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Low-Temperature Chromophore Isomerization Reveals the Photoswitching Mechanism of the Fluorescent Protein Padron

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Supporting Information

ABSTRACT: Photoactivatable fluorescent proteins are essential players in nanoscopy approaches based on the super-localization of single molecules. The subclass of reversibly photoswitchable fluorescent proteins typically activate through isomerization of the chromophore coupled with a change in its protonation state. However, the interplay between these two events, the details of photoswitching pathways, and the role of protein dynamics remain incompletely understood. Here, by using a combination of structural and spectroscopic approaches, we discovered two fluorescent intermediate states along the on-switching pathway of the fluorescent protein Padron. The first intermediate can be populated at temperatures as low as 100 K and results from a remarkable trans−cis isomerization of the anionic chromophore taking place within a protein matrix essentially deprived of conformational flexibility. This intermediate evolves in the dark at cryotemperatures to a second structurally similar but spectroscopically distinct anionic intermediate. The final fluorescent state, which consists of a mixture of anionic and neutral chromophores in the cis configuration, is only reached above the glass transition temperature, suggesting that chromophore protonation involves solvent interactions mediated by pronounced dynamical breathing of the protein scaffold. The possibility of efficiently and reversibly photoactivating Padron at cryotemperatures will facilitate the development of advanced super-resolution imaging modalities such as cryonanoscopy.

Reversibly photoswitchable fluorescent proteins (RSFPs) have received particular attention because of their utility in nanoscopy,[1] contrast-enhancing imaging schemes,[2] and biotechnological applications.[3] RSFPs can be repeatedly photoswitched between a fluorescent on state and a nonfluorescent off state by illumination with visible light of appropriate wavelengths. The molecular mechanisms of the switching processes, however, remain incompletely understood, and no intermediates along the switching reaction pathways have been experimentally identified. Here, by using a combination of low-temperature X-ray crystallography, in crystallo optical spectroscopy, and molecular dynamics simulations, we investigated the on-switching pathway of the fluorescent protein Padron[4] and discovered two fluorescent intermediates along the pathway. Upon illumination at 100 K, the trans anionic chromophore characteristic of the Padron off state, B_{trans}, activates to a first intermediate I_{cis}, which displays a spectroscopically distinct cis anionic configuration. I_{cis} relaxes in the dark to a second intermediate $B_{cis,LT}$, also cis anionic, which upon an increase in temperature above the glass transition temperature (T_g) in turn evolves to AB_{cis}, a mixture of B_{cis}, the fluorescent on state, and A_{cis}, a nonfluorescent protonated form of the chromophore. The observation of I_{cis} reveals that, remarkably, trans−cis chromophore isomerization can take place in Padron at 100 K, a temperature at which protein dynamics is essentially arrested. Furthermore, the data show that trans−cis chromophore isomerization can occur in Padron through a mechanism entirely decoupled from a protonation change of the chromophore benzylidene moiety. Thus, Padron is capable of dramatic fluorescence photoactivation at cryotemperatures.

Whereas most RSFPs display negative photoswitching, that is, fluorescence off-switching results from illumination at wavelengths absorbed by the protein in its fluorescent state, Padron and some other RSFPs[5,6] display positive switching, that is, illumination at such wavelengths enhances fluorescence on-switching. The X-ray structures of Padron in its off and on states [Figure S1 in the Supporting Information (SI)] closely resemble those of Padron0.9 (a mutant of wild-type Padron that favored crystallization)[7] and reveal trans and cis configurations of the chromophore, respectively, with surprisingly little rearrangement of the chromophore pocket between the two states. In its trans configuration and at physiological pH, a nonfluorescent anionic form of the chromophore (B_{trans}; λ_{abs} = 504 nm) is maintained by H-bonding of the benzilidene phenolate to Tyr159 and a water molecule. Upon illumination of B_{trans} at 523−532 nm at 100 K, a blue-shifted absorbance peak grows (λ_{abs} = 481 nm; Figure 1A), reminiscent of low-temperature photoconversion processes that have been described in green fluorescent protein and some mutants.[8] Time-resolved cryocrystallographic data (Tables S1 and S2 in the SI) reveal a concomitant trans−cis isomerization of the chromophore (Figure 2 and Figure S2), with

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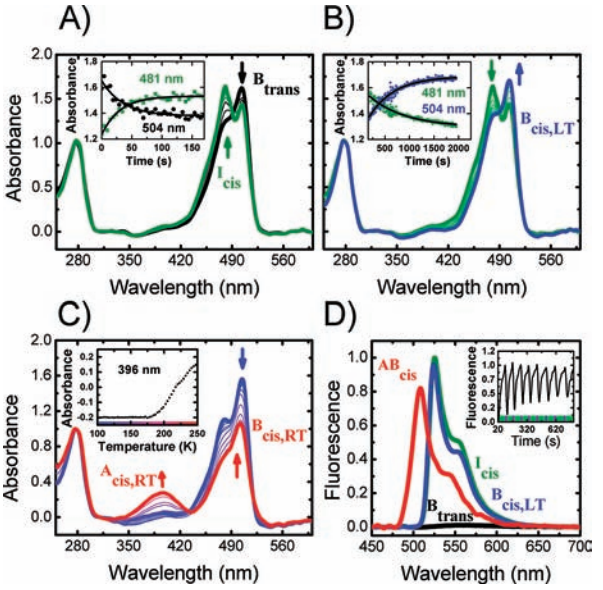

Figure 1. Spectroscopic signature of Padron along its off−on photoswitching pathway, recorded in crystallo. (A, B, C) Absorbance spectra. (A) Illumination of the Padron off state (B_{trans}, black line) at 523 nm (0.15 kW/cm^2) at 100 K yields a (partially populated) first intermediate (I_{cis}, green line). Inset: evolution of the peak absorbances of B_{trans} (black ●) and I_{cis} (green ■). Fits with monoexponential kinetic models are also shown with black lines. (B) Spontaneous relaxation of I_{cis} in the dark at 100 K yields a second intermediate ($B_{cis,LT}$, blue line). Inset: evolution of the peak absorbances of I_{cis} (green ●) and $B_{cis,LT}$ (blue ■). Fits with monoexponential kinetic models are also shown with black lines. (C) A subsequent increase in the temperature (100 K → 240 K) transforms $B_{cis,LT}$ into AB_{cis} (mixture of $A_{cis,RT}$ and $B_{cis,RT}$, red line) at ∼180 K. Inset: rise of the absorbance band of the neutral chromophore during temperature elevation. In panels A-C, intermediate spectra are shown as thin lines. (D) Emission spectra of Padron in B_{trans} (black line), I_{cis} (green line), $B_{cis,LT}$ (blue line), and AB_{cis} (red line). Excitation at 488 nm (2.5 mW/cm^2). Inset: Padron reversible photoswitching at 100 K upon alternate actinic irradiation at 532 nm (1.2 kW/cm^2, green bars) and 405 nm (0.03 kW/cm^2, violet bars) and with excitation at 488 nm (2.5 mW/cm^2). All spectra except intermediate spectra in (C) were collected at 100 K. Absorbance spectra were normalized at 280 nm. Spectra recorded in solution are presented in Figure S4. Fluorescence spectra were normalized according to the results in solution.

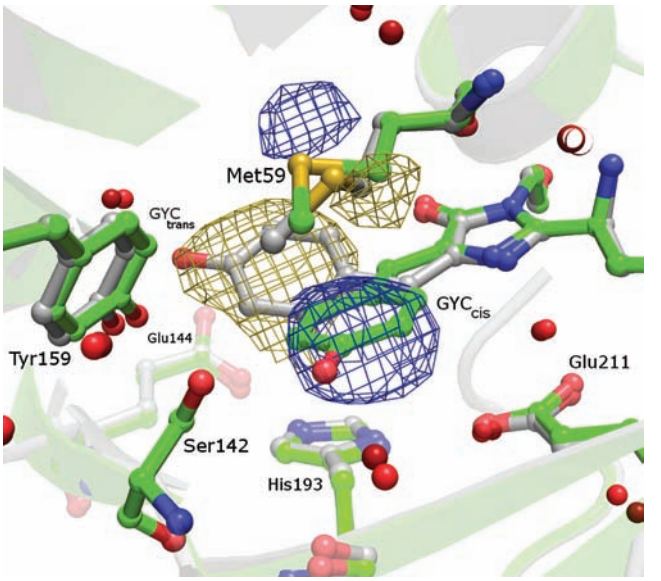

Figure 2. Low-temperature chromophore isomerization in Padron. Crystal structures in states B_{trans} (nonfluorescent, white color) and I_{cis} (fluorescent, green color) are shown, with the chromophore and key residues of the chromophore pocket (except Arg66, omitted for clarity) represented in ball-and-stick mode and the protein matrix in cartoon mode. Isomerization is evident from the experimental difference electron density map (yellow, -4.5σ; blue, $+4.5\sigma$) computed from data sets recorded on the same crystal before and after actinic illumination at 532 nm at 100 K.

only minor structural changes of the protein matrix detectable at the resolution of our data (2.7 Å). A clear displacement of Met59, located above the chromophore, is seen, accompanied by smaller motions of Met93 and Ile195 flanking Met59, which suggests that isomerization might couple with a slight deformation of the β-barrel. Molecular dynamics simulations at 100 K are consistent with these findings. They reveal that upon excitation in the rigid protein matrix, pronounced twisting of the B_{trans} chromophore around the imidazolinone bridge bond occurs, exclusively in the upper half of the chromophore pocket. This also accounts for the nonfluorescence of this anionic chromophoric state. The simulations suggest that residual backbone dynamics are necessary to break the H-bonding of the hydroxybenzilidene moiety to complete the isomerization and that a significant motion of Met59 is required (see Figure S3 and the discussion in the SI).

The formed I_{cis} intermediate is brightly fluorescent at λ_{em} = 524 nm and relaxes in minutes in the dark at 100 K to a second intermediate $B_{cis,LT}$, which has an absorbance signature similar to but narrower than that of B_{trans} (λ_{abs} = 504 nm) and fluoresces like I_{cis} (λ_{em} = 524 nm) (Figure 1B,D and Figure S4).

Crystallographic analysis of $B_{cis,LT}$ shows no detectable structural difference in comparison with I_{cis}, consistent with a low free-energy barrier between the two states that likely involves only minor conformational rearrangements. Altered electrostatics or H-bonding networks in the strained protein matrix following chromophore isomerization are likely to be responsible for the blue-shifted absorption spectrum of I_{cis}. Interestingly, illumination of I_{cis} at 405 nm is able to switch the chromophore back to a nonfluorescent (presumably trans) state. Thus, alternate excitation of Padron at 532 and 405 nm at 100 K results in partially reversible fluorescence photoswitching (Figure 1D). This photocycle apparently involves B_{trans}, I_{cis}, and $B_{cis,LT}$ as the main players (for details, see the SI and Figure S5). We note that illumination of Padron at 100 K at 488 nm (the wavelength typically used for room-temperature microscopy of this protein) still activates fluorescence (Figure S4) but prevents efficient population of I_{cis}, presumably because at this wavelength I_{cis} displays a strong extinction coefficient and thus either converts to $B_{cis,LT}$ or switches back to B_{trans}.

Like the B_{trans} → AB_{cis} process at room temperature (RT), the reaction B_{trans} → I_{cis} at 100 K involves a one-photon absorption process (Figure S6) that displays a quantum yield of $\sim 5 \times 10^{-6}$ instead of $\sim 2 \times 10^{-4}$ at RT. The I_{cis} → $B_{cis,LT}$ relaxation rate increases with temperature, following an Arrhenius law over the temperature range 120−160 K with a low free-energy barrier of ∼3.8 kJ/mol (Figure S7). Above a temperature of ∼180 K, typical of T_g in proteins, the fluorescence emission is blue-shifted to 510 nm (Figure 1D and Figure S4), and the absorbance band at 504 nm decreases concomitantly with a significant rise of a broad absorption band centered at ∼396 nm (Figure 1C). This

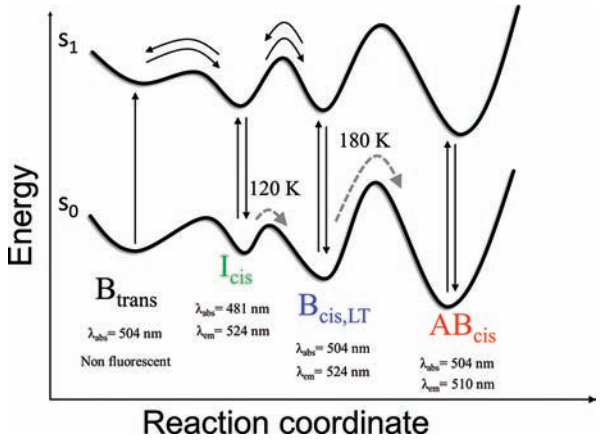

Figure 3. Scheme of the proposed mechanism for off−on Padron photoswitching.

reveals relaxation of $B_{cis,LT}$ to the mixture of the neutral (A_{cis}; λ_{abs} = 396 nm) and anionic (B_{cis}; λ_{abs} = 502 nm) species of the chromophore normally observed at room temperature in the Padron on state. The structural similarity of $B_{cis,LT}$ and the on-state mixture AB_{cis} at the resolution of our diffraction data (2.35 Å) suggests that the relaxation process does not involve pronounced conformational rearrangements, although dynamical breathing must be required for efficient proton transfer (probably from the bulk solvent) following isomerization, in line with recent proposals.[7]

A proposed model for off−on photoswitching in Padron is recapitulated in Figure 3. This model brings new insight into the photoswitching mechanisms in RSFPs. First, photoswitching has to date been described as correlated changes in chromophore protonation and isomerization,[5,9−11] but the interplay between these two changes has remained debated.[12−17] Our data strongly suggest that in the case of Padron, protonation of the benzylidene moiety of the chromophore is not needed in the process of chromophore trans−cis isomerization but rather follows that step. Different scenarios might hold in other RSFPs. Second, the occurrence of chromophore isomerization in Padron at cryotemperatures, which involves a much larger displacement than in other photoreceptors,[18,19] is remarkable in terms of protein dynamics and is unique among RSFPs. In Dronpa (see Figure S8 and the discussion in the SI),[10] mTFP0.7,[9] and IrisFP,[11] cis−trans isomerization involves major conformational rearrangements of residues Glu144, His193, Glu211, and Arg66 (Padron numbering), all situated in the lower half of the chromophore pocket. These structural changes are incompatible with the essentially stalled protein dynamics at 100 K. In contrast, Padron seems to be tuned in such a way that isomerization may occur in the upper half of the pocket, involving only subtle motions of Met59 and the neighboring protein scaffold, compatible with low-temperature protein dynamics. The positively switchable RSFP asFP595 also exhibits only minor conformational rearrangements upon chromophore isomerization,[5] and it will be interesting to investigate the cryoswitching properties of this protein, particularly in view of the anionic-to-zwitterionic activation mechanism suggested by theoretical investigations.[20]

The possibility of photoactivating Padron at cryotemperatures opens the door to the development of cryonanoscopy, which would offer a number of advantages, including reduced photobleaching, sample preservation, freeze-trapping of transient cellular states, and potential correlative light cryoelectron microscopy studies. Furthermore, brighter fluorescence activation is obtained than at ambient temperature, since no fluorescence loss follows from substantial chromophore protonation as observed upon photoswitching at room temperature and at physiological pH.

■ ASSOCIATED CONTENT

Supporting Information. Crystal growth, X-ray data collection and structure determination, spectroscopy setup and methods, molecular dynamics simulations, details of the low-temperature Padron photocycle, and illustrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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