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Gas-Phase Photochemistry of the Photoactive Yellow Protein Chromophore *trans-p*-Coumaric Acid

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Abstract: The photoisomerization of trans-*p*-coumaric acid (*trans*-CA) triggers a photocycle in photoactive yellow protein that ultimately mediates a phototactic response to blue light in certain purple bacteria. We have used fluorescence excitation and dispersed emission methods in a supersonic jet to investigate the nature of the electronic excited states involved in the initial photoexcitation and subsequent photoisomerization of *trans*-CA. We observed three distinct regions in the fluorescence excitation spectrum of *trans*-CA. Region I is characterized by sharp features that upon excitation exhibit *trans*-CA S_1 emission. In region II, features increase in width and decrease in intensity with increasing excitation energy. Upon excitation, we observed dual emission from the S_1 state of *trans*-CA and what may be the S_1 state of *cis*-CA. The onset of dual emission corresponds to an isomerization barrier of about 3.4 kcal/mol. Finally, the extremely broad absorption feature in region III is excitation to the S_2 electronic excited state and excitation results in *trans*-CA S_1 emission. Furthermore, we collected CA from the molecular beam after laser excitation in each of the three regions as further evidence of the photoisomerization process. The relative amounts of *trans*- and *cis*-CA in the collected molecules were measured with high-pressure liquid chromatography. Although *trans*-CA was excited in all three regions, a significant *cis*-CA peak appeared only in region II, though a small cis peak was observed in region III.

Introduction

Photoinduced trans-to-cis isomerizations of small chromophores in photosensory proteins are often utilized by nature to transform light energy into a cellular response, for example rhodopsin in vision[1] and bacteriorhodopsin in photosynthesis.[2] In the photoactive yellow protein (PYP), the photoisomerization of its chromophore triggers a photocycle in the protein, causing large protein conformational changes that are thought to ultimately mediate the negative phototaxis to blue light exhibited by certain purple bacteria.[3] PYP is found in the cytosol of the halophilic phototrophic purple bacterium *Ectothiorhodospira halophila* and related bacterial species.[4,5] The chromophore of PYP is *trans-p*-coumaric acid, linked to a cysteine in the protein via a thioester bond.[6,7]

The photocycle of PYP consists of two well-defined intermediates[5,8] and possibly several others.[9,10] In the ground state of PYP, denoted pG_{446} (446 nm is the absorption maximum), the coumaric acid exists as the deprotonated trans isomer. Irradiation causes excitation to the S_1 excited electronic state of the chromophore followed by a trans-to-cis isomerization that generates the first well-defined intermediate pR_{465}, but no significant protein conformational changes have occurred at this stage. A few milliseconds later, a blue-shifted intermediate is formed (pB_{355}) in which large protein conformational changes have exposed the chromophore to solvent and the chromophore has become protonated. This intermediate is very long-lived and is thought to be the signaling state. A few hundred milliseconds later, pG_{446} is recovered.

PYP is a good model system for studying how photoreceptors convert light energy into biological responses because it contains only one isomerizable double bond, as compared to the complication of several isomerizable double bonds in the chromophore of rhodopsin.[11,12] In addition, the crystal structure has been determined at very high resolution for the ground state [13,14] and several photocycle intermediates.[14−16] Despite the wealth of experimental and theoretical research done on PYP,

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there are many aspects of the excited electronic states involved in the initial photoexcitation and subsequent photoisomerization that are not well understood. For example, several solution experiments on PYP have shown that different results are obtained upon excitation of PYP at its absorption maximum versus excitation on the blue edge of its absorption.[9,17−19] This wavelength-dependent behavior could be due to multiple electronic excited states or ground-state inhomogeneities. In this contribution, we describe results of electronic spectroscopy and dynamics of *trans-p*-coumaric acid (*trans*-CA) using fluorescence spectroscopy in a supersonic jet. In PYP the chromophore is lodged in a hydrophobic pocket of the protein during the initial excitation and photoisomerization, and therefore we believe that gas-phase studies of the chromophore might be useful. For example, in solution the chromophore shows an 8000 cm^{-1} fluorescence Stokes shift[20] compared to about 2000 cm^{-1} in the protein.[21]

However, aside from the fact that our studies of the PYP chromophore do not include the protein environment, there are two differences between the *trans*-CA model chromophore in this study and the chromophore in the protein. First, we have investigated the neutral phenol form of the molecule while the chromophore exists as the phenolate anion in the pG and pR states of the protein. The crystal structure[13] reveals several amino acid residues in the chromophore pocket that form hydrogen bonds with the phenolate anion which helps stabilize the negative charge. In addition, electronic structure calculations have shown that the electrostatic environment of the protein surroundings plays a significant role in stabilizing the negative charge.[22] Thus, it is likely that in the environment of the protein, the effect of the charge on the spectroscopic properties of the chromophore is reduced by the opposite electrostatic effect of the surrounding protein. In future studies, we plan to form gas-phase complexes of *trans*-CA and basic solvent molecules to determine the effect of deprotonation on the excitation and emission spectra.

The second difference is that *trans*-CA in this study exists as the free acid while the PYP chromophore is attached to a cysteine in the protein via a thioester bond. Calculations have shown that sulfur substitution in the molecule has little effect on bond lengths or angles; it just red-shifts the electronic transition.[23] Furthermore, solution studies[20] of the free acid chromophore showed the same decay rate upon excitation as that in the denatured protein, suggesting that *trans*-CA is a good model system for the chromophore in PYP.

In this study, we attempt to answer the following questions: what is the nature of the initially excited state, $\pi-\pi^*$ or $n-\pi^*$? Is there a barrier to trans-to-cis isomerization in the S_1 state? Does the cis isomer form via an avoided crossing of the excited state with the cis ground state, as has been suggested for stilbene and bacteriorhodopsin? Can we shed any light on why excitation at the high energy end of the absorption of PYP has resulted in behavior different from that by excitation at the absorption maximum in several solution experiments?

Experimental Section

The spectra presented were recorded using fluorescence excitation and dispersed fluorescence techniques described in detail elsewhere.[24] *trans-p*-Coumaric acid was purchased from Aldrich and used without further purification. *trans*-CA was first mixed in a 1:1 ratio with uracil to prevent decomposition.[25] The mixture was heated to 170 °C to attain a sufficient vapor pressure, and the nozzle temperature was kept about 20° higher than the sample temperature. The molecules were seeded into helium gas at a stagnation pressure of 3−4 atm, and the mixture was expanded into a vacuum chamber through a 50−100 μm orifice. Excitation spectra were measured by monitoring the total fluorescence as a function of excitation wavelength using a photomultiplier tube. The dispersed fluorescence spectra were measured by dispersing the emission obtained at fixed excitation wavelength through a monochromator with 4 Å/mm reciprocal dispersion and detecting the fluorescence with a photomultiplier tube.

In addition to spectroscopic experiments, the condensable material from the molecular beam was collected and analyzed by HPLC. In the typical molecular beam collection experiment, an approximately 6 cm × 6 cm square of aluminum foil was placed in the path of the molecular beam about 9 in. from the nozzle. The sample was prepared and heated to the temperature described above. The unfocused excitation laser, about 2 mm in diameter, was tuned to a particular transition and the laser excited molecular beam collected for 1 h. The residue was washed off the foil with methanol and injected into the HPLC.

HPLC experiments were performed on an HP 1090 liquid chromatography system (Agilent Technologies) equipped with a reverse-phase, C18 column (Rainin Microsorb MV). The solvent system contained 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile. *cis*- and *trans*-CA were separated using a linear gradient of 0−60% acetonitrile in 60 min and detected by monitoring the absorbance of the eluent at 220 nm.

Several diagnostic experiments were also run to ensure that the features observed in the HPLC traces were due to laser excitation of the molecular beam, and not of the residue already deposited on the foil. These will be discussed in the Results and Discussion section.

Ground-state vibrational calculations were performed using the Gaussian 98 package.[26] The B3LYP method was used with a 6-31G* basis set for geometry optimizations and frequency calculations of *p*-cresol and *p*-coumaric acid.

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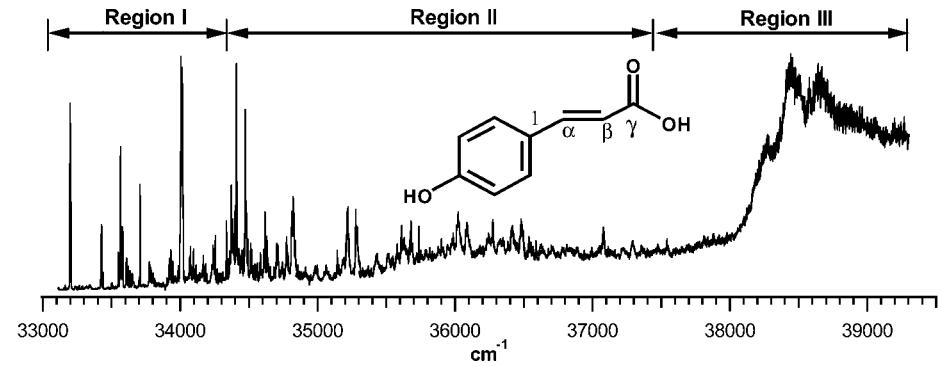

Figure 1. Fluorescence excitation spectrum and labeled structure of *trans*-p-coumaric acid.

Results and Discussion

We have investigated the electronic structure and photoisomerization dynamics of *trans*-CA, the chromophore of photoactive yellow protein, using fluorescence excitation and dispersed emission methods in a supersonic jet. As further evidence of the photophysical picture deduced from the spectroscopic data, the molecular beam excited at different laser frequencies was collected on aluminum foil and the residue chromatographed by HPLC. These results are presented below.

A. Fluorescence Excitation and Emission Data The fluorescence excitation spectrum of *trans*-CA acid is shown in Figure 1. To facilitate discussion, the spectrum has been divided into three distinct regions. Region I, at the red end of the spectrum, is characterized by sharp well-resolved peaks. Region II contains features that increase in broadness and decrease in intensity with increasing excitation energy. Finally, the bluest region III exhibits an extremely broad absorption feature.

Region I. In the first region, features in the fluorescence excitation spectrum (Figure 2A) are sharp and well-resolved. The furthest red feature occurs at 33 200 cm^{-1} and is assigned as the origin of the S_1-S_0 electronic transition. The laser was scanned about 1500 cm^{-1} further to the red (not shown) where only one low-intensity feature appeared that may be a minor rotamer or hot band. The origin transition is split into two peaks by about 4 cm^{-1}. This is due to the fact that the −OH group on the benzene ring can be found in two orientations relative to the acrylic acid group, as seen in several substituted alkylphenols.[27,28] In the protein, the pG_{464} and pR_{446} intermediates would only exist as single conformers because the chromophore is found as the phenolate anion. However, two stable conformers may exist for the pB_{355} intermediate, which is protonated.

Upon excitation of the origin, the emission spectrum labeled 1 in Figure 2B is obtained. The origin is the strongest feature in this spectrum, and the less intense features to the red represent vibrational features. The fact that the origin is the strongest transition in the spectrum suggests that the ground and excited S_1 state are similar in geometry, according to the Franck−Condon principle. Furthermore, the electronic transition is likely $\pi-\pi^*$ in nature since moving a single electron in a large delocalized system to an antibonding orbital does not often cause large changes in geometry or force constants of the molecule. On the other hand, $n-\pi^*$ transitions tend to be localized and are often associated with geometry changes that would shift

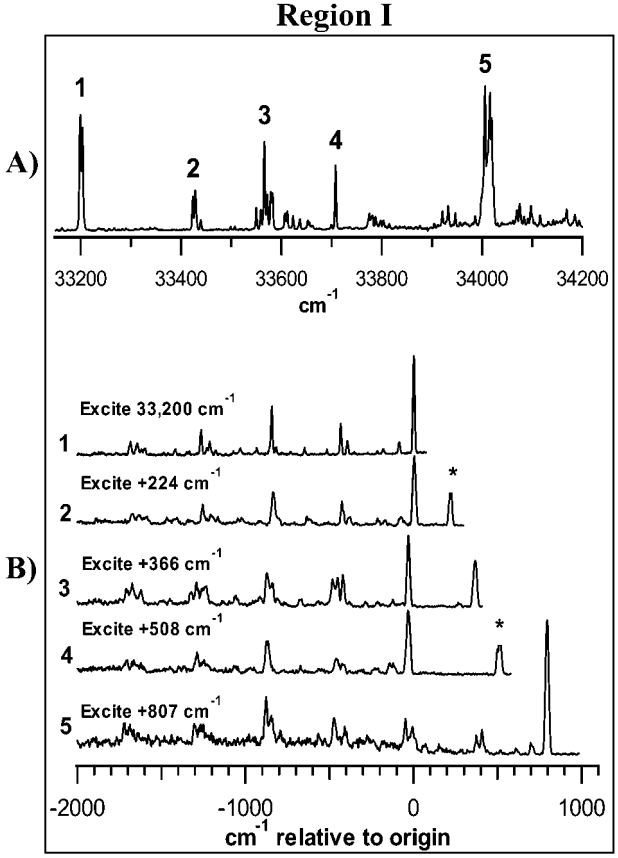

Figure 2. (A) Expanded fluorescence excitation spectrum of *trans*-CA in region I. (B) Emission spectra resulting from excitation of numbered features in top figure. The *x* axis is in cm^{-1} relative to the origin transition (33 200 cm^{-1}). In spectra 2 and 4, the feature marked with an asterisk at the excitation wavelength is due entirely to scattered light;[41] in the rest of the spectra, less than 10% of the intensity at the excitation wavelength is a result of scattered light.

the excited state geometry significantly along some coordinate relative to the ground state, in which case the emission would appear broad and red-shifted. For example, the CO bond length in formaldehyde changes by 0.12 Å upon $n-\pi^*$ electronic excitation, while the average bond length in benzene only changes by 0.03 Å upon $\pi-\pi^*$ excitation.[30]

Figure 3 shows the emission spectrum obtained by exciting the origin of *p*-cresol compared to excitation of the origin of *trans*-CA. The two emission spectra are similar, suggesting that

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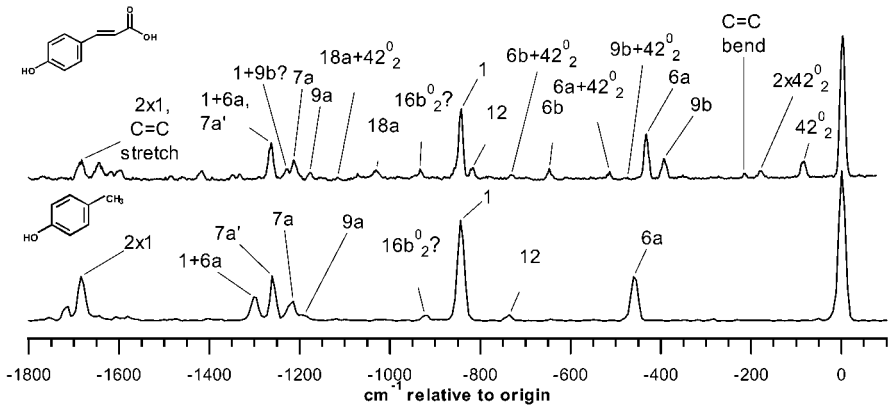

Figure 3. Emission spectrum and vibrational assignments from Table 1 resulting from excitation of the origins of *trans*-CA (top) and *p*-cresol (bottom). The *x* axis is in cm^{-1} relative to the origin transitions of both molecules, 33 200 and 35 313 cm^{-1}, respectively.

Table 1. Vibrational Assignments of *trans-p*-Coumaric Acid

trans-CA S_0 (cm^{-1})	*trans*-CA calculation[a] (cm^{-1})	*trans*-CA S_1 (cm^{-1})	mode assignments[b]	*p*-cresol S_0 (cm^{-1})	*p*-cresol calculation[d] (cm^{-1})	styrene[e] (cm^{-1})
82	39	>224 ?	42^0_2 [c]	-	-	90
180	-	?	42^0_4 [c]	-	-	199
216	199	224	ethylene bend	-	-	
393	389	366	9b	-	422	227
432	495	379	6a	460	459	443
475	-	?	$9b+42^0_2$	-	-	
513.6	-	?	$6a+42^0_2$	-	-	
646.6	630	508?	6b	-	648	624
731	-	?	$6b+42^0_2$	-	-	710
816	772	807	12	736	736	996
841	841	817	1	845	833	858
930	490	?	$?16b^0_2$	923	504	
1030	1003	969?	18a	-	1009	1011
1115	-	?	$18a+42^0_2$	-	-	
1177	1178	1138	9a	1190	1170	1204
1213	1219	1210	7a	1214	1217	1204
1228	-	?	1+9b?	-	-	
1262.6	1295	1274	7a′	1261	1258	
1344	-	?	$7a'+42^0_2$	-	-	
1416	685	1418	4^0_2	-	700	
1686	-	1614	2×1	1685	-	1540

[a] Vibrational frequencies were computed using B3LYP/6-31G* methods in Gaussian 98.[26] All calculated vibrations except ν_{42} were scaled by a factor of 0.9622 so that the ν_1 vibrations matched. [b] All modes are labeled as their corresponding modes in benzene using Wilson notation.[29] [c] This mode is labeled as the corresponding mode in styrene. [d] Calculations taken from ref 31. [e] Assignments from ref 34.

most of the vibrational activity upon electronic excitation of *trans*-CA occurs in the phenol ring. Ground-state vibrational frequencies were calculated using Gaussian 98[26] for both *p*-cresol and *trans*-CA to attempt to assign the new features that appear in the *trans*-CA spectrum relative to the *p*-cresol spectrum. The emission spectrum of *p*-cresol in a supersonic jet [27] and the infrared spectrum [31,32] have been previously assigned, although we disagree with some of the jet assignments.[33] The ground-state and excited-state vibrational assignments shown in Table 1 are based on calculations and comparison to vibrational frequencies in *p*-cresol and styrene. The excitation and emission spectra become congested at higher vibrational energies so that it is difficult to distinguish between fundamental vibrations and combination bands above about 1200 cm^{-1}. The lowest frequency vibration occurs at 82 cm^{-1} in the ground state. We assign this as the second overtone of the C_1-C_α torsional mode on the basis of the results of the calculation and the existence of a similar feature in styrene at 90 cm^{-1}.[34] The fundamental of this vibration is calculated to be 39 cm^{-1}. We have not definitively assigned the C_1-C_α torsional mode in the S_1 state. Since the lowest-energy vibrational feature in the S_1 state occurs at 224 cm^{-1}, the frequency of the 88 cm^{-1} mode must be significantly higher in the excited state. This is not surprising since the two bonds adjacent to the ethylene bond are expected to take on double bond character in the $\pi-\pi^*$-type transition.[23] The feature in the emission spectrum at 216 cm^{-1} is an in-plane ethylene bending mode, with a corresponding mode in the S_1 state at 224 cm^{-1}. Most of the new features in the *trans*-CA emission spectrum that do not appear in the *p*-cresol spectrum may be assigned to combination bands between fundamental vibrations with the 82 cm^{-1} torsional mode. However, a few fundamental modes such as 9b and 6b that do not appear in the emission spectrum of cresol may also be assigned with the aid of the calculations. Most of the assigned S_0 vibrations can be correlated to vibrations in the S_1 state.

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(33) Most of our assignments for *p*-cresol agree with those of Song and Hayes.[27] However, they assign two features in the emission spectrum as fundamentals of $\nu4$ and $\nu5$, two nontotally symmetric out-of-plane vibrations in which only even overtones are allowed by symmetry.
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Similarly, *trans*-urocanic acid[35] and styrene[34] exhibit vibrational activity localized in the imidazole and benzene moieties, respectively. However the excitation and emission spectra of stilbene,[36,37] which has higher C_{2h} symmetry, are dominated by strong progressions in the ethylene in-plane bend vibrations.

Excitation of vibrational features in region I results in emission spectra very similar to origin emission; a few representative spectra are shown in Figure 2B (spectra 2−5). The fact that the emission spectra are very similar in this region means that all of the vibrations excited emit to the same ground state. The majority of the vibrational peaks excited show relaxed emission; the excitation energy originally contained in one mode has been distributed to many low-frequency modes prior to emission. This redistribution of vibrational energy is called intramolecular vibrational redistribution, or IVR. IVR is a relaxation process described by radiationless transition theory.[38] The initially excited vibrational mode (bright state) may mix via anharmonic couplings to low-frequency vibrational modes (dark states) that do not have significant Franck−Condon overlap with the ground state. IVR occurs at excitation energies where the density of states is high enough that there are random states nearly isoenergetic with the bright state. The Franck−Condon factors of most vibrational modes in a molecule obey $\Delta v = 0$ propensity rules. When many dark states are coupled to the bright state, the emission is largely determined by the Franck−Condon factors of the dark states, that is the emission follows $\Delta v = 0$ propensity rules. Therefore, emission is not to the zero-point level of the ground state, as would be the case in unrelaxed emission, but to vibrational levels in the ground state with the same vibrational quantum numbers as those of the emitting vibronic levels, resulting in relaxed emission. Relaxed emission occurs from all but two vibrational modes in region I. The vibration at 807 cm^{-1} (Figure 2B, spectrum 5) exhibits unrelaxed emission, that is emission occurs from the initially excited vibrational mode to the zero-point level in the ground state (i.e., no IVR has occurred), and the vibration at 366 cm^{-1} (Figure 2B spectrum 3) shows both relaxed and unrelaxed emission. This implies that IVR is mode-selective in this region.

Region II. The fluorescence excitation spectrum of region II is shown in Figure 4A and is comprised of features that decrease in intensity and increase in width as the excitation energy increases.

If the broadening is due to shortening of the lifetime, some other radiationless process is competing with fluorescence.

Excitation in region II results in dual emission, as shown in Figure 4B (spectra 6−9). The emission is comprised of the same *trans*-CA S_1 emission features observed in region I (spectrum 1), although broadened significantly. In addition, blue-shifted components (marked with arrows) are observed, the bluest occurring at about 34 000 cm^{-1}. These blue-shifted features are relatively easily observed initially, but at higher excitation energies they have become so broad that they are difficult to distinguish from the background features. Similar dual emission is observed in *trans*-urocanic acid,[35] except that the blue-shifted components are weaker in *trans*-CA.

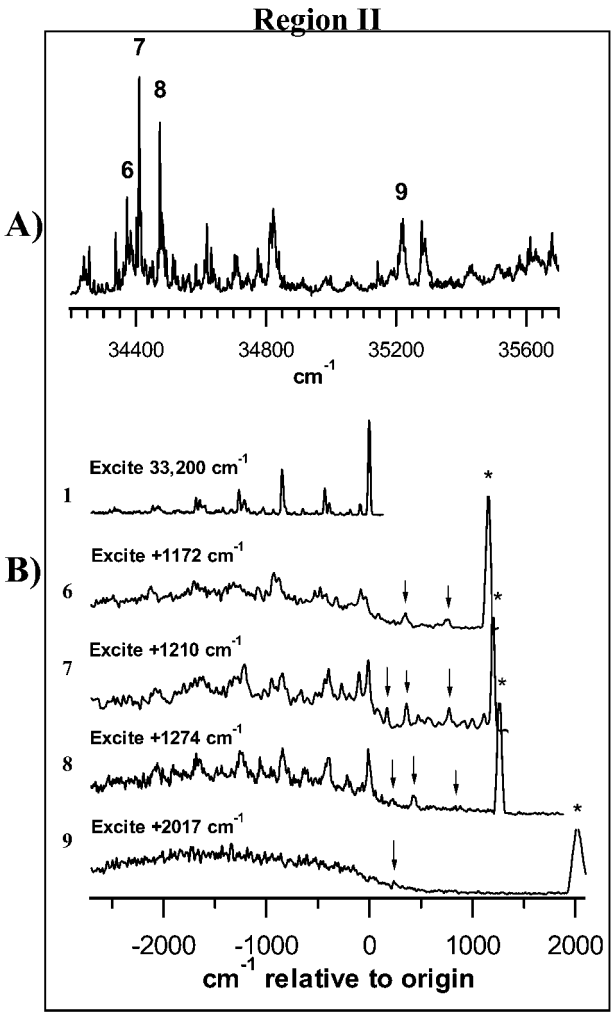

Figure 4. (A) Expanded fluorescence excitation spectrum of *trans*-CA in region II. (B) Emission spectra 6−9 result from excitation of numbered features in top figure. Emission spectrum 1 results from exciting the origin in region I for comparison. The *x* axis is in cm^{-1} relative to the origin transition 33 200 cm^{-1}. Features marked with asterisks are entirely due to scattered laser light.[41] Features marked with arrows are blue-shifted emission from the *cis*-CA S_1 state.

Dual emission signifies coupling to another excited electronic state, which may be the *cis*-CA S_1 state. The onset of emission spectra exhibiting dual emission first occurs for excitation around 34 400 cm^{-1} (origin + 1200 cm^{-1}) at which point a barrier in the S_1 state has been surpassed to allow coupling to another state. We propose that there is an asymmetric double minimum potential in the torsional coordinate around the ethylenic double bond with the *trans*-CA minimum separated by a roughly 1200 cm^{-1} barrier from the *cis*-CA minimum. In the absence of tunneling, pseudo-eigenstates (neglecting time-dependent processes such as spontaneous emission) below the barrier would be pure trans or pure cis and would exhibit either trans-like or cis-like emission spectra. Above the barrier, the eigenstates would be mixed linear combinations of trans and cis states. Therefore, their emission spectra would have both trans-like and cis-like components; that is, dual emission as observed. It should be noted that this mixing above the barrier is not a relaxation process and does not require a high density of states in the torsional coordinate. It occurs because above the barrier any eigenstate spans the whole of the torsional coordinate space between trans and cis. This is the same for

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any double-minimum problem where, above the barrier, the system samples the entire space on both sides of the barrier even for discrete and well-separated eigenstates.

We believe that the state to which the *trans*-CA S_1 state is coupling is the *cis*-CA S_1 state. First, the features in the excitation spectrum in this region become broad and decrease in intensity, the same behavior that was observed in frequency domain studies of *trans*-stilbene[34,36] and diphenylbutadiene.[39] The broadening is due to IVR, which is a prerequisite for isomerization to occur since the energy deposited in the initially excited mode must flow into the reactive mode. The 1200 cm^{-1} barrier that we observe is also similar to the isomerization barrier estimated for *trans*-stilbene.

The difference between our study and the stilbene studies is that dual emission is not observed in *trans*-stilbene. Isomerization is thought to occur via a conical intersection of the S_1 potential surface at a twisted geometry around the ethylenic bond with the S_0 surface of *cis*-stilbene. We emphasize that our data does not rule out that this internal conversion mechanism of isomerization is occurring in addition to the adiabatic cis/trans isomerization in S_1 that we believe we are observing.

The process of rotational isomerization of the carboxylic acid moiety about the $C_\beta-C_\gamma$ bond is another process that could be consistent with our data. However, we believe that the barrier to the rotational isomerization of the carboxylic acid moiety in the S_1 state would be much higher than we observe, since DFT calculations have shown that the $C_\beta-C_\gamma$ bond gains bonding character in the excited state.[23] It is also unlikely that the higher-energy s-trans isomer of *trans*-CA is significantly populated in the jet and that dual emission might be a result of excitation of both species. If a new isomer were being selectively excited, a distinct emission spectrum would result and not the dual emission that we observe. There is always some possibility that the excitation laser could be simultaneously exciting two overlapping features and hence produce dual emission. However, it would be highly coincidental that all of the many features that produce dual emission involve overlapping transitions.

If the electronic state that the *trans*-CA S_1 state is coupling to is the *cis*-CA S_1 state, then blue-shifted emission features may be emission from the S_1 state of *cis*-CA to its ground state. The only way to definitively assign the blue-shifted features is to perform spectroscopy on the *cis*-CA isomer. However, HPLC analysis of *cis*-CA after it is heated to the temperatures necessary to attain a significant vapor pressure in the molecular beam shows that it either isomerizes to *trans*-CA or decomposes to a product with a retention time similar to that of *trans*-CA.

If the blue-shifted emission originates from the *cis*-CA S_1 state and excess vibrational energy has been distributed into many low-frequency modes as in *trans*-CA, then the bluest feature observed in the emission spectrum should be at the frequency of the *cis*-CA origin. The *cis*-CA origin would then be at 34 000 cm^{-1}, about 800 cm^{-1} blue-shifted from the *trans*-CA origin. Since the trans-to-cis isomerization barrier occurs 34 400 cm^{-1} above the *trans*-CA ground state, the cis-to-trans barrier would be only 400 cm^{-1} if the *cis*-CA ground state were equal in energy to the *trans*-CA ground state. However, calculations[23] have predicted that the *cis*-CA ground state is higher in energy, that is, less stable, than the *trans*-CA ground state; therefore, it is likely that the cis-to-trans isomerization barrier is very small.

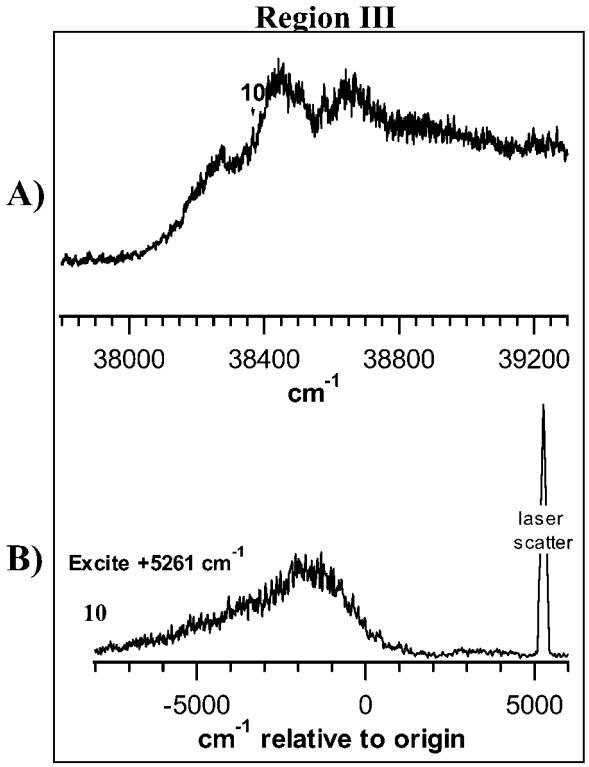

Figure 5. (A) Expanded fluorescence excitation spectrum of *trans*-CA in region III. (B) Emission spectrum 10 results from excitation of numbered feature in top figure. The *x* axis is in cm^{-1} relative to the origin transition 33 200 cm^{-1}.

Additional experiments were performed which provide further evidence that cis/trans isomerization is occurring in Region II. These are discussed in the HPLC results section.

Region III. The fluorescence excitation feature in Region III is extremely broad (>1500 cm^{-1}), as shown in Figure 5A. We believe that this broad feature represents absorption to the S_2 electronic excited state of *trans*-CA. The broadness indicates that absorption occurs to a point on the S_2 potential energy surface that contains a large density of states. This implies that the S_2 state is significantly shifted in one or more coordinates relative to the ground state. If absorption occurred to a single level, the absorption feature would be sharp, its width increased only by lifetime broadening. The broadness we observe is due to many overlapping fluorescence features from the many states excited during absorption. The fact that the S_2 geometry is different from that of the ground state indicates that the electronic transition may be $n-\pi^*$ in nature. However, $n-\pi^*$ states typically have extremely small oscillator strengths which is not the case here. What is likely occurring is a mixing of the $n-\pi^*$ state with a close lying $\pi-\pi^*$ state, which would lend intensity to the weak $n-\pi^*$ transition. In addition, calculations[40] predict an $n-\pi^*$ state on the blue end of the absorption spectrum of PYP. The presence of this state has been hypothesized in several time-resolved studies where different time constants were

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(40) Molina, V.; Merchan, M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4299−4304.

(41) Scattered light is measured by comparing the signal intensity at the excitation wavelength when the excitation laser is on-resonance with a transition and a few cm^{-1} off-resonance from a transition.

determined upon excitation on the blue edge of the PYP absorption compared to excitation at the absorption maximum.[9,17−19]

Excitation in region III results in the emission spectrum shown in Figure 5B. The emission is very broad but occurs in the same wavelength region as *trans*-CA S_1 emission. It is very difficult to deduce whether there are any blue-shifted emission features in this spectrum. If emission is occurring from what we believe is the *cis*-CA S_1 state, it is likely that the features would be broadened to such an extent that their intensities would lie below the noise level.

According to Kasha's rule, emission does not occur from electronic states lying higher in energy than S_1 because the density of states available at those energies makes it probable that another radiationless process will occur faster than fluorescence. The two most common radiationless processes that occur from S_2 are internal conversion to the S_1 state or intersystem crossing to a nearby triplet state. Since the emission appears to be S_1 emission, it is likely that internal conversion is occurring from S_2 to S_1 prior to emission.

The photophysics that can be deduced from the fluorescence excitation and emission data can be summarized as follows: In region I, exciting the sharp, well- resolved features in the excitation spectrum results in emission from the *trans*-CA S_1 state. In region II, the broadening of features in the excitation spectrum and the dual emission signify that some process, perhaps isomerization, is competing with fluorescence after a barrier of about 3.4 kcal/mol is overcome. Finally in region III, the broad feature indicates absorption to the S_2 electronic state; upon excitation, internal conversion to S_1 occurs prior to emission, and it is difficult to tell whether the spectrum contains blue-shifted features that may be indicative of isomerization.

B. Chromatography Results The major piece of evidence we have that photoisomerization is occurring is the dual emission occurring in region II. As further proof that isomerization is occurring in region II, and to determine whether isomerization is occurring in region III where the blue-shifted features may be too weak to observe, the molecular beam was laser-excited in each of the three regions and collected on aluminum foil. The resulting residue was chromatographed and compared to control experiments. The spectroscopy experiments would lead us to predict that solely a *trans*-CA feature should be observed in region I, both *cis*- and *trans*-CA peaks in region II, and *trans*-CA and perhaps *cis*-CA features in region III.

Control experiments were performed to measure the retention times of *cis*- and *trans*-CA. The results of the control experiments are shown in Figure 6A. The HPLC trace at the top of Figure 6A results from the *trans*-CA out of the bottle, mixed with uracil, and dissolved in methanol sample. The HPLC trace in the bottom of Figure 6A shows the result of irradiating a solution of *trans*-CA in methanol with a standard UV lamp for about 48 h. Two peaks appear, the signature *cis*-CA peak appears at higher retention times. A mass spectrum was taken of the *trans*- and *cis*-CA mixture formed upon irradiation in methanol which showed no additional photoproducts were formed.

Laser excitation of the molecular beam in each of the three regions results in the traces shown in Figure 6B. Excitation of the origin (33 200 cm^{-1}) in region I, below the isomerization barrier, produces only the *trans*-CA peak. Excitation in region II (+1200 cm^{-1}) (where dual emission is observed, suggesting

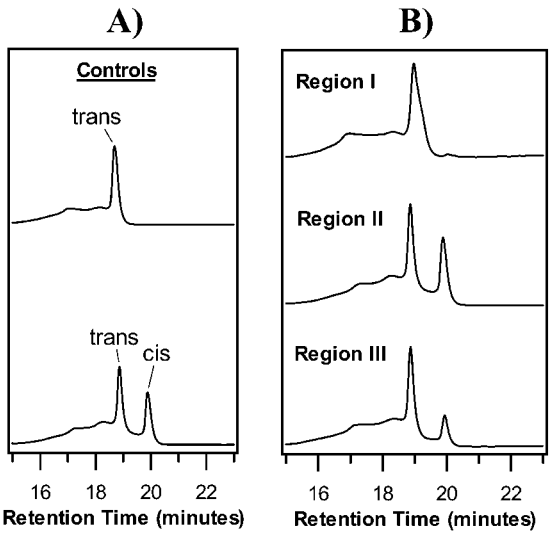

Figure 6. (A) HPLC control experiments. (top) *trans*-CA plus uracil dissolved in methanol (bottom) *trans*-CA in MeOH irradiated with UV lamp for 48 h. (B) HPLC molecular beam collecting experiments. (top) Excitation in region I at origin (center) Excitation in region II at +1210 cm^{-1} (bottom) Excitation in region III at +5261 cm^{-1}.

isomerization) results in both *trans*- and *cis*-CA peaks. In region III, blue-shifted features were not observed in the emission spectra, and it was not clear whether they were not there or whether the broadness of the emission would be indistinguishable from the noise. The HPLC of the molecular beam collected during excitation in Region III does show a characteristic *cis*-CA peak, although the intensity is significantly lower than in the isomerization region. The experiments described above were performed at least twice, and at least one other representative feature in each region was excited.

These results were surprising initially because the number of molecules actually excited by the laser in the interaction region is miniscule. Because the excitation laser is pulsed at 20 Hz, the laser interacts with the continuous molecular beam for only 10 ns of every 50 ms which amounts to only 0.002% of the molecules being excited. Furthermore, the laser beam is only about 2 mm in diameter so that the portion of the molecular beam excited is small compared to the diameter of the molecular beam.

The first suspicion was that perhaps the molecules were isomerizing on the aluminum foil via scattered light in the chamber, and not actually in the gas phase. A couple of experiments were performed to rule this out. First, a film of *trans*-CA was deposited on aluminum foil and directly exposed to laser light. Exciting wavelengths in regions I and II resulted in no *cis*-CA peaks in the HPLC spectrum. Next, the molecular beam containing *trans*-CA was allowed to flow for 1 h in the absence of the excitation laser to deposit a film on the aluminum foil. Then, the molecular beam was stopped, and the excitation laser tuned to a wavelength in the isomerization region was directed into the chamber for 1 h. No *cis*-CA peak appeared in the HPLC. Similarly, a film was deposited in the manner described above: the oven containing the *trans*-CA sample was cooled to room temperature, and the laser was directed into the chamber with only an unseeded molecular beam flowing. The *cis*-CA formed was negligible.

The next suspicion was that perhaps scattered laser light in the chamber was responsible for exciting *trans*-CA molecules

outside of the interaction region. When the molecular beam hits shock waves or the walls of the vacuum chamber, the gas molecules become randomized static gas. If the dwell time of the *trans*-CA molecules in the chamber after the beam becomes randomized is longer than milliseconds, then it is possible that each *trans*-CA molecule is exposed to a pulse of excitation light. Experiments were done using various iris sizes to restrict the diameter of the laser beam, and therefore decrease the amount of scattered light in the chamber. The *cis*-CA peak did decrease if the diameter of the laser was made as small as possible. Therefore, we conclude that *trans*-CA is indeed isomerizing in the gas phase with the help of light-scattering from the walls of the chamber and from the molecular beam itself.

These results show that our interpretation of the fluorescence excitation and emission data is correct. Furthermore, we were able to determine that isomerization is indeed occurring in region III, although to a lesser extent than in region II.

Conclusions

The initial excitation of the chromophore in photoactive yellow protein involves a $\pi-\pi^*$ transition to the S_1 state of *trans*-CA. At excitation energies greater than 1200 cm^{-1} above the *trans*-CA electronic origin, cis/trans isomerization occurs. The isomerization barrier is about 3.4 kcal/mol. Although *cis*-CA may be forming by curve crossing of the S_1 potential surface with the S_0 surface of *cis*-CA, which is the commonly discussed mechanism for *trans*-stilbene and *trans*-retinal, another isomerization mechanism may be occurring that involves isomerization into the *cis*-CA S_1 state followed by fluorescence emission. Furthermore, a close-lying second electronic excited state that may be masked in solution studies was observed which provides a second, although less efficient, pathway to isomerization. It is important that nature chose a chromophore with close-lying excited states[22,40] so that light may be absorbed over a larger wavelength region and increase the efficiency of the photocycle.

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