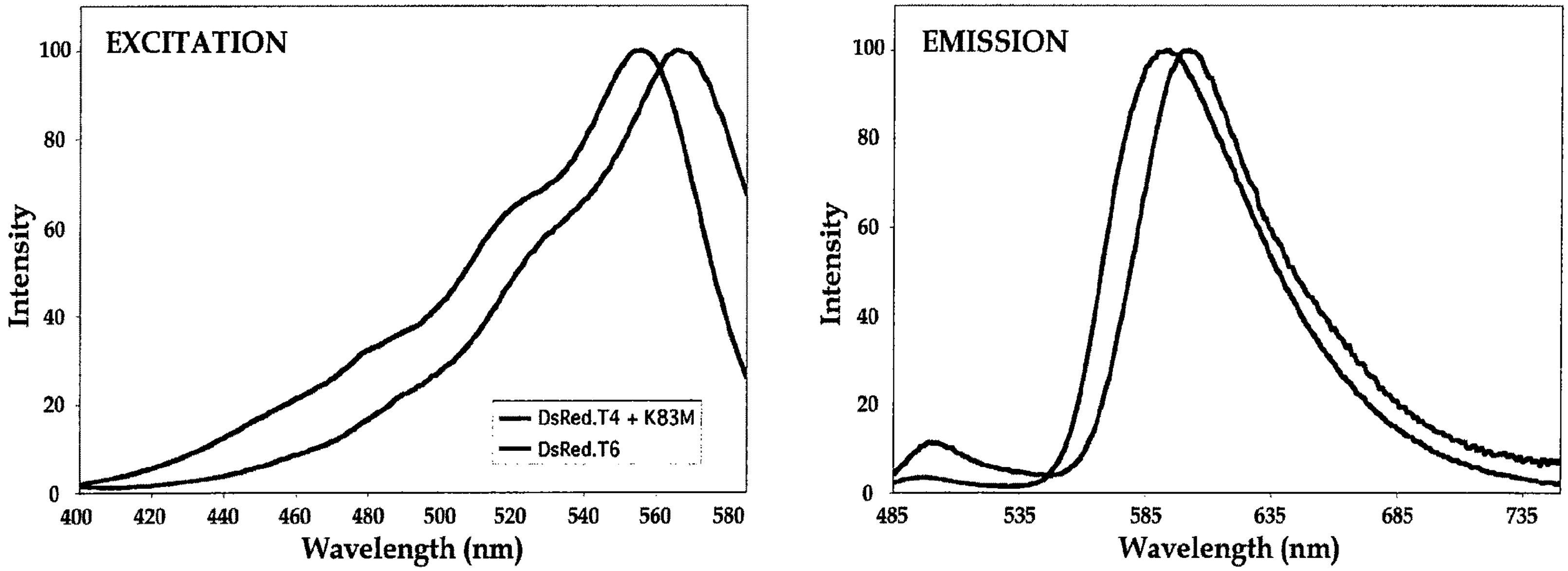
EXCITATION
Intensity
0
20
40
60
80
100
400
420
440
460
480
500
520
540
560
580
Wavelength (nm)
DsRed.T4 + K83M
DsRed.T6
EMISSION
Intensity
0
20
40
60
80
100
485
535
585
635
685
735
Wavelength (nm)

FIG 2

MONOMERIC RED FLUORESCENT PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 11/100,988, filed Apr. 7, 2005, which claims priority to U.S. Provisional Application No. 60/560,340, filed Apr. 7, 2004, each of which is incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under MCB-9875939, awarded by the National Science Foundation, and under RPG-00-245-01-CSM, awarded by the American Cancer Society. The government has certain rights in the invention.

INTRODUCTION

Fluorescent proteins such as green fluorescent protein (GFP) are invaluable tools used in pure and applied research. Although fluorescent proteins have been widely available for a relatively short time, they have had an important impact on biomedical research, contributing to our understanding of basic cellular and developmental processes that underlie health and disease. GFP and its relatives are widely used for medically-oriented research. For example, GFP has been used to analyze bacterial gene expression during infection, to visualize tumor cell behavior during metastasis, and to monitor GFP fusion proteins in gene therapy studies. Fluorescent proteins are also useful in high-throughput screens for drug discovery.

A red fluorescent protein produced by the coral *Discosoma* and designated DsRed (wild-type DsRed) is potentially useful as a fluorescent reporter protein or as a fusion tag. A red fluorescent protein is particularly attractive because of its suitability for use in conjunction with fluorescent proteins having different fluorescent properties, such as GFP. However, wild-type DsRed suffers from certain drawbacks.

First, the maturation process that yields the red fluorophore is slow, with a half-time of ~12 h at 37° C. Second, wild-type DsRed occurs as a stable tetramer of four very similar polypeptides, which makes its use as a fluorescent reporter in a fusion protein problematic. For example, tetramerization of the DsRed fusion protein may interfere with or perturb the function or localization of the protein. In addition, DsRed tetramers undergo higher-order aggregation. Fusion of DsRed to membrane proteins or to oligomeric proteins often produces large aggregates. Efforts to develop variants of DsRed having a reduced tendency to form tetramers have met with limited success, in that the variants suffer from disadvantages such as undesirable shifts in fluorescence or more rapid photobleaching.

There is, therefore, ongoing interest in developing new fluorescent protein labels with improved characteristics as experimental and clinical tools.

SUMMARY OF THE INVENTION

The present invention provides polynucleotide encoding a variant polypeptide of wild-type DsRed or the rapidly maturing DsRed.T4, a tetrameric variant that has the substitutions described herein below. The variant polypeptide has reduced oligomerization relative wild-type DsRed and has a fluorescence spectra similar to the fluorescence spectra of the wild-type DsRed, the variant polypeptide comprising the amino acid substitutions K83M, K163H, and Y193H and further comprises at least of one amino acid substitutions E26Y, K92T, V96S, T106E, T108Q, I125K, S131A, I180V, and M182K.

In another aspect, the invention provides isolated polynucleotides encoding a variant polypeptide of the rapidly DsRed.T4 and exhibiting reduced oligomerization relative to DsRed.T4 and detectable red fluorescence, the variant polypeptide comprising at least one of amino substitutions K83M or K83L; K163Q, K163M, or K163H; and Y193H, and further comprising at least three amino acid substitutions selected from E26Y, K92T, V96S, T106E, T108Q, I125K, S131A, I180V, and M182K; and further comprising at least three amino acid substitutions selected from the group consisting of R149K, R153Q, H162S, L174T, E176D, Y192N, R216H, H222S, L223G, and F224S.

In other aspects, the invention provides genetic constructs comprising the polynucleotides, vectors comprising the constructs, cells comprising the constructs, variant polypeptides encoded by the polynucleotides, and methods of obtaining expression of the polynucleotides.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts the hydrophobic interface (FIG. **1**A) and the polar interface (**1**B) involved in tetramerization of DsRed, the latter of which also includes the hydrophobic tail (residues 222-225).

FIG. **2** compares the excitation and emission spectra for a derivative of tetrameric variant DsRed.T4 having a K83M substitution, and a derivative of DsRed.T4 having K83M, K163H, and Y193H substitutions.

DETAILED DESCRIPTION

The present invention provides polypeptide variants of wild-type *Discosoma* sp. red fluorescent protein (DsRed). Wild-type DsRed forms oligomers (e.g., dimers or tetramers) under physiologic conditions, which, in the native polypeptide, appears to play a role in its fluorescence activity. A coding sequence for wild-type *Discosoma* sp. red fluorescent protein (DsRed) is shown in SEQ ID NO:1, and the amino acid sequence of DsRed is shown in SEQ ID NO:2. The polypeptide variants of the present invention have a reduced tendency to oligomerize relative to wild-type DsRed. This reduced tendency to oligomerize may be assessed by any suitable means, whether in vivo or in vitro, as described below.

Polypeptide variants were genetically engineered, as described in detail below, by altering a sequence encoding a rapidly maturing tetrameric variant of wild-type DsRed, designated DsRed.T4 (SEQ ID NO:3). DsRed.T4 is described in further detail in co-pending U.S. patent application Ser. No. 10/844,064, which is incorporated by reference in its entirety. Relative to the wild-type DsRed of SEQ ID NO:2, DsRed.T4 contains the following substitutions:

P(-4)L	H41T
R2A	N42Q
K5E	V44A
N6D	A145P
T21S	T217A

Relative to either wild-type DsRed or DsRedT4, the polypeptides of the present invention exhibit a reduced tendency to oligomerize or form tetramers, and exist primarily as monomers under physiologic conditions. The polypeptide variants exhibit detectable red fluorescence. By "detectable red fluorescence" it is meant that the fluorescence overlaps the emission spectra of wild-type DsRed is distinguishable over background. Preferably, the emission spectra is similar to that of wild-type DsRed.

To develop monomeric DsRed variants, a polynucleotide sequence encoding DsRedT4 was modified using the following general approach. As one skilled in the art will appreciate, one could also begin with a polynucleotide encoding another DsRed, for example, wild-type DsRed.

Mutations were introduced into a polynucleotide sequence encoding DsRedT4 to disrupt the tetramerization interfaces that form between DsRed polypeptides. Amino acids suspected of contributing to oligomerization were replaced by amino acids that are likely to reduce oligomerization.

Crystal structures of DsRed reveal residues that may be involved in tetramerization. The region that we have designated the "hydrophobic interface" primarily engages in hydrophobic interactions that exclude the solvent (FIG. **1**A). The region that we have designated the "polar interface" primarily engages in polar interactions, although this interface also includes some hydrophobic residues (Leu-174 and the C-terminal tail) that interact with the opposing subunit (FIG. **1**B).

Two considerations guided selection of residues to be mutated and the specific substitutions to be tested. First, certain residues are more likely than others to make a significant energetic contribution to oligomerization. For example, a statistical study revealed that isoleucine side chains are often important for protein-protein interactions (Bogan and Thorn, 1998). In designing the variants of the present invention, it was assumed that surface-exposed hydrophobic residues are likely to promote oligomerization, and therefore, such residues were considered prime candidates for mutagenesis. Thus, it is likely that generation of a stable monomer requires modification of residues Ile-180 and Ile-125 (FIG. **1**A). Second, the DsRed protein will tolerate substitutions more readily at some positions than at others. Many mutations at the tetramerization interfaces will have secondary effects on the folding and/or maturation of the protein. To make educated guesses about which residues can be mutated and which alternative residues can be substituted, we used sequence alignments between DsRed, GFP, and the other known fluorescent proteins (Matz et al., 1999; Wall et al., 2000).

In some polypeptide variants, mutations in two of the tetramerization interfaces resulted in a loss of tetramerization, which can be measured in vitro or in vivo as described in the Examples, and loss of fluorescence. It was, therefore, speculated that tetramerization may be important for fluorescence in wild-type DsRed, possibly because the autocatalytic formation of the chromophore may stabilize the folded structure of DsRed or because oligomerization may increase the quantum yield.

To restore fluorescence to an oligomerization-disrupted or monomeric DsRed variants, site-directed mutagenesis was performed to introduce specific changes that were expected to stabilize the immature and/or mature forms of the protein. Two general classes of mutations were introduced in an attempt to restore fluorescence after disrupting the tetramer: (1) mutations that may stabilize folding intermediates to permit the protein to mature even in the absence of oligomerization; and (2) mutations expected to rigidify mature DsRed to enhance quantum yield.

In a second strategy to restore fluorescence to an oligomerization-disrupted or monomeric DsRed variant, a wide range of mutations were introduced into either the entire DsRed coding sequence or into a selected segment of the coding sequence. The variants thus generated were screened for improved fluorescence.

The effect of various mutations has, in some cases, been demonstrated, and in other cases, has been presumed or inferred. The mutations can be divided into the following six categories of effects:

1. Mutations that presumably stabilize immature folding intermediates. In tetrameric DsRed variants, K83 and K163 face the interior of the protein. The presence of these highly polar internal residues is likely to destabilize folding intermediates. Therefore, K83M and K163H substitutions were introduced to reduce the polarity at these positions. The K163H substitution is relatively conservative because histidine is still somewhat polar. The K83M substitution introduces a hydrophobic residue, and red-shifts the fluorescence spectra. However, it was discovered by random mutagenesis that a Y193H substitution reverses the spectral changes caused by K83M, and also enhances brightness. Residue 193 is close to residue 83 of the folded protein, so the Y193H substitution may compensate for the reduced polarity caused by K83M. Variants containing the trio of substitutions K83M, K163H, Y193H seem to be stabilized, as indicated by their ability to tolerate interface mutations that are not tolerated by DsRed.T4.
2. Mutations that disrupt the hydrophobic tetramerization interface. This category comprises E26Y, K92T, V96S, T106E, T108Q, I125K, S131A, I180V, and M182K, each of which is predicted to disrupt intersubunit interactions at the hydrophobic interface.
3. Mutations that disrupt the polar tetramerization interface. This category comprises R149K, R153Q, H162S, L174T, E176D, Y192N, R216H, H222S, L223G, F224S, and L225Q, each of which is predicted to disrupt intersubunit interactions at the polar interface. The hydrophobic C-terminal tail of DsRed is considered to be part of the polar interface.
4. Mutations that enhance monomer fluorescence, presumably by stabilizing and/or rigidifying the protein. This category comprises V71A, C117T, V175C, S179T, S203N, and G219A. Note that C117T also eliminates a surface cysteine residue that might otherwise be oxidized within the secretory pathway.
5. Mutations that improve expression in *E. coli*. When a variant such as DsRed.T4 is produced in *E. coli* using its own start codon, the protein levels are much lower than when the same protein is produced using an N-terminal hexahistidine tag, which suggests that the 5' end of the gene is important for expression in bacteria. The coding sequences were altered such that the putative translation products contained substitutions in residues 2-4 and screened for strong expression in *E. coli*. A polypeptide having the substitutions A2D, S3N, and S4T was found to be strongly expressed. These N-terminal mutations probably enhance translation in *E. coli*, an effect that may not occur with expression in eukaryotic cells. In addition, the N-terminal mutations might have some beneficial effect on the maturation and/or stability of DsRed.
6. Mutations that reduce the general "stickiness" of the protein. We introduced a number of surface mutations that lie outside of the tetramerization interfaces that should reduce the tendency of the protein to adhere to other macromolecules. Specifically, we targeted basic residues (especially arginines) and large, hydrophobic residues. The substitutions in this category include R13Q, R36K, K47Q, M141A, and 1210V. We have shown that these substitutions do not signifi-

cantly reduce fluorescence, and expect that variants containing one or more of these substitutions may exhibit reduced aggregation with other macromolecules. Reduced aggregation may be evaluated by comparing the solubility of DsRed variants to that of wild-type DsRed.

An example of a DsRed monomeric variant containing numerous mutations and identified as DsRed.M1 (SEQ ID NO:5) is described below. Of course, useful monomeric variants of the presently claimed invention having fewer mutations than those contained in the DsRed.M1 may be developed using the guidance and teaching herein of this disclosure.

DsRed.M1 was further modified by introducing D6N, a reversion to the native sequence, in order to reduce the number of acidic residues at the N-terminus The additional substitutions K121H, K168E, D169G, D115G, and G116N were made to improve brightness or fluorescence.

Once a DsRed monomeric variant having desirable characteristics is identified, one may, of course, use any polynucleotide sequence encoding the variant to express the variant. For example, the polynucleotide encoding the variant may be modified for optimal expression in a particular organism in view of the preferred codon usage of that organism. The polynucleotide may be operably linked to an inducible or constitutive promoter functional in the intended cell or organism. The polynucleotide may be linked in-fram to a second polynucleotide sequence encoding a polypeptide of interest to form a sequence encoding a fusion protein in which the polypeptide of interest is labeled with the DsRed monomeric variant at its N- or C-terminus.

EXAMPLES

Selection of Amino Acid Substitutions to Reduce Tetramerization

Site-directed mutagenesis using standard methods well-known to one of ordinary skill in the art was used to disrupt the two tetramerization interfaces of DsRed. Various substitutions at each position were made, and fluorescence was evaluated as described below.

The Hydrophobic Interface

Extensive mutagenesis of the hydrophobic interface was performed, beginning with the DsRed.T4 variant. DsRed.T4 contains a T215 substitution. In addition, Met-182, Ile-180, Val-96 and Ile-125 were substituted with more polar residues, and a hydrogen-bonding residue was removed through an S131A substitution. The DsRed.T4 variant having these substitutions was designated DsRed.D1. This variant is probably dimeric. An additional V104A substitution is tolerated in the DsRed.D1 background. It is of note that, in the related fluorescent protein HcRed, a single Leu-to-His mutation in the putative hydrophobic interface at the position corresponding to Ile-125 of DsRed was reportedly sufficient to convert HcRed into a dimer (Gurskaya et al., 2001). Bacterial colonies producing DsRed.D1 are somewhat less fluorescent than those producing DsRed.T4, but the signal with DsRed.D1 is still strong.

The Polar Interface

Initial attempts to mutagenize the polar interface yielded reduced the fluorescence. For example, Leu-174 forms hydrophobic interactions with the opposing subunit, but all of the Leu-174 substitutions that we initially tried, including a conservative change to Val, virtually abolished fluorescence. It was thus concluded that Leu-174 is required for fluorescence and should not be mutated. Similar results were obtained with His-162, which associates with its counterpart on the opposing subunit in an unusual stacking interaction, and with His-222, which inserts into a groove in the opposing subunit. Conservative mutations of these His residues to Ser or Asn severely diminished the fluorescence. Indeed, mutagenesis of most the key residues at the polar interface were found to impair fluorescence.

Assessing Oligomeric State of DsRed Variants

The oligomeric state of a DsRed variant may be assessed by nondenaturing SDS-PAGE or size exclusion chromatography.

For nondenaturing SDS-PAGE, one µg of each purified DsRed variant or wild-type DsRed was mixed with SDS-containing sample buffer on ice and immediately electrophoresed at 4° C. in a 10% polyacrylamide gel, followed by staining with Coomassie Blue. Additional aliquots of wild-type DsRed and DsRed variants were denatured by boiling prior to electrophoresis.

Gel filtration chromatography may be used to indicate whether a fluorescent protein exists predominantly as a monomer, dimer or tetramer (Gurskaya et al., 2001). Optionally, gel filtration will be conducted using the Pharmacia FPLC system. An extension of this method known as small-zone size-exclusion gel filtration chromatography could be used to measure association constants (Raffen and Stevens, 1999).

Oligomerization states and association constants of our DsRed variants may be evaluated using analytical ultracentrifugation (Laue and Stafford, 1999). This approach was used by Baird et al. (2000) for their initial demonstration that DsRed is a tetramer. A Beckman XL-A analytical ultracentrifuge will be used. Velocity sedimentation will be used to ascertain which DsRed species are present (monomers, dimers and/or tetramers), and then equilibrium sedimentation will be used to measure the oligomer association constants (Laue and Stafford, 1999).

Analytical ultracentrifugation will be carried out in collaboration with Borries Demeler (University of Texas Health Sciences Center at San Antonio), who has extensive experience with analytical ultracentrifugation (eg., Demeler and Saber, 1998) and is the author of the state-of-the-art UltraScan II software (www.ultrascan.uthscsa.edu).

An in vivo assay for DsRed oligomerization may be used in a genetic screen. For example, a yeast two-hybrid system such as that reported to have has been used to verify the oligomerization of wild-type DsRed (Baird et al., 2000) may be used to assess the tendency of DsRed variants to oligomerize.

Another approach used to monitor DsRed oligomerization in vivo was to fuse GFP or DsRed to the protein Gos1p in *S. cerevisiae*. Gos1p is a membrane protein anchored to the cytoplasmic face of yeast Golgi cisternae. The GFP-Gos1p fusion protein gives a fluorescence pattern that is typical for the Golgi in *S. cerevisiae*, whereas a wild-type DsRed Gos1p fusion generates large red blobs in the cells, presumably because multiple Golgi cisternae become crosslinked via DsRed tetramerization. Thus, visualizing DsRed-Gos1p fusions provides an in vivo assay for DsRed oligomerization.

In another screening assay, randomly mutagenized DsRed proteins will be fused to the C-terminus of glutathione S-transferase (GST; Smith and Johnson, 1988). Because GST is a dimer (McTigue et al., 1995), the fusion of GST to an oligomeric DsRed variant will generate crosslinked aggregates that will be insoluble upon gentle detergent lysis of the cells. By contrast, the fusion of GST to a monomeric DsRed variant will generate a soluble protein. The concept of using this method to evaluate the oligomeric state of DsRed variants was tested using an expression and detergent lysis protocol similar to that used to evaluate solubility or aggregation of

DsRed, fluorescent DsRed.T1, a tetrameric variant, was efficiently extracted from the bacterial cells whereas the fluorescent GST-DsRed.T1 was quantitatively retained in the pellet. It is expected that this assay will provide a sensitive screen for a monomeric DsRed.

Optimizing Spectral Properties

In an earlier stage of this project, we attempted to create a bright, red-shifted DsRed variant. We began with DsRed.T4 and introduced a K83M substitution, which had been shown to red-shift the fluorescence spectra of wild-type DsRed (Baird et al., 2000). In the DsRed.T4 background, K83M red-shifted the spectra (FIG. **2**), but substantially reduced the intrinsic brightness of the protein. Screening of randomly mutagenized variants was undertaken to identify variants having restored fluorescence. Surprisingly, an L174Q mutation was found to increase brightness. Subsequent tests confirmed that in the context of K83M, other substitutions at the polar interface, including H162S and H222S, preserve or restore colony fluorescence.

Without being limited as to theory, we speculate that the K83M mutation allows DsRed to tolerate changes at the polar interface by stabilizing the immature form of the DsRed, thereby allowing for correct folding. The cores of most proteins are hydrophobic, but in the case of DsRed, K83 is one of several charged residues that face the interior of the protein (Wall et al., 2000; Yarbrough et al., 2001). It may be that immature DsRed is stabilized by tetramerization, particularly by interactions at the polar interface, and that the K83M substitution renders the immature protein sufficiently stable to fold even in the absence of interactions at the polar interface. Based on this hypothesis, we predict that in the absence of K83M, mutations such as L174Q will decrease the yield of mature DsRed but will not reduce the intrinsic fluorescence of the mature protein. Meanwhile, regardless of the reason, K83M has enabled us to mutagenize the polar interface.

To address the reduced fluorescence found with variants having a K83M substitution, two rounds of random mutagenesis and screening were undertaken. Two additional substitutions (K163H and Y193H) that significantly increase the brightness of the purified protein were identified. These new mutations are distinct from L174Q, and they alter residues that face the interior of the protein. The variant of DsRed.T4 having the K83M, K163H, and Y193H substitutions was designated DsRed.T6. As judged by colony fluorescence, DsRed.T6 is comparable to DsRed.T4 in brightness. Interestingly, the spectral red-shifting observed with K83M is largely reversed by the two additional substitutions in DsRed.T6 (FIG. **2**). As can be seen from FIG. **2**, the K83M substitution red-shifts the excitation and emission peaks by ~20 nm. The two additional substitutions present in DsRed.T6 reverse most of this red-shifting, and also suppress the green emission. We have also introduced K83M, K163H, and Y193H substitutions into the DsRed.D1 background and the resulting variant, designated DsRed.D3, has strong fluorescence and tolerates mutations at the polar interface. We plan is to start with DsRed.D3 and mutate most or all of the residues that contribute to the polar interface.

Preferably, the monomeric DsRed variant of the present invention retains the spectral properties of the tetramer, namely, bright red fluorescence with minimal green emission. However, fluorescent bacterial colonies expressing polypeptides containing substituted amino acids for those of the wild-type DsRed that ordinarily form the hydrophobic tetramerization interface appear somewhat dimmer than colonies of bacteria expressing tetrameric variants. Two kinds of changes may account for decreased fluorescence of the bacterial colonies expressing mutant DsRed. First, a mutation may reduce

the intrinsic brightness of DsRed by lowering the extinction coefficient and/or the quantum yield. Second, a mutation might slow DsRed maturation and/or reduce the percentage of the DsRed molecules that eventually become fluorescent. To counteract such effects, random mutagenesis strategy will be used to identify brighter variants of the monomers.

Minimizing the Green Emission of Monomeric DsRed

A predicted side effect of disrupting the DsRed tetramer will be a loss of FRET and a consequent increase in the green emission. To alleviate this problem, mutations that increase the ratio of red to green molecules in mature DsRed may be introduced. Substitutions of K83M, K163H, and Y193H in DsRedT6 correlate with brighter red fluorescence and reduced green emission, relative to that of the DsRed.T4+K83M variant.

In addition to mutations identified in these random screens, directed mutations aimed at weakening the tetramerization may fortuitously reduce the percentage of green molecules in mature DsRed. For instance, the A145P substitution that we incorporated into DsRed.T3 and DsRed.T4 to lower the green emission was originally generated during our attempts to mutagenize the polar interface. More recently, we found that an H222S substitution at the polar interface decreases the green emission.

Screening for Reduced Blue Excitation

Monomeric variants of interest having significant green emission, may be further mutagenized and screened for reduced green emission or reduced blue excitation. A 488-nm laser is used to excite fluorescence in bacteria containing mutant DsRed proteins, and the bacterial cells are then sorted by flow cytometry to identify clones with reduced green emission. This approach would be more difficult with a monomeric DsRed, because undesirable mutations that caused protein aggregation would suppress the green emission due to FRET. Alternatively, mutants may be screened for reduced blue excitation. When total fluorescence is measured, the signal obtained by excitation with blue light should correlate with the percentage of the DsRed molecules having a green fluorophore, regardless of whether the emission spectrum has been modified by FRET.

The assay for reduced blue excitation is based on our standard slide projector method, except that the plates will be photographed with a digital camera. We will photograph each plate under two conditions. First, the total fluorescence after excitation with blue light will be recorded by illuminating through a 485±11 nm bandpass filter and capturing the emission signal through a Kodak Wratten filter #12, which passes wavelengths above 520 nm (Cronin and Hampton, 1999). The image of the plate will be colorized green using Adobe Photoshop. Second, the total fluorescence after excitation with yellow light will be recorded by illuminating through a 520±20 nm bandpass filter and capturing the emission signal through a Kodak Wratten filter #22, which passes wavelengths above 550 nm. This image of the plate will be colorized red. The red image will be due almost exclusively to DsRed molecules with the red fluorophore, whereas the green image will include a strong contribution from DsRed molecules with the green fluorophore. Upon merger of the red and green images, most of the colonies will appear yellow, but colonies having an increased ratio of red to green molecules will appear orange.

Evaluating Solubilities

One approach to evaluating solubilities of the fluorescent proteins in is as follows. *E. coli* cells carrying inducible expression vectors encoding hexahistidine-tagged wild-type DsRed or DsRed variants were grown to an optical density (OD) (λ=600 nm) of 0.5, induced for 7 h, lysed with B-PER

II detergent (Pierce) and centrifuged for 20 min at 27,000×g. Equivalent amounts of the pellet and supernatant fractions were subjected to SDS PAGE followed by immunoblotting with an anti-hexahistidine antibody. The percentage of each protein in the supernatant fraction was then quantified for each protein and the percentage of protein molecules extracted (i.e., solubilized) was determined. Typically, only about 25% of the wild-type DsRed molecules are solubilized. Suitably, at least 30% of DsRed variant molecules are solubilized. Preferably, at least 50% of DsRed variant molecules are solubilized. More preferably, at least 70% of DsRed variant molecules are solubilized.

Another approach to evaluate variants for reduced aggregation is by nondenaturing SDS-PAGE. One μg of each purified DsRed variant was mixed with SDS-containing sample buffer on ice and immediately electrophoresed at 4° C. in a 10% polyacrylamide gel, followed by staining with Coomassie Blue. Additional aliquots of wild-type DsRed and DsRed variants were denatured by boiling prior to electrophoresis. Migration of proteins as a diffuse band that may reflect the formation of higher-order oligomers, whereas formation of a sharp band or bands of the appropriate size suggests reduce aggregation.

Creation and Characterization of a Red Fluorescent DsRed Monomer

A coding sequence and the amino acid sequence of one mutant according to the present invention, designated DsRed.M1, is shown as SEQ ID NO:4 and SEQ ID NO: 5, respectively. DsRed.M1 contains the following 37 substitutions relative to DsRed.T4:

A2D*
S3N
S4T
R13Q
E26Y
R36K
K47Q
V71A
K83M
K92T
V96S
T106E
T108Q
C117T
I125K
S131A
M141A
R149K
R153Q
H162S
K163H
L174T
V175C
E176D
S179T
I180V
M182K
Y192N
Y193H
S203N
I210V
R216H
G219A
H222S
L223G
F224S
L225Q

*A2D is a second mutation of residue 2, which was an arginine in wild-type DsRed.

Oligomeric State: DsRed.M1 behaves functionally as a monomer. The protein appears to be monomeric as judged by nondenaturing SDS-PAGE or size exclusion chromatography.

Brightness: The DsRed.M1 mature protein exhibits detectable red fluorescence, but is less bright than tetrameric variants, such as DsRed.T4. DsRed.M1 is also less bright than a monomeric red fluorescent DsRed protein designated mRFP1, which was generated by Tsien et al. (US 2003/0170911 A1)

Spectral Properties: In contrast to mRFP1, which has a red-shift, DsRed.M1 has excitation and emission spectra similar to those of the tetrameric DsRed variants. Surprisingly, DsRed.M1 has negligible green emission. The lack of green emission is fortunate, and rather unexpected, given that the tetrameric DsRed variants exhibit green emission that is largely suppressed by intersubunit resonance energy transfer.

Photostability: mRFP 1 photobleaches much faster than the tetrameric DsRed variants, an effect that may correlate with the altered spectral properties of mRFP1. It is expected that DsRed.M1 will be more photostable than mRFP1 because the spectral properties of DsRed.M1 are similar to those of the tetrameric variants.

Maturation: DsRed.M1 appears to retain the rapid maturation of the parental DsRed.T4, which is considerably faster than that wild-type of DsRed.

Each publication cited in herein or in the appendix is incorporated by reference in its entirety. Also incorporated by reference in its entirety is WO 03/054158A2.

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SEQUENCE LISTING

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Thr Lys Gly Gly Pro Leu Pro Phe Ala Trp Asp Ile Leu Ser Pro Gln
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Phe Gln Tyr Gly Ser Lys Val Tyr Val Lys His Pro Ala Asp Ile Pro
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Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg Val
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Met Asn Phe Glu Asp Gly Gly Val Val Thr Val Thr Gln Asp Ser Ser
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Leu Gln Asp Gly Cys Phe Ile Tyr Lys Val Lys Phe Ile Gly Val Asn
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Leu
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Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg Val
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Met Asn Phe Glu Asp Gly Gly Val Val Thr Val Thr Gln Asp Ser Ser
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Leu Gln Asp Gly Cys Phe Ile Tyr Lys Val Lys Phe Ile Gly Val Asn
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Tyr Tyr Val Asp Ser Lys Leu Asp Ile Thr Ser His Asn Glu Asp Tyr
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Leu
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Met Asn Phe Glu Asp Gly Gly Val Val Glu Val Gln Gln Asp Ser Ser
            100                 105                 110

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Asp Tyr Met Lys Leu Ser Phe Pro Glu Gly Phe Thr Trp Glu Arg Ser
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Leu Gln Asp Gly Thr Phe Ile Tyr Lys Val Lys Phe Lys Gly Val Asn
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Pro Ser Thr Glu Lys Leu Tyr Pro Gln Asp Gly Val Leu Lys Gly Glu
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Phe Lys Thr Val Tyr Lys Ala Lys Lys Pro Val Gln Leu Pro Gly Asn
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His Tyr Val Asp Ser Lys Leu Asp Ile Thr Asn His Asn Glu Asp Tyr
        195                 200                 205

Thr Val Val Glu Gln Tyr Glu His Ala Glu Ala Arg His Ser Gly Ser
    210                 215                 220

Gln
225
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We claim:

1. An isolated polynucleotide encoding a variant polypeptide of the rapidly maturing Discosoma red fluorescent protein T4 (DsRed.T4) consisting of SEQ ID NO:3, wherein the variant polypeptide has reduced oligomerization relative to wild-type DsRed, and matures more rapidly than wild-type DsRed, and wherein the variant polypeptide comprises substitutions: (A) at least one amino acid substitution selected from the group consisting of K83M, K83L, K163Q, K163M, K163H, and Y193H, (B) at least three amino acid substitutions selected from the group consisting of E26Y, K92T, V96S, T106E, T108Q, I125K, S131A, I180V, and M182K, (C) at least three amino acid substitutions selected from the group consisting of R149K, R153Q, H162S, L174T, E176D, Y192N, R216H, H222S, L223G, and F224S, and (D) at least one amino acid substitution selected from the group consisting of V71A, C117T, S175C, S179T, S203N, and G219A.

2. A variant polypeptide of DsRed.T4 consisting of SEQ ID NO:3 wherein the variant polypeptide has reduced oligomerization relative to wild-type DsRed and matures more rapidly than wild-type DsRed, and wherein the variant polypeptide comprises substitutions: (A) at least one amino acid substitution selected from the group consisting of K83M, K83L, K163Q, K163M, K163H, and Y193H, (B) at least three amino acid substitutions selected from the group consisting of E26Y, K92T, V96S, T106E, T108Q, I125K, S131A, I180V, and M182K, and (C) at least three amino acid substitutions selected from the group consisting of R149K, R153Q, H162S, L174T, E176D, Y192N, R216H, H222S, L223G, and F224S.

3. A polynucleotide encoding a fusion protein comprising the variant polypeptide of claim **2**.

4. A fusion protein comprising the variant polypeptide of claim **2**.

* * * * *