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Ultrafast Carbonyl Motion of the Photoactive Yellow Protein Chromophore Probed by Femtosecond Circular Dichroism

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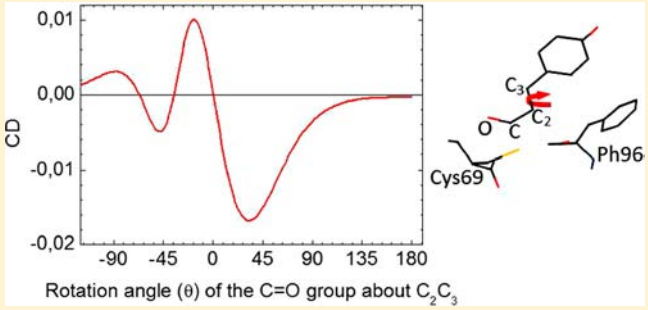
Supporting Information

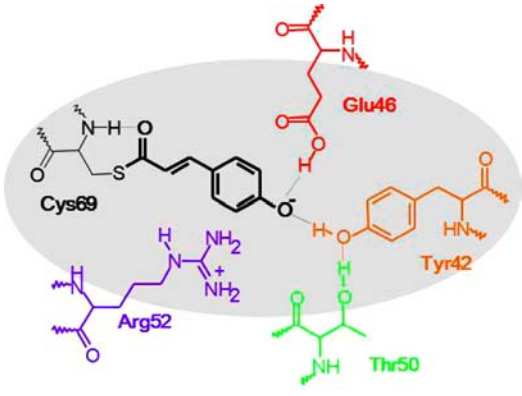
ABSTRACT: Motions of the *trans-p*-coumaric acid carbonyl group following the photoexcitation of the R52Q mutant of photoactive yellow protein (PYP) are investigated, for the first time, by ultrafast time-resolved circular dichroism (TRCD) spectroscopy. TRCD is monitored in the near-ultraviolet, over a time scale of 10 ps. Immediately after excitation, TRCD is found to exhibit a large negative peak, which decays within a few picoseconds. A quantitative analysis of the signals shows that, upon excitation, the carbonyl group undergoes a fast (≪0.8 ps) and unidirectional flipping motion in the excited state with an angle of *ca.* 17−53°. For the subset of proteins that do not enter the signaling photocycle, TRCD provides strong evidence that the carbonyl group moves back to its initial position, leading to the formation of a nonreactive ground-state intermediate of trans conformation. The initial ground state is then restored within *ca.* 3 ps. Comparative study of R52Q and wild-type PYP provides direct evidence that the absence of Arg52 has no effect on the conformational changes of the chromophore during those steps.



1. INTRODUCTION

Mimicking the capabilities of biomolecules to convert light into energy for the engineering of artificial nanodevices is a current scientific challenge.[1−3] This goal however requires a previous detailed understanding of the natural transduction mechanisms at work at the molecular scale. Over the past decade, photoactive yellow protein (PYP) has become a model system for studying light conversion processes in biological photoreceptors.[4−8] Recently, PYP has been also used for the development of new protein labeling probes for fluorescence imaging.[9] PYP is the putative photoreceptor involved in the negative phototactic motion toward blue light of several halophilic purple bacteria.[10] The photoresponse is mediated by the deprotonated *trans-p*-coumaric acid (see Scheme 1), covalently linked to the protein via a thioester bond. Upon blue-light irradiation, the protein undergoes a photocycle which triggers signal transduction. It is known that the first step of the photocycle involves the trans−cis isomerization of the chromophore, through the flipping motion of the thioester carbonyl group (C═O) on the femto-picosecond time scale, leaving intact the geometry on phenolate side (for reviews see refs 4 and 5 and references therein). This reaction leads to the formation of the first photocycle intermediate, known as I_0,

Scheme 1. Schematic Representation of the PYP Active Site in the Ground State



with a yield of *ca.* 0.3.[11−21] Consequently, a progressive reorganization of the hydrogen-bond (HB) network around the chromophore, meant to accommodate the *cis* conformation of

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I_0, occurs on the nanosecond time scale, and leads to the formation of a relaxed *cis* isomer called I_1.[16,17,22,23]

It is well established that the isolated deprotonated chromophore of PYP does not form any stable *cis* isomer in basic aqueous solution.[24−30] In the native protein, calculations predicted that the isomerization could be promoted by the presence of the positively charged residue Arg52, located nearby the chromophore.[31−33] Such a crucial role of R52 has been however discarded on the basis of experimental studies of the R52Q and R52A mutants, in which Arg52 is replaced by neutral residues. These experiments provided evidence that, despite the absence of the charged residue, the earliest steps of the photocycle of the two mutants remain similar to that of the native protein.[13,34]

There is however clear experimental evidence that the protein environment plays a crucial role on the isomerization process of PYP.[13,35−39] In particular, alteration of the HB network of the active site by mutation was observed to induce a marked decrease of the isomerization yield.[13,34,39] Both UV−visible and IR time-resolved absorption spectroscopy provided evidence that blue light absorption opens two competitive channels:[13,18,19,40] a reactive channel leads to the formation of I_0 and a nonreactive one restores the initial ground state (pG) in *ca.* 3−6 ps, through the formation of a ground-state intermediate, named GSI[18] or X.[13] In the following, we will use the notation GSI to refer to this transient. Although the existence of GSI is well established, its nature remains until now disputed.[13,18,19,40] A distorted vibrationally hot trans species[13,17] or an intermediate with a conformation close to *cis*[18,40] have been conjectured.

While UV−visible transient absorption spectroscopy allowed the characterization of the different electronic states involved in the PYP photocyle, it did not provide direct structural information on the chromophore.[18,35,37,41−43] In contrast, important structural information has been obtained by time-resolved crystallography, time-resolved IR, and resonance Raman spectroscopy.[16,17,20−23,40,44−47] Notably, time-resolved crystallography showed that the chromophore C═O explores various environments, during the photoisomerization process,[20,21,23,44] but the chromophore structural changes, in the subpicosecond time scale, remains elusive, so far. In this regard, circular dichroism (CD) spectroscopy can provide a very sensitive probe of the molecular conformation.[48,49] In particular, it is well-known that the sign of CD signals associated with the absorption bands of carbonyl groups can reveal very precise information about their environment, thanks to the octant rule.[50] With our expertise in time-resolved CD experiments (TRCD) with subpicosecond time resolution,[51] we carried out the first study of this type on the R52Q mutant of PYP, in which Arg52 has been replaced by a neutral glutamine. Measurements on wild-type PYP (WT) were also done for comparison. The detailed analysis of our results allowed us to follow the motion of the chromophore C═O after excitation, both in the excited state pG* and the intermediate GSI.

2. MATERIALS AND METHODS

2.1. Sample Preparation. WT and R52Q were produced as previously described.[52,53] Samples were prepared in Tris-HCl buffer (10 mM) at pH 8.1. For steady-state and time-resolved spectroscopy, the sample absorbance was adjusted to about 1 (for 1 mm optical path) at the absorption maximum. Steady-state CD spectra were measured with a Jasco J710 spectropolarimeter.

2.2. Time-Resolved CD. Subpicosecond time-resolved experiments were carried out with the setup described in ref 54. The pump beam (420 nm) was directly obtained by frequency-doubling a 1 kHz Ti:sapphire laser. The pulse energy was about 1 μJ. The pulses were focused to a 208 × 137 μm^2 elliptical spot in a 1 mm path length silica cell. The probe, tuned between 300 and 355 nm, was generated through optical parametric amplification of a white-light continuum followed by second-harmonic generation stage. It was focused to a 83 × 34 μm^2 elliptical point on the sample. The two laser beams passed through a common polarizer to ensure exactly parallel polarizations, and their superposition was assured with a 50 μm pinhole at the sample position. Pump−probe cross-correlation has been measured to be *ca.* 800 fs, and represents the instrument response function (IRF). TRCD measurements were performed by evaluating the variation of the laser beam ellipticity with a Babinet−Soleil compensator and a crossed analyzer, as a function of the pump−probe delay.[48] Measurements were alternatively conducted with and without the pump to enable us to extract the CD changes (δCD) and absorption changes ($\delta\alpha$L = ln(10) × δOD) induced by the excitation. In the following, we note $\delta CD = (\delta\alpha_L - \delta\alpha_R)$, where $\delta\alpha_{L,R}$ is the absorption coefficient change for left or right circular polarization, respectively, and L is the cell optical path. Note that $\delta\alpha$L and δCD are unitless. Since the amplitude of the CD is only a tiny fraction (a few 10^{-3}) of the absorption, TRCD experiments are quite difficult to carry out. More specifically, measurements of the TRCD with a Babinet−Soleil compensator, which avoids artifacts correlated to the use of electro-optic modulators, are very time-consuming. Typically, 8-h acquisitions were necessary to obtain the CD curves presented in this article, while the acquisition time of usual transient absorption signals was 10-fold reduced.

3. RESULTS AND DISCUSSION

3.1. Steady-State CD Spectroscopy. The steady-state absorption and CD spectra of R52Q are displayed in Figure 1.

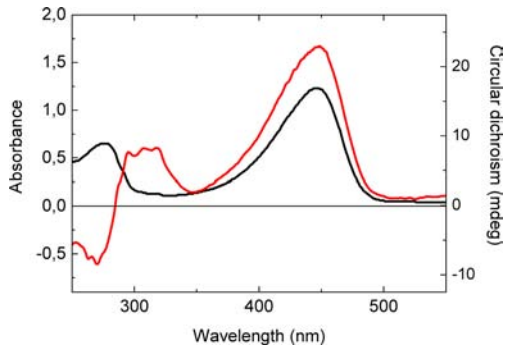

Figure 1. Absorption (black) and CD (red) spectra of R52Q.

Interestingly, the spectra of R52Q are indiscernible from their wild-type counterpart (see Supporting Information, Figure S1), emphasizing the fact that this mutation neither brings any dramatic change to the chromophore electronic state nor to its conformation. Similar CD spectra have been previously reported for WT.[55−57] Comparative study with the apoprotein showed that the negative CD signal below 300 nm arises from the protein backbone, whereas the positive signal above 300 nm is mainly due to the chromophore.[55−57] More specifically, the CD structure in the 300−320 nm range has been assigned to the chromophore C═O group, on the basis of the two following observations.[55] A strong CD signal associated with a weak absorption, in this spectral range, is known to be a typical feature of n−π^* transitions of C═O groups.[49,55,58] In addition, the positive sign of the CD which is in accordance with the

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octant rule, is associated to the presence of the N atom (N69) borne by the Cys69 residue nearby.[55,58]

3.2. Transient Absorption and Transient CD Spectroscopy. We performed time-resolved experiments on WT and R52Q. Both samples yielded comparable results. The first experiments were actually done on WT, with results shown in Figure S2, in the Supporting Information. As explained in the Supporting Information, because of the novelty and difficulty of these experiments, it happened that some improvements were made when we turned to the R52Q mutant. For the quantitative analysis of the experimental TRCD results, in the following we will thus mainly concentrate on R52Q whose transient signals exhibit a better signal-to-noise ratio. At this point, it has to be recalled that the photoinduced spectroscopic behavior of R52Q has been found to be very similar to that of WT, even at early times. The photophysical processes at work in both proteins are believed to be essentially identical. The main difference is that deletion of R52 in R52Q slows the excited-state deactivation down and decreases the quantum yield of isomerization of the chromophore.[13,34−36,38] Because of the softening of the active site HB network, the nonreactive branch of the photocycle was found to be reinforced in R52Q: about 80% of the excited chromophores restore the initial ground state (pG) without initiating the photocycle versus 70% in WT.[13]

We carried out time-resolved measurements at five different probe wavelengths: 301, 318, 332, 351, and 355 nm. This spectral range corresponds to the spectral domain relevant for the chromophore C═O transition. This feature is clear from the steady-state CD spectrum of R52Q (Figure 1) and WT. Previous experiments[13,18,19,35] have also shown that the excited-state absorption of WT and R52Q extends to the UV domain and one can infer, from theoretical works carried on WT, that this transition involves the chromophore C═O orbitals.[31,59]

For each probed wavelength, we performed two types of experiments. First, a "regular" pump−probe transient absorption experiment, much easier to do than TRCD, allowed us to obtain a precise estimation of the relaxation time scales of the protein. A typical differential absorbance ($\delta\alpha$L) decay measured at 332 nm, for R52Q, is given in Figure 2. At all probed wavelengths, immediately after excitation, we observed positive $\delta\alpha$L signals, dominated by excited-state absorption (ESA). Similar decays have been observed in previous experiments in the same spectral region.[13,35] The kinetic traces at the five probed wavelengths were globally fitted with one exponential function and a step function, convoluted to a Gaussian function (fwhm = 0.8 ps) corresponding to the IRF. The fit yields a relaxation time of 3.2 ± 0.6 ps; the small plateau corresponds to the residual contribution of long-lived species. We here give as the fitting error twice the standard error, that is, the half-width of the 95% confidence interval. As illustrated in Figure 3 inset,

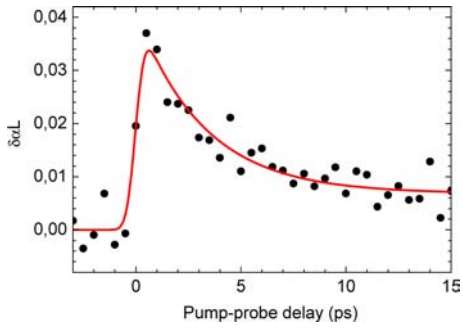

Figure 2. Transient absorption change in R52Q at 332 nm. The solid red line is the fit with an exponential function and a step function, convoluted with a Gaussian function (fwhm = 0.8 ps) corresponding to the IRF.

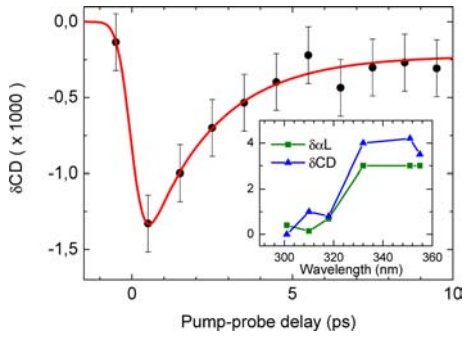

Figure 3. Transient CD change in R52Q at 332 nm. The CD is expressed as CD = $(\delta\alpha_L - \delta\alpha_R)$L. The vertical bars are representative error bars. The solid red line is the fit with an exponential function and a step function, convoluted with a Gaussian function (fwhm = 0.8 ps) corresponding to the IRF. The inset compares the maximum absorption and the CD changes, in arbitrary units.

the amplitude of the $\delta\alpha$L signals is wavelength-dependent. One notes it increases at long wavelengths, in fair complementarity with previous transient absorption experiments,[13,18,19] which showed an ESA band between 350 and 400 nm. We can thus confidently assign the positive $\delta\alpha$L observed between 301 and 355 nm to the blue wing of this ESA band. The residual $\delta\alpha$L signal observed after 10 ps (see Figure 2) can be assigned to the end of the ESA decay as previously observed for WT and R52Q.[13,35] It is worth noting that the two-photon ionization processes of the chromophore, observed in the previous studies of WT and R52Q,[13,18,35] lead to the formation of long-lived radicals absorbing below 360 nm. Interestingly, the comparison of the $\delta\alpha$L signals at 351 and 355 nm with those previously published in refs 13 and 35 indicates that the relative amplitudes of the exponential and the step functions are similar. We thus deduce that the contribution of radicals to the $\delta\alpha$L signals in the present experimental conditions represents less than 6% of the excited population.

Second type of experiments we performed is TRCD. Typical results obtained for R52Q at 332 nm are displayed in Figure 3. The error bar indicates the typical experimental uncertainty. It is noteworthy that the figure displays the change of CD signal (δCD) as a function of pump−probe delay. It is therefore zero at negative delays and gives the amplitude and the sign of the CD changes induced by the pump at positive delays. For all probed wavelengths, the same behavior has been found. As shown in Figure 3, immediately after excitation, a large *negative* signal is observed. The fit of δCD with the sum of one exponential function and a step function, convoluted to a Gaussian function (fwhm = 0.8 ps) representing the IRF, gives a relaxation time of 2.1 ± 0.8 ps. Although this time constant slightly differs from that found for $\delta\alpha$L (3.2 ps), the 95% confidence intervals overlap, and direct comparison of both signals on a normalized scale, as shown in Figure 4, confirms that the relaxation times are indeed comparable. Another interesting feature, shown in the inset of Figure 3, is that the amplitudes of $\delta\alpha$L and δCD signals display the same wavelength dependence, immediately after excitation. These

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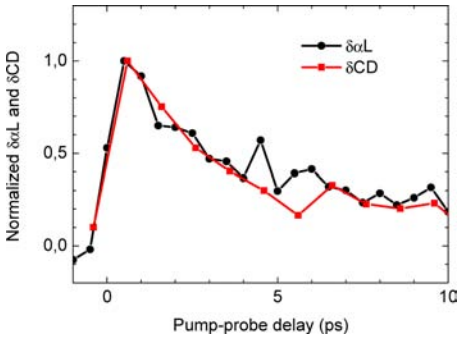

Figure 4. Normalized time-resolved absorption and CD changes of R52Q at 332 nm.

two observations clearly indicate that both signals stem from the same electronic states.

3.3. Chromophore C═O Motion in the Excited State. The most striking feature of the transient CD of R52Q is the strongly negative value of the signal observed immediately after excitation, at all probed wavelengths. It is worth noting that measurements carried on WT (see Supporting Information, section 1) show a very similar behavior, which confirms that photophysical processes of the same nature occur in both proteins at early times. In addition, femtosecond fluorescence spectroscopy demonstrated that the frequency of one out-of-plane bending mode of the chromophore, supposed to promote the flip of the C═O, is not altered by this mutation.[60] All these observations point to the fact that similar conformational changes are involved in the excited-state deactivation of R52Q and WT.

As detailed in the Supporting Information (section 2), we calculated the CD contribution of the pG* state (CD_{pG*}) to δCD signal of R52Q, at 332 nm, for the following two cases. In the first case, the excited-state is considered to be structurally homogeneous, while in the second case, the excited state is assumed to be structurally heterogeneous. Estimating that the fraction of excited molecules (η) is equal to 6.6%, we found that CD_{pG*} and the average value of CD_{pG*} ($<CD_{pG*}>$) are *ca.* −0.015 for the homogeneous and the heterogeneous cases, respectively. Although, this value may be overestimated due to the uncertainty on η and some slight contribution from the sample anisotropy, it comes out that the CD of pG* is not only of opposite sign from that of the initial ground state pG ($CD_{pG} = 2.7 \times 10^{-4}$), known to be slightly positive in this spectral region,[55] but also much larger in absolute amplitude. It is well established that CD signals are closely connected to the C═O environment. The octant rule allows acquisition of the absolute sign of the CD by considering the environment of a C═O bond.[50] The principle is the following: given an atomic group A located in the vicinity of the C═O bond, and defining its position relative to the middle of the C═O bond with vector $\vec{R}_{AC}$ its contribution to the CD scales as[58]

$$\Theta = \frac{-xyz}{R_{AC}^4} \quad (1)$$

In this expression, R_{AC} is the distance between A and C and *x, y, z* are the coordinates of the normalized vector $\vec{R}_{AC}/R_{AC}$. Product *xyz* expresses the "octant rule", which assigns a different sign for the various octants, whereas the inverse dependence with the fourth power of the distance implies that only the closest groups play a role. To investigate the CD in the ground state of WT, we examined the crystallographic structure of WT, 1UWN, obtained from the Protein Data Bank (PDB).[61] We determined that two main contributions to the ground-state CD exist. As mentioned above, the dominant one comes from the N69 atom, which yields a positive contribution to the CD. The contribution of the S atom of Cys69 (S69) is also found to be very important, and to give a negative contribution to the CD. The N69 contribution is however found to be stronger. The total steady-state CD of WT is thus expected to be slightly positive, as experimentally observed.[55] Since the R52Q mutation has no effect on the steady-state CD spectra of the protein, it can be deduced that the local environment of the chromophore C═O remains similar to that of WT, as far as Cys69 is concerned. This is consistent with the X-ray structure of R52Q (2D02 from PDB).[62]

To determine the role of the relative position of the chromophore C═O group with respect to the protein backbone, we then computed the CD, obtained by summing the contributions of N69 and S69 atoms with eq 1, as a function of the C═O rotation about the C_2-C_3 axis (0° corresponds to the ground-state configuration). The result is displayed in Figure 5. Two observations are worthwhile. First,

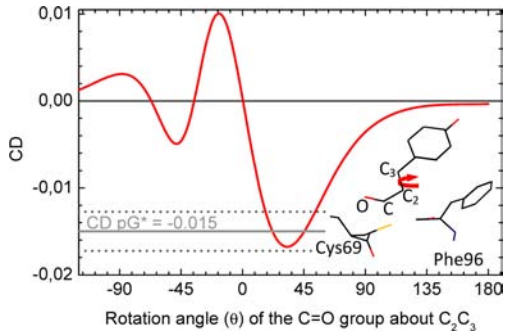

Figure 5. Calculated variation of CD associated to the pG state of R52Q and WT as a function of the rotation angle of C═O about the axis C_2-C_3. Amplitude of CD at 0° is fixed to 2.7×10^{-4} for the sake of comparison with the CD of the excited state pG* at 332 nm indicated by the solid gray line. The dotted gray lines represent a tolerance of ±15% on the position of the solid gray line. The inset shows the relevant residues. A positive angle corresponds to a rotation of C═O toward the Phe96 residue.

we observe a very strong negative differential CD signal when the chromophore C═O is rotated by about 30°. As shown in the inset of Figure 5, this rotation corresponds to a motion of the C═O toward the reader; it leads to a decrease of the distance between the chromophore C═O and the Ph96 residue, in agreement with the photoisomerization trajectories presented in ref 31. In contrast, the rotation in the opposite direction induces significantly smaller variations of the CD signal, the larger ones of which are associated to positive signals. To reach quantitative conclusions, the calculated CD amplitudes of Figure 5 have been scaled so as to yield a value of 2.7×10^{-4} for an angle of 0°, which corresponds to the trans structure of pG. CD_{pG*} at 332 nm estimated considering a homogeneous structural excited state is indicated by the solid gray line. It crosses the calculated CD curve (red) at $\theta = +22°$ and +45°. We next took into account both the error on the experimental determination of CD_{pG*} and the error on the calculated CD by allowing an effective tolerance of ±15% on the level of CD_{pG*} in Figure 5, the calculated CD curve being kept fixed. The dotted gray lines thereby obtained cross the calculated curve at values which define a confidence interval for

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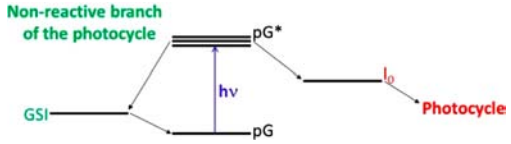
the torsion angle: we obtained $+17° < \theta < +53°$. It is clear from Figure 5 that the remarkably large negative average value of CD_{pG^*} ($\langle CD_{pG^*} \rangle$) found considering a structural heterogeneous excited state can be reached only if the distribution of the chromophore C═O angles is located in the same confidence interval. We infer that upon excitation the main part of excited-state population undergoes the same structural changes. The excited state can be thus considered as structurally homogeneous. Interestingly, this flipping motion of the chromophore C═O has two effects. It drives N69 further away from the chromophore C═O and it puts S69 in a more favorable position for the octant rule, increasing tremendously its negative contribution to the CD signal. It should be noted that the distance between S69 and the chromophore C═O does not change much during this rotation, which thus preserves the covalent link between the chromophore and the backbone. Second, one can see in Figure 5 that, according to our calculations, a large rotation of the C═O group (>90°) is not expected to have a significant effect on the CD signals. The δCD associated to the chromophore in the full *cis* conformation ($\theta = 180°$) is therefore expected to be very small (about -3.5×10^{-4}). On the contrary, a partial flipping of the chromophore C═O ($+17° < \theta < +53°$) yields a large negative δCD. It is finally reasonable to interpret the strong negative δCD signal, observed immediately after excitation of WT and R52Q, as due to a fast ($\ll$0.8 ps) unidirectional partial flip of the chromophore C═O group in the excited state. Although the use of the octant rule could be questioned in the case of a molecule in the excited state, this fast motion of the C═O is consistent with theoretical works,[29,31] which predicted a partial isomerization of the chromophore in the pG* state, preserving the HB between the C═O group and Cys69.[31] The existence of this HB in pG* was also confirmed by transient infrared spectroscopy.[40]

3.4. Chromophore C═O Motion Following Excited-State Deactivation. To explore the C═O motion following the excited-state deactivation, we compared the kinetics of the $\delta\alpha$L and δCD signals. The main difficulty here is that the transitions from pG* to GSI and I_0 and from GSI to pG occur on the same time scale, in *ca.* 3−6 ps.[13,18,40] It is thus not straightforward to disentangle these relaxation processes from the experimental data. For this purpose, we therefore performed a simulation of the $\delta\alpha$L and δCD decays with the kinetic model previously developed in refs 13, 18 and 40, illustrated in Scheme 2.

Scheme 2. Kinetic Model for the Excited-State Deactivation of WT and R52Q[13,18,40]



With this model, we simulated the temporal evolution of the concentrations of the various species (pG*, GSI, I_0, and I_1), on the time scale of 10 picoseconds, and calculated the corresponding $\delta\alpha$L and δCD traces (see Supporting Information, section 3). Values of the absorption coefficients of pG* and GSI were estimated from the present experiment and previous time-resolved transient absorption measurements.[13,35] It is also known from previous measurements that the absorption coefficients of the *cis* intermediates I_0 and I_1 are similar to that of pG, in the spectral region below 370 nm, corresponding to the n−π* transition of the C═O group.[13,19,63−65] Such an observation can be explained by the weak coupling of the lone pair orbital of the C═O group with the rest of the chromophore, in the ground state. Furthermore, transient infrared spectroscopy brought evidence that the first photocycle intermediates I_0 and I_1 display the full *cis* geometry.[16,17] In the light of our calculations (Figure 5), we deduced their corresponding CD, and from our estimate of the CD amplitude associated with the excited state, pG*, we performed a simulation of the transient CD decays, considering the following two cases: (1) In the first case, the CD associated with GSI has the same value as that of the excited state ($CD_{GSI} = CD_{pG^*}$) which supposes that the chromophore C═O remains twisted in GSI. (2) In the second case, the CD associated with GSI has the same value as that of the initial ground state pG ($CD_{GSI}=CD_{pG}$), which implies that the chromophore C═O has recovered its original position in GSI. In such a case, the contribution of GSI to the TRCD signal becomes negligible. Note that a similar behavior would also be expected if GSI displayed a full *cis* conformation. It is however well established from previous studies that the absorption coefficient of the *cis* isomer is smaller (by about a factor of 2) than that of the trans isomer, in the visible spectral region.[13,18,63−65] The absorption spectrum of GSI was however found to be similar to that of pG, both in shape and intensity, except for a 10-nm red shift.[13,18] These features strongly suggest that the conformation of the chromophore in GSI, if distorted, remains close to the initial trans configuration. Moreover, transient infrared spectroscopy showed the presence a HB between the chromophore C═O and Cys69 both in states pG* and GSI, which excludes that these intermediates have the full *cis* conformation.[40]

Both simulated transient CD curves have been plotted together with the simulated transient absorption in Figure 6.

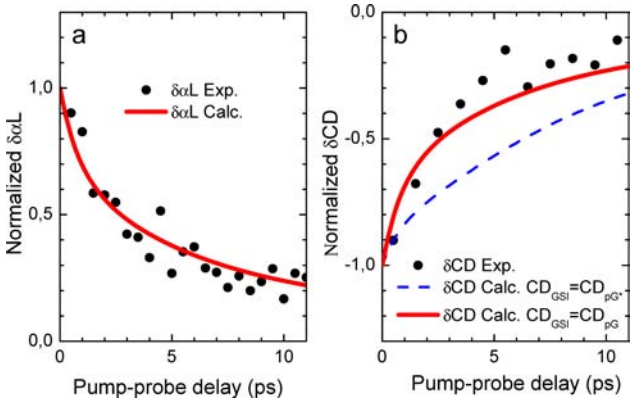


Figure 6. (a) Normalized experimental (black dots) and calculated $\delta\alpha$L (red line). (b) Normalized experimental (black dots) and calculated δCD with the hypothesis that $CD_{GSI} = CD_{pG^*}$ (dashed blue line) and with the hypothesis that $CD_{GSI} = CD_{pG}$ (red line).

The simulations show that the contributions to the transient absorption and the TRCD signals mainly arise from pG* and GSI while those of I_0 and I_1 are negligible, on the time scale of 10 ps. On the other hand, it is clear that the two hypotheses predict a very different behavior for the relaxation of the transient CD signal. If the geometry of the chromophore C═O in GSI is close to that of pG, there is no clear difference

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between the transient CD and the transient absorption kinetics. In contrast, the transient CD is expected to exhibit a much slower decay than the transient absorption if the geometry of the chromophore C═O in GSI remains close to that of the excited state; a CD relaxation time of *ca.* 9 ps is then obtained (see Supporting Information, Table S3). Such a slow decay, and significant difference with the transient absorption decay, is not compatible with our experimental findings (see Figures 4 and 6). This conclusion applies for all probed wavelengths. We can therefore conclude that the conformation of the chromophore C═O in GSI is very close to that of the initial ground state pG.

3.5. Nature of GSI and Role of R52. In a previous report, some of us attributed GSI to a distorted vibrationally hot trans ground state.[13] Conversion of GSI to pG in about 3−6 ps was then associated to a combination of vibrational relaxation and conformational relaxation of the chromophore along the isomerization coordinate.[13,17] It appears from the present measurements that large conformational changes probably do not occur in GSI, as far as the C═O group is concerned. We therefore think that the main difference between GSI and pG should be sought in a combination of chromophore vibrational relaxation and relaxation of the chromophore environment. As a matter of fact, relaxation of GSI is known to be quite sensitive to the chromophore surrounding, its rate constant being significantly reduced in mutants whose HB network around the chromophore is weakened.[13] Femtosecond transient IR spectroscopy as well as picosecond Raman spectroscopy indeed showed that the HB network of the protein active site is significantly perturbed, although not disrupted, in the excited state of the protein.[40,45−47] Particularly noteworthy, Raman spectroscopy revealed changes in the spectrum of a tryptophan residue located 12 Å apart from the chromophore, in the time scale of 3 ps. This highlights the fast propagation of a perturbation within the protein structure, induced by photon absorption.[45]

Finally, calculations predicted that the isomerization pathway could be promoted by the presence of the positively charged Arginine-52, lying nearby the chromophore.[28,31,32] Such a dramatic effect however was not supported by experimental studies of the R52Q and R52A mutants, in which Arg52 is replaced by neutral residues.[13,34] TRCD measurements provide here the direct evidence that the absence of the arginine has no influence on the conformational changes of the chromophore in the protein excited state leading to the same deactivation process in R52Q than in the native wild-type protein.

4. CONCLUSIONS

We have presented the first study of the R52Q mutant of PYP by time-resolved CD spectroscopy, in the spectral region of the near UV. Upon excitation, the transient CD signals are found to be largely negative and to decay in *ca.* 2.1 ps. Quantitative analyses of the signals, based on the octant rule and a kinetic model developed in previous studies,[13,40] allow us to follow the photoinduced motion of the chromophore carbonyl. We in particular propose an improved description of the initial events occurring in the nonreactive part of the PYP photocycle. Upon excitation, the chromophore undergoes a fast (≪0.8 ps) and unidirectional flipping motion of its C═O group, with an angle of *ca.* 17°−53°. For the chromophores which do not enter the photocycle, the C═O group flips back to its initial trans position leading to the intermediate GSI, before restoring the initial ground state pG, within *ca.* 3 ps. The conversion of GSI to pG could be due to vibrational relaxation of the chromophore and restoration of the initial hydrogen-bond network, which is known to be significantly altered in the excited state of the chromophore. Transient CD measurements carried on the wild-type PYP yielded similar results to R52Q providing evidence that Arginine-52 has no influence on the conformational changes of the chromophore in the excited state.

■ ASSOCIATED CONTENT

Supporting Information

(1) Comparison of WT and its R52Q mutant; (2) determination of the absorption and the circular dichroism of the excited molecules for R52Q; (3) simulations of transient CD and transient absorption decays of R52Q. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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