Reducing the Environmental Sensitivity of Yellow Fluorescent Protein

MECHANISM AND APPLICATIONS*

Yellow mutants of the green fluorescent protein (YFP) are crucial constituents of genetically encoded indicators of signal transduction and fusions to monitor protein-protein interactions. However, previous YFPs show excessive pH sensitivity, chloride interference, poor photostability, or poor expression at 37 °C. Protein evolution in *Escherichia coli* has produced a new YFP named Citrine, in which the mutation Q69M confers a much lower pK_a (5.7) than for previous YFPs, indifference to chloride, twice the photostability of previous YFPs, and much better expression at 37 °C and in organelles. The halide resistance is explained by a 2.2-Å x-ray crystal structure of Citrine, showing that the methionine side chain fills what was once a large halide-binding cavity adjacent to the chromophore. Insertion of calmodulin within Citrine or fusion of cyan fluorescent protein, calmodulin, a calmodulin-binding peptide and Citrine has generated improved calcium indicators. These chimera can be targeted to multiple cellular locations and have permitted the first single-cell imaging of free [Ca^{2+}] in the Golgi. Citrine is superior to all previous YFPs except when pH or halide sensitivity is desired and is particularly advantageous within genetically encoded fluorescent indicators of physiological signals.

Yellow fluorescent proteins (YFPs) were created (1) by mutating Thr^{203} of the *Aequorea victoria* green fluorescent protein (GFP) (2) to aromatic amino acids, typically Tyr. The resulting π-π stacking and increased local polarizability immediately adjacent to the chromophore are believed to be responsible for the ~20-nm shift to longer excitation and emission wavelengths (3). However, the changes in internal hydrogen bonding and steric packing also made the fluorescence more vulnerable to photobleaching (4, 5), decolorization by protonation (6–10), and quenching by many anions (10–12), of which chloride is the physiologically most relevant. These sensitivities can be exploited for specialized applications such as measuring fluorescence recovery after photobleaching and sensing pH and halide concentrations, but are deleterious for using YFPs either as simple fusion tags or as acceptors for fluorescence resonance energy transfer (FRET). YFPs are becoming very popular in such roles, particularly as partners for cyan fluorescent protein (CFP) mutants of GFP (2, 5, 13–15). CFPs and YFPs are spectroscopically well enough separated to be easily distinguishable in either excitation or emission spectra, yet the emission wavelengths of CFPs and excitation wavelengths of YFPs overlap well enough to make them good partners for FRET. They have largely superseded the initial pairing of blue mutants and improved green forms of GFP (16), because the blue mutants were too dim and photobleachable, and because shorter wavelengths generically excite more autofluorescence and raise more concerns of phototoxicity.

Measurements of FRET between CFP and YFP are becoming increasingly common to monitor protein-protein interactions nondestructively in live cells (5, 13, 17). The potential partners are fused to CFP and YFP, respectively, and coexpressed in cells. Because FRET requires that the CFP and YFP be within a few nanometers of each other, it can detect proximity at molecular dimensions, with 2 orders of magnitude higher spatial resolution than simple co-localization of the two colors. This approach has been used to monitor interactions of nuclear receptors and coactivators (18), nuclear transport factors (19), protein kinase A and anchoring proteins (20), G-protein subunits (21), G-protein-coupled receptors (22), and cytokine receptors (23). FRET can also detect intramolecular conformational changes, particularly within genetically encoded fluorescent indicators for a wide variety of intracellular analytes and processes such as Ca^{2+} (8, 24–26), (Ca^{2+})_2-CaM (27), Zn^{2+} (5), NO (28), cGMP (29, 30), protease activation (31, 32), and protein kinase A-dependent phosphorylation (33).

Genetically encoded indicators offer the major advantages of versatile and modular construction, applicability to intact transgenic organisms, and precise targetability to specific tissues, organelles, and subcellular microenvironments. These advantages are particularly important for Ca^{2+} indicators, which have been the subject of more effort than any of the other
indicator classes. Both ratiometric and non-ratiometric indicators of Ca$^{2+}$ have been constructed from CFP, GFP, or YFP (2) as fluorophores and calmodulin as calcium binding moiety in several configurations. In cameleons (26), an N-terminal CFP is fused to calmodulin, the calmodulin-binding peptide M13 from myosin light chain kinase, and a C-terminal YFP. Binding of Ca$^{2+}$ to calmodulin leads to a conformational change that enhances the fluorescence resonance energy transfer (FRET) from the shorter wavelength emitting CFP to the longer wavelength emitting YFP. Subsequent modifications in the YFP acceptor protein led to improved cameleons with decreased sensitivity to cytoxic pH changes (8). The YFP portion of these improved cameleons (termed EYFP V68L/Q69K) had a $\kappa_{\text{c}}$ of 6.1, rendering it largely insensitve to pH changes near neutrality. However, due to poor folding at 37 °C, specific targeting was hard to achieve.

In a different approach, calmodulin was directly inserted into the backbone of YFP in place of Tyr$^{145}$ to generate a medium affinity Ca$^{2+}$ indicator termed camgaroo-1 that increased fluorescence intensity ~8-fold upon saturation with Ca$^{2+}$ (34). A problem with this non-ratiometric indicator was that the fluorescence of the indicator in transfected cells at resting Ca$^{2+}$ levels was almost zero, making it difficult to identify transfected cells for experiments. Also, the protein did not express well at 37 °C.

In an effort to overcome these problems, we undertook an expression screen in Escherichia coli and identified an improved mutant of YFP, consisting of GFP with mutations S65G/V68L/Q69M/T72A/S203Y. For brevity we have named this mutant Citrine, consisting of GFP with mutations S65G/V68L/Q69M/S72A/T203Y. Significant improvement in Ca$^{2+}$ binding, but it unfortunately expressed poorly at 37 °C.

RESULTS

Pretreatment of cameleons (34) in the vector pRSET B (Invitrogen) was subjected to an error-prone PCR, transformed the resulting library into HeLa cells (3 x 10$^5$ per 35-mm dish) with Lipofectin (Life Technologies, Inc.). After 2 days of expression cells were suspended in Hanks' balanced saline solution, normalized at $A_{560}$ and measured in the fluorescence spectrometer.

Single Cell Imaging—Single HeLa cells were imaged with a charge-coupled device camera (Photometrics, Tucson, AZ) as described (26) at room temperature 1-5 days after transfection. The excitation filter for ratiometric imaging was 440DF10 with a 455DCLP dichroic mirror. The emission filters were 480DF30 (CFP) or 535DF25 (Citrine). Experiments were processed digitally using Metafluor software version 2.75 or 4.01 (Universal Imaging, West Chester, PA). For imaging cameleons-2, a 480DF30 excitation filter was used in combination with a fluorescein dichroic mirror and emission filter 535DF25.

Citrine was expressed in E. coli JM109(DE3) and the protein purified as previously described (34). Following enterokinase (Inviron) catalyzed proteolysis of the 6-His tag, 1 ml of Ni-NTA-agarose (Qiagen) was added to bind residual uncleaved protein and 6-His peptides and the solution was gently agitated (4°C for 2 h). Agarose resin was removed by filtration and the protein was concentrated to 20 mg/ml with a Micon-30 (Amicon). Citrine was crystallized by hanging drop vapor diffusion at 4°C by addition of equal volumes of protein and crystallization buffer (7% PEG 3400, 50 mM NH$_4$OAc, 50 mM NaOAc, pH 5.0). Crystals were visible after 3-4 days and grew to ~0.5 x 0.2 x 0.2 mm within 14 days. The crystals belong to space group P212121 with unit cell dimensions of a = 55.20 Å, b = 61.76 Å, and c = 50.88 Å and one monomer asymmetric unit. X-ray intensity data on a single crystal were collected at room temperature on a Mar 345 image plate detector (Mar Research) with a multilayer mirror monochromated CuK$_\alpha$ beam from a Rigaku FR rotating anode x-ray generator with mirrors. The crystal diffracted to 2.2 Å resolution with an $R_{	ext{merge}}$ of 5.5 and 99.3% completeness with 63% redundancy. All data were integrated and scaled with DENZO/SCALEPACK (37). The Wilson B-factor is 29.7 Å$^2$.

Refinement and Analysis—The atomic coordinates of the Protein Data Bank (PDB) entry 2YFP (3) with all solvent molecules, the chromophore, and residue Gln 69 removed were used as the starting model for refinement. The B factor for all atoms was set to 25 Å$^2$. One round of rigid body refinement, simulated annealing, and individual B factor refinement in CNS (38) resulted in an $R_{	ext{factor}} = 24$% and an $R_{	ext{free}} = 29$%. Refinement proceeded with alternate rounds of manual adjustment in XTALVIEW (39) and simulated annealing/B factor refinement in CNS. The stereochemistry of the model was evaluated with PROCHECK (40). The most favored regions of the Ramachandran plot contained 89.6% of the nonglycine residues with the remaining 10.4% in the additional allowed regions. Cavity volumes were determined with MMSFS (41).
fluorescence. Sequencing of the brightest clones (camgaroo-2) revealed just one new mutation, replacement of residue 69 (Gln in wild type, Lys in EYFP V68L/Q69K) by Met. The excitation and emission maxima as well as the response to \textit{in vitro} titration with \textit{Ca}^{2+} \((5.3 \pm 0.3 \mu M)\) apparent dissociation constant, Hill coefficient 1.24, fluorescence enhancement of \(-7\)-fold (Fig. 1A) were much the same as for camgaroo-1. However, camgaroo-2 produced far brighter expression in HeLa cells grown at 37 °C, where it filled the cytosol and nucleus uniformly (Fig. 1B). Stimulation of the cells with histamine produced only about \(5\%\) intensity increase (Fig. 1C), consistent with the bias of camgaroo towards higher amplitude \([\text{Ca}^{2+}]\) transients. A saturating elevation of cytosolic \([\text{Ca}^{2+}]\) induced with iomycin increased the fluorescence about \(6\)-fold (Fig. 1C). We also targeted camgaroo-2 to mitochondria using the pECFP-Mito vector (CLONTECH), which uses the targeting sequence of subunit VIII of cytochrome \(c\) oxidase. Transfected cells showed a pattern typical of mitochondria (Fig. 1D), indistinguishable from that of the accepted mitochondrial marker rhodamine 123 (data not shown). Camgaroo-2 is functional in mitochondria because a response to histamine was detected and iomycin produced a significant fluorescence increase, although lower in dynamic range than in the cytosol (Fig. 1E).

The desirable effects of mutation Q69M in camgaroo-2 prompted transfer of this same mutation into EYFP V68L/Q69K not containing any inserted proteins. This improved variant of YFP, \textit{i.e.} Citrine, has excitation and emission peaks of 516 and 529 nm, respectively, a quantum yield of 0.76, and an extinction coefficient of \(7.7 \times 10^4\) (Table I). These properties are comparable to those of previous YFPs. One unexpected spectroscopic difference is that Citrine photobleaches at about half the rate as EYFP V68L/Q69K (Fig. 2A). Based on the illumination intensity of 1.9 W/cm\(^2\), we estimate the photobleaching quantum yield of Citrine to be about \(2.3 \times 10^{-5}\), in surprisingly good agreement with an estimate of \(2.6 \times 10^{-5}\) obtained at much higher illumination intensities (35). The corresponding value for EYFP V68L/Q69K is \(5 \times 10^{-5}\) from Fig. 2A and Ref. 42. Citrine also has a considerably lower \(pK_a\), 5.7, than previous YFPs such as EYFP V68L/Q69K (Fig. 2B and Table I) making it less sensitive to fluctuations in intracellular pH. Cytosolic pH can range from \(~7.3\) to \(~6.8\), depending on cell type and stimulation (43), so cytosolic Citrine should not be expected to vary in fluorescence during normal physiological stimulation. Furthermore, pH titrations were the same in 100 mM potassium chloride and 100 mM sodium gluconate (Fig. 2B), indicating that Citrine is not perturbed by chloride. The \(pK_a\) values of all previous YFPs increase with increasing halide concentrations (10–12). For example, Fig. 2B also shows the chloride dependence of EYFP V68L/Q69K, which is actually one of the less halide-sensitive YFPs. Citrine folded efficiently at 37 °C, and with appropriate targeting sequences, could be expressed in the endoplasmic reticulum of HeLa cells. In contrast, EYFP V68L/Q69K did not tolerate attachment of ER-targeting sequences, and remained mostly nonfluorescent, with sporadic cells showing cytosolic fluorescence (data not shown).

In addition, circular permutations of Citrine were observed to develop fluorescence at 37 °C (Table I), in contrast to comparable permutations of EYFP V68L/Q69K that become fluorescent only at 20 °C or less. In summary, the Q69M mutation improves many of the shortcomings of YFP including pH and chloride sensitivity as well as the inability to fold well in organelles or as a circular permutation.

To investigate why the mutation Q69M improves YFPs chloride and pH resistance, we determined the x-ray structure of Citrine at 2.2-Å resolution (Table II and PDB accession code 1HU). As expected, the effect of the Q69M mutation on the overall structure of YFP is minor. The root mean square deviation between Citrine and the same protein with Gln at 69 (PDB accession code 1YFP) is 0.32 Å (3). In the immediate vicinity of the chromophore and the adjacent Met\(^{69}\) residue, small positional shifts (on the order of 0.3 Å when compared with 1YFP) resulting from the introduction of the bulky methionine side chain are apparent (Fig. 3A). There is a localized slight outward displacement of the two closest strands of the β-barrel due to steric contact of the side chains of residues Val\(^{150}\) and Leu\(^{201}\) with the methionine. Additional residues in the local environment, including the chromophore and its \(\pi\)-stacked partner Tyr\(^{203}\) have undergone compensatory shifts and thus the majority of the packing interactions and hydrogen bond network are unchanged.

In previous YFPs, the \(pK_a\) of the chromophore and the halide binding constant are interdependent such that protonation and halide binding facilitate each other. To explain this effect, it has been proposed that in the presence of halide, the anionic form of the chromophore is destabilized through suppressed de-localization of the negative charge (10). Conversely, neutralization of the chromophore would reduce electrostatic repulsion of an adjacent anion. Previous x-ray structural studies on YFP have shown that iodide binds in a large cavity adjacent to the chromophore and in close contact to the heterocyclic carbonyl oxygen of the chromophore (10). In the absence of halide, the
Table I: Spectral properties and pK\textsubscript{a} of selected YFP variants

<table>
<thead>
<tr>
<th>Protein</th>
<th>(\lambda_{ex})</th>
<th>(\lambda_{em})</th>
<th>(e)</th>
<th>Quantum yield</th>
<th>pK\textsubscript{a,\textsubscript{e}} (147 mM Cl\textsuperscript{-})</th>
<th>pK\textsubscript{a,\textsubscript{e}} (no Cl\textsuperscript{-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrine</td>
<td>516</td>
<td>529</td>
<td>77 \times 10\textsuperscript{2}</td>
<td>0.76</td>
<td>5.7</td>
<td>5.7</td>
</tr>
<tr>
<td>Citrine-C48L/C70V</td>
<td>516</td>
<td>529</td>
<td>69 \times 10\textsuperscript{2}</td>
<td>0.72</td>
<td>5.7</td>
<td>ND</td>
</tr>
<tr>
<td>cpCitrine\textsuperscript{b}</td>
<td>506</td>
<td>524</td>
<td>20 \times 10\textsuperscript{2}</td>
<td>0.10</td>
<td>7.7</td>
<td>ND</td>
</tr>
<tr>
<td>EYFP V68L/Q69K</td>
<td>516</td>
<td>529</td>
<td>62 \times 10\textsuperscript{2}</td>
<td>0.71</td>
<td>6.1</td>
<td>6.0</td>
</tr>
<tr>
<td>cpEYFP V68L/Q69K</td>
<td>506</td>
<td>524</td>
<td>18 \times 10\textsuperscript{2}</td>
<td>0.09</td>
<td>8.9</td>
<td>ND</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Excitation maximum (nm).
\textsuperscript{b} Emission maximum (nm).
\textsuperscript{c} Extinction coefficient (M\textsuperscript{-1} cm\textsuperscript{-1}).
\textsuperscript{d} Determined in 147 mM chloride.
\textsuperscript{e} Determined in 147 mM gluconate.
\textsuperscript{f} ND, not determined.
\textsuperscript{g} Circularly permuted EYFP V68L/Q69K.
\textsuperscript{h} Circularly permuted cpEYFP V68L/Q69K.

Table II: Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Resolution (Å)</th>
<th>No. of reflections\textsuperscript{a}</th>
<th>Completeness (%)\textsuperscript{b}</th>
<th>Rmerge, (%)\textsuperscript{a,b}</th>
<th>Refinement Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12106 (1181)</td>
<td>99.3 (99.7)</td>
<td>5.5 (14.4)</td>
<td>R\textsubscript{cryst} (%)\textsuperscript{c} 16.4 (19.5)</td>
</tr>
<tr>
<td>No. of solvent molecules</td>
<td>97</td>
<td>No. of solvent molecules</td>
<td>97</td>
<td>Average B factor (Å\textsuperscript{2})</td>
<td>26.3</td>
</tr>
<tr>
<td>Side chain</td>
<td>29.4</td>
<td>Solvent</td>
<td>35.5</td>
<td></td>
<td></td>
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<tr>
<td>Side chain</td>
<td>29.4</td>
<td>Solvent</td>
<td>35.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root mean square deviation bond length (Å)</td>
<td>0.009</td>
<td>Root mean square deviation bond angle (°)</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Numbers in parentheses refer to the highest resolution shell.
\textsuperscript{b} Rmerge = \sum |I(hkl) - I(merged)|/\sum I(hkl).
\textsuperscript{c} R\textsubscript{free} was calculated using about 10% of the reflections which were omitted from the refinement.

binding cavity (55 Å\textsuperscript{3}) is partially occupied by the side chain of Gln\textsuperscript{69} (Fig. 3B). In order to form the anion binding cavity, the side chain of this residue must undergo a conformational change and swing out of the cavity thereby expanding the cavity size (91 Å\textsuperscript{3}) and positioning the nitrogen of the carboxamide such that it is no longer accessible to a sphere with radius 1.2 Å (Fig. 3A). In the x-ray structure of Citrine, the Met is well ordered (B\textsubscript{av} = 17.5 Å\textsuperscript{3}) and there is no unexplained differences density in the region of the cavity. This suggests that the Met side chain is tightly packed into the cavity and likely unable to undergo a conformational change that would be analogous to that observed for Gln\textsuperscript{69} between the free and iodide bound forms of EYFP (10). Even if such a conformational change was permitted, it is unlikely that the thioether side chain of Met could contribute to the formation of a halide-binding site since it is incapable of hydrogen bonding in the same manner as the carboxamide nitrogen of a Gln side chain. The benefits of Q69M are not generalizable across GFP colors, because this mutation prevents CFPs from becoming fluorescent (results not shown). CFPs have bulkier chromophores based on Trp rather than Tyr at position 66, so their fluorescence would be analogous to that observed for Gln\textsuperscript{69} between the free and iodide bound forms of EYFP (10). Even if such a
Citrine represents a third generation of YFPs or yellow mutants of green fluorescent protein. The first generation was exemplified by S65G/S72A/T203Y (26) and "10C" (1), S65G/Q69K with Citrine did not alter the Ca\(^{2+}\)-dependent FRET (Fig. 4). To test this we targeted YC3.3 to the Golgi by fusing the trans-Golgi marker α-mannosidase II (6). GT-YC3.3 was saturated at resting conditions (Fig. 5C), indicating a high concentration of free Ca\(^{2+}\) in the Golgi. Histamine (100 μM) caused a very small decrease. The Golgi calcium store could be depleted with several washes of ionomycin/EGTA and was refilled upon readmitting extracellular calcium (Fig. 5C), demonstrating the feasibility of single cell imaging of free calcium concentrations in the Golgi of mammalian cells. It has to be kept in mind that ionomycin does not perform optimally in acidic compartments. Also it should be noted that YC3.3 is near its lower pH limit under these conditions. Further improvements in pH resistance are still desirable, especially if one wants to study even more acidic compartments of interest such as secretory vesicles. YC3.3 was similarly well expressed in the ER (data not shown).

**DISCUSSION**

Citrine represents a third generation of YFPs or yellow mutants of green fluorescent protein. The first generation was exemplified by S65G/S72A/T203Y (26) and "10C" (1), S65G/V68L/S72A/T203Y. These proteins proved to be quite sensitive to pH (e.g. pH 6.9–7.1) (6), halides such as Cl\(^{−}\) (11) and...
at lowering the chromophore $pK_a$ than an awkward fit with a positively charged side chain. The apparent photobleaching of YFPs probably consists of two components, a reversible proton redistribution or tautomeration and a truly irreversible covalent reaction (4, 5). Either or both would be hindered by better packing of the hydrophobic core and elimination of a cavity next to the chromophore.

Despite the inferiority of Q69K, it was an essential stepping stone in the evolution of better properties by random mutagenesis and screening, because direct alteration of the Gln codon CAG to the Met codon ATG would require two base changes in a single codon, a very unlikely event. It was fortunate that there was an easy evolutionary path from CAG to the Lys codon AAG and then to ATG. Many other examples of optimal sequences may remain relatively inaccessible to random mutation due to barriers created by the genetic code.

We have demonstrated the application of Citrine in a series of genetically encoded Ca$^{2+}$ indicators based on Citrine, all of which were improved in relation to their predecessors. Camgaroo-2 may constitute an alternative to cameleons in confocal microscopy given that it can be conveniently excited at the 488 nm argon laser line, or in cases in which targeting of cameleons are not successful. For example, we and others have found targeting of cameleons to mitochondria to be difficult (45), whereas camgaroo-2 was easy to send to the mitochondria with the targeting sequence of cytochrome c oxidase subunit VIII. Single cell imaging of mitochondrial calcium offers exciting new prospects for studying its dynamics in this organelle as well as to address aspects of heterogeneity of the mitochondrial population (46). Camgaroos lack a CaM-binding peptide and therefore have lower Ca$^{2+}$ affinities than the newest generic design of GFP-based Ca$^{2+}$ indicators, “G-CaMP” (47) or “pericam” (45). These indicators are chimeras of the CaM-binding peptide M13, circularly permuted GFP or YFP, and CaM. However, many of these molecules still do not express well at 37 °C, so annealing mutations corresponding to Q69M might well be worth incorporating.

Our new improved cameleons expressed well at 37 °C and were successfully targeted to the ER and Golgi. Cytosolic pH fluctuations are readily transmitted to the ER (48), therefore it was important to be able to express a pH-resistant functional indicator in this organelle, which had not been possible with previous versions of cameleons. Similarly, previous cameleons did not allow imaging free calcium in the Golgi due to the mild acidity of the compartment, which quenched other YFPs. Little is known about calcium regulation in the Golgi. One study using targeted aequorin identified the Golgi as a major calcium store within the cell (49), but aequorin has many disadvantages, such as lack of intrinsic fluorescence and requirement for an exogenous cofactor, that limit its use as a reliable calcium probe. We believe that Citrine should supersede previous YFPs within fusions for multicolor observation of protein trafficking, protein-protein interaction, and intramolecular conformational change, especially within genetically encoded Cu$^{2+}$ indicators.

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REFERENCES

Environmental Insensitive Yellow Fluorescent Protein