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Reverse pH-Dependence of Chromophore Protonation Explains the Large Stokes Shift of the Red Fluorescent Protein mKeima

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Genetically encoded fluorescent probes of the green fluorescent protein (GFP) family, including members of classes hydrozoa or anthozoa and their engineered variants,[1,2] are widely used for *in vivo* imaging.[3] Red fluorescent proteins (RFPs) have received particular attention, because their emission is well separated from the green-yellow autofluorescence of cells and because they are particularly useful in multicolor imaging schemes.[4]

Recently, Kogure et al. have developed an RFP named Keima that exhibits the largest Stokes shift (180 nm) observed to date.[5] When used in combination with, e.g., a cyan fluorescent protein, Keima offers the unique opportunity to perform dual color fluorescence cross-correlation spectroscopy using a single laser line to excite both fluorophores, thus considerably facilitating the technique. Here we used X-ray crystallography in combination with (*in crystallo*) UV−vis absorption, fluorescence, and Raman spectroscopy to investigate the molecular mechanism of the large Stokes shift in mKeima, the monomeric variant of Keima.

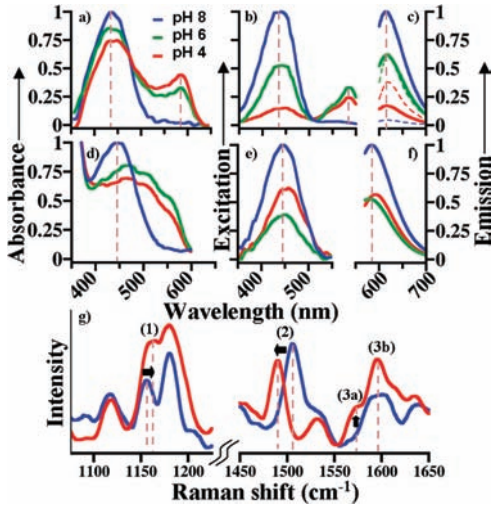

Figure 1. (a, d) Absorption, (b, e) excitation, and (c, f) emission spectra of (a−c) mKeima and (d−f) mKeima_Ser142Ala. Excitation spectra were measured with emission set to 620 nm for (b) and 590 nm for (e). Emission spectra were measured with excitation set to 440 and 590 nm in (c) (dashed lines). (g) *In crystallo* Raman spectra of mKeima: (1) imidazolinone C−C stretching mode, (2) imidazolinone/exocyclic C═C delocalized mode, and (3a−b) in-plane phenyl ring stretching modes.

The pH-dependent absorption spectra of mKeima, in both solution or the crystals display two main bands centered at 440 and 590 nm (Figures 1 and S1). Excitation spectra demonstrate that both bands elicit red fluorescence at 620 nm (Figure 1). Thus, it appears that the large Stokes shift of mKeima does not occur significantly in acidic conditions. Moreover, the data suggest that the neutral (phenol) form of the chromophore is favored at basic pH, approximately pH 8, while the anionic (phenolate) form dominates at approximately pH 4. This unexpected finding is based on the assumption that, as generally observed in GFP-like proteins, the more blue-shifted absorption band (here 440 nm) originates from the protonated form of the chromophore. Such an assumption is justified by the fact that shifting the electron density from the hydroxybenzylidene to the imidazolinone moiety of the chromophore, occurring upon $S_0 \rightarrow S_1$ transition, is energetically more demanding when the former group is protonated.[6] *In crystallo* Raman spectra support such a "reverse protonation" effect in mKeima (Figures 1 and S2 and Supporting Information (SI)). Upon lowering the pH, they reveal a softening of the imidazolinone/exocyclic C═C mixed mode ((2) in Figure 1g) and a hardening of the imidazolinone C−C stretching mode (1) consistent with more delocalized electrons in a deprotonated chromophore.[7] Moreover, the 1570 cm^{-1} phenyl mode (3a) appearing at pH 4 is also characteristic of a phenolate.[8]

To investigate the molecular origin of the "reverse protonation", we solved the X-ray structure of mKeima at three different pH values, 3.8, 5.6, and 8.0. All three structures show that the post-translational modifications of the chromophore-forming sequence Gln62-Tyr63-Gly64 result in a conventional two-ring conjugated chromophore. Interestingly, our study reveals a *cis* conformation of the chromophore at pH 3.8 and a mostly *trans* conformation at pH 8.0, while at pH 5.6 both the *trans* and *cis* isomers are found in approximately equal amounts (Figure 2). Assuming that *trans* and *cis* conformations of the chromophore correlate with neutral and anionic states, as observed in photoactivatable fluorescent proteins,[9−11] these results are in agreement with our spectroscopic findings (Figure 1). Similar structural observations were also reported for mKate, although the pH-dependence was not reverted in this case.[12]

Monomeric Keima displays high sequence identity (87%) with a fluorescent variant (His146Ser) of the chromoprotein Rtms5 from the reef building coral *Montipora efflorescens*;[13,14] however, this variant does not exhibit a large Stokes shift. As the only difference between Rtms5-His146Ser and mKeima in the immediate chromophore environment is the mutation Asn157Asp, we focused our attention to this residue. In all three structures, the carboxylate group of Asp157 is found hydrogen bonded to the hydroxyl group of Ser142, and these two residues form a pair devoid of interactions with residues other than the chromophore and water molecules. The unusual behavior of mKeima can now be understood: at pH

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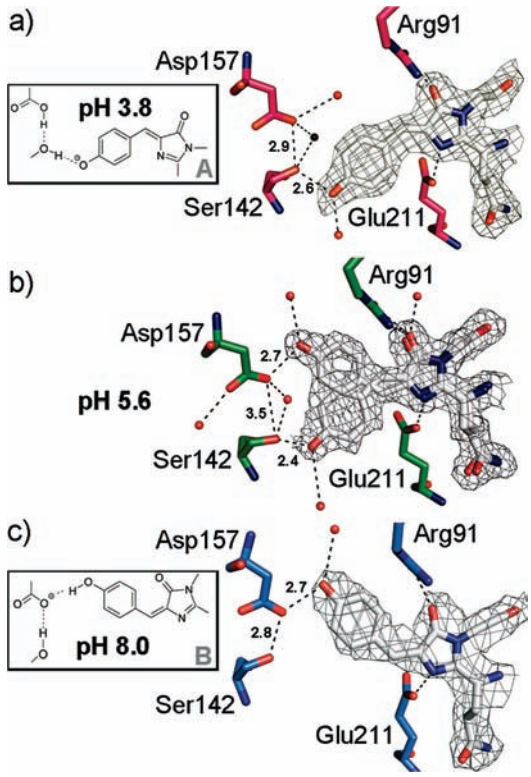

Figure 2. $2F_o - F_c$ electron density maps of the chromophore of mKeima at (a) pH 3.8, (b) pH 5.6, and (c) pH 8.0 contoured at 1σ, with the final models superimposed. H-bond distances in Å. Insets, schematic representations of the protonation states of the mKeima chromophore.

8.0, Asp157 is deprotonated, and the hydroxyl group of Ser142 is required to H-bond the two residues. This group is therefore not available to stabilize the anionic chromophore, whereas the negative charge on Asp157 promotes the binding of its phenolic form in the *trans* conformation (Figure 2, inset A). On the contrary, at pH 3.8, Asp157 is protonated, which serves to provide H-bonding to Ser142. As a result, the hydroxyl moiety of Ser142 becomes available to bind the anionic chromophore in its *cis* conformation (Figure 2, inset B). A mixture of the two configurations is observed at pH 5.6, close to the pK_a of Asp157. Upon photon absorption at high pH, due to the electron density shift toward the imidazolinone moiety, the hydroxybenzylidene becomes exceedingly acidic. Thus, we propose that the strong fluorescence of mKeima at such pH arises from excited state proton transfer (ESPT), much like what is observed in *av*GFP at low pH.[15] In such a scheme, Asp157 would act as the proton acceptor, in a similar manner as in the His148Asp mutant of *av*GFP.[16] We note that such a short-range ESPT mechanism probably involves very minor conformational rearrangements, as the large Stokes shift is maintained at 100 K, a temperature at which large structural changes are blocked (Figure S1).[17] Thus, the molecular origin of the large Stokes shift in mKeima is not linked to solvent reorganization such as demonstrated in mPlum.[18]

Our results can be related to those reported on *as*FP499, an anthozoan FP that possesses the same chromophore tripeptide as mKeima and displays H-bonding interaction between Ser143 and Asp158, two residues equivalent to Ser142 and Asp157 in mKeima.[19] Interestingly, a "reverse chromophore protonation" effect was also noticed in *as*FP499, although the *cis* chromophore is apparently not able to isomerize to a *trans* conformation due to steric hindrance. However, *as*FP499 does not display a very large Stokes shift at high pH because it lacks the acylimine bond extending the π-conjugating system and characterizing RFPs.

We anticipated that stabilization of the *cis* conformer in mKeima could essentially abolish the large Stokes shift, whereas stabilization of the *trans* conformer could extend it to a broader pH range. Consequently, we decided to produce the mutant Asp157Asn, which has its ionizable carboxyl group replaced by a nonionizable carboxiamide. With Asn in this position, the chromophore exists exclusively in the anionic form (Figure S3) engaged in a H-bond with Ser142. We also engineered the Ser142Ala mutant, on the basis that an Ala residue at position 142 is expected to prevent any interaction with the hydroxybenzylidene moiety of the chromophore. Although the brightness of the mutant decreased at high pH as compared to the parent protein, its performance was indeed higher at low pH, in the range 4−6 (Figure 1).

By combining X-ray crystallography and spectroscopy, we have suggested the molecular mechanism responsible for the large Stokes shift of mKeima. This allowed us to rationally design the Ser142Ala mutant, which will potentially improve the performance of mKeima in acidic cellular compartments such as lyzosomes.

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Supporting Information Available: Crystal growth, X-ray and spectroscopic data collection, structure determination, and illustrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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