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Ground state isomerization of a model green fluorescent protein chromophore

Xiang He, Alasdair F. Bell, Peter J. Tonge*

Department of Chemistry, State University of New York at Stony Brook, Stony Brook, NY 11794, USA

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Abstract The relationship between ground state *cis–trans* isomerization and protonation state is explored for a model green fluorescent protein chromophore, 4-hydroxybenzylidene-1,2-dimethylimidazolinone (HBDI). We find that the protonation state has only a modest effect on the free energy differences between *cis* and *trans* isomers and on the activation energies for isomerization. Specifically, the experimental free energy differences are 3.3, 8.8, and 9.6 kJ/mol for cationic, neutral, and anionic forms of HBDI, respectively, and the activation energies are 48.9, 54.8, and 54.8 kJ/mol for cationic, neutral, and anionic forms, respectively. Furthermore, these activation energies are much smaller than might be expected based on comparison with similar systems. These results suggest that there may be a sub-population of the chromophore, which is nearly equally accessible to all three protonation states, through which thermal isomerization may proceed.

Key words: Green fluorescent protein; Ground state isomerization; 4-Hydroxybenzylidene-1,2-dimethylimidazolinone

1. Introduction

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has developed into an important tool for monitoring the location and movement of proteins within living cells [1–3]. This is because upon proper folding and in the presence of oxygen, GFP will convert a Ser65-Tyr66-Gly67 tripeptide sequence into a 4-hydroxybenzylidene-imidazolinone fluorophore without the assistance of any cofactors. Thus, by genetically fusing the DNA sequence of GFP to that of the protein of interest, it is possible to express a target protein inside living cells with a fluorescent protein tag. Using fluorescence microscopy the GFP can then be monitored, generating information on the mobility and localization of the target protein and also on protein–protein interactions through double labeling FRET measurements [1–3].

One profitable approach to understanding the complex photophysical properties of GFPs has been the synthesis and characterization of model compounds [4–8]. Comparisons between the properties determined for model compounds in solution and for the chromophore within the protein matrix have provided important insights into the role of protein–chromophore interactions in modulating and controlling the chromophore's photophysics. A number of studies investigating the steady state spectroscopy of model compounds have provided information on the absorption and emission of light by the chromophore, the pK_as of different protonation states as well as the structure of the chromophore [4–8]. More recently, time resolved absorption techniques have been used to study the mechanism of excited state deactivation [9–11] and gas phase absorption spectra of anionic and cationic forms of 4-hydroxybenzylidene-1,2-dimethylimidazolinone (HBDI) have been reported [12,13]. Importantly, model GFP chromophores have very low fluorescence quantum yields at room temperature (<0.001) whereas the protein has a fluorescence quantum yield of about 0.8. It has been suggested that this may be due to the steric restrictions imposed by the protein environment [9–11].

The focus of the current study is the ground state isomerization of a model chromophore, HBDI, and the effect of protonation on this process. We have experimentally determined the free energy differences and the activation energies for *cis–trans* isomerization for the cationic, neutral, and anionic forms of HBDI. These studies reveal that (i) for the cationic form of HBDI there is a substantial population (20%) of the *trans* isomer even at room temperature in water and (ii) the activation energies for *cis–trans* isomerization are similar for all three protonation states and are lower than would be expected. These observations provide evidence for a sub-population in the ground state through which thermal isomerization proceeds.

2. Materials and methods

2.1. Nuclear magnetic resonance (NMR) spectroscopy

The synthesis of HBDI has been reported elsewhere [7]. Briefly, HBDI was prepared as described by Kojima et al. [20] and recrystallized from ethanol/methylene chloride (10:1). The only modification to this synthetic route was that 4-hydroxybenzaldehyde was used as a starting material instead of 4-acetoxybenzaldehyde. ^{1}H NMR spectra were obtained on Varian InvSOO and Inv600 spectrometers. The acidic and basic solutions for NMR measurements were prepared by adding DCl and NaOD, respectively, into D_2O. For cationic, neutral, and anionic forms the pD values were 0.2, 5.0, and 12, respectively. The pD values were calculated using the relationship pD = pH+0.4. Concentrations of HBDI were in the range of 0.5–10 mM and all measurements were made at 25°C.

2.2. Determination of activation energies

A 200 W mercury lamp (Oriel) was used as the light source for photoisomerization. Optical bandpass filters for 365, 405 and 437 nm (10 nm bandwidth) were used for neutral, cationic, and anionic

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*Corresponding author. Fax: (1)-631-6325797.
E-mail address: peter.tonge@sunysb.edu (P.J. Tonge).

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forms of HBDI, respectively. Irradiation times were on the order of 3–5 min with irradiation powers between 5 and 10 mW. Concentrations of HBDI were in the range of 10–20 μM (0.2–0.4 abs unit). The relaxation from *trans* to *cis* isomers of neutral, anionic, and cationic forms of HBDI in the dark was monitored by following the absorption spectrum as a function of time. The rate constants were determined by plotting the ratio of the relaxed *trans* isomer population over the total photogenerated *trans* isomer population as a function of time. The activation energies were determined by plotting the rate constants determined at a range of different temperatures (4–65°C) as a function of $1/T$. All absorption measurements were made on a Varian Cary 100 UV-vis spectrometer.

3. Results

3.1. *Ground state free energy differences between* cis *and* trans *isomers*

To probe the effect of protonation on *cis–trans* isomerization we have synthesized the model compound HBDI as shown in Fig. 1 [7]. In the current study we define the *cis* isomer of the bridging double bond as having the hydrogen atom on the bridging methylene group and the carbonyl oxygen on the same side of the double bond. For HBDI in water, the macroscopic pK_as of the model compound have values of 1.4 and 8.0, respectively [14]. Since no zwitterion can be detected, the two macroscopic pK_as have been assigned to ionization of the imidazolinone N3 (pK_a 1.4) and the phenolic hydroxyl group (pK_a 8.0). The various ionization states have distinct absorption spectra with the cationic, neutral, and anionic forms of the chromophore having absorption maxima at 391, 368, and 425 nm, respectively. HBDI is also non-fluorescent, with a fluorescence quantum yield of only < 0.001 at room temperature in water [6].

For studies on the role of protonation on ground state isomerization of HBDI we have chosen to use NMR spectroscopy to characterize the *cis* and *trans* isomers as shown in Fig. 2. Our NMR assignments are summarized in Table 1 and are based on investigations of the geometric isomerism of benzamidocinnamates and their related azlactones [15]. The most striking observation is the apparent doubling of resonances in the spectrum of the cationic form of HBDI. According to our NMR assignments, this indicates that both the *cis* and *trans* isomers are present in significant quantities in the cationic form of HBDI at room temperature in water. Based on the integration of the NMR peaks we estimate that about 20% of the cationic HBDI adopts the *trans* isomer under the conditions reported here. In addition, although it appears from Fig. 2 that both the neutral and anionic forms exist as a single isomer, small sub-populations of the *trans* isomer are observed. We estimate that there is approximately 2.6% and 2.1% of the *trans* isomer for neutral and anionic forms, respectively. To exclude the possibility that the acidic conditions used to produce the cationic form are not damaging or altering the model compound, we monitored changes in the ^{1}H NMR spectrum as a function of pD. When the pD is raised from 0.2 to 12, the protonation state changes and the population of *trans* isomer slowly converts to the *cis* isomer. However, upon returning the pD to 0.2, the 20% population of *trans* isomer is regenerated, demonstrating the reversibility of isomerization. Based on these populations of the *trans* isomer we calculate the free energy difference between the *cis* and *trans* isomers to be 3.3, 8.8, and 9.6 kJ/mol for cationic, neutral, and anionic forms, respectively. The fact that this free energy difference is markedly smaller for the cationic form is probably due to steric repulsion between the aromatic H6 proton and the proton on the imidazolinone N3 nitrogen in the *cis* isomer that is relieved in the *trans* isomer. This interaction is not present in either the neutral or anionic forms of HBDI.

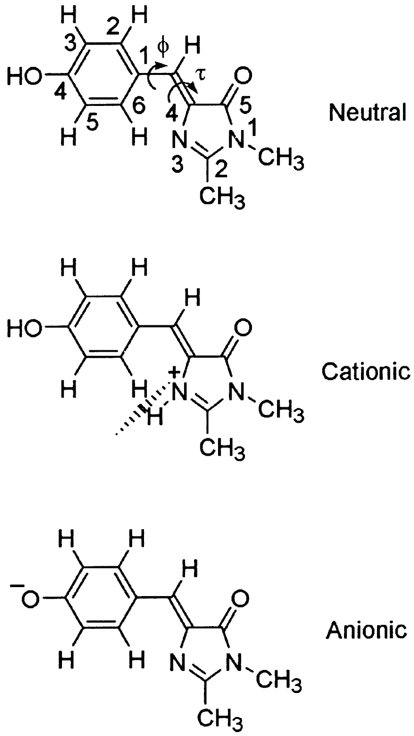

Fig. 1. The different protonation states and numbering scheme for the model GFP chromophore, HBDI.

3.2. *Activation energy of ground state* cis–trans *isomerization*

The other key parameter for characterizing ground state isomerization is the activation energy as this will determine the rate of isomer interconversion. In order to evaluate the activation energies we use light to perturb the equilibrium between *cis* and *trans* isomers, in favor of the *trans* isomer, and then follow the return to equilibrium using absorption spectroscopy. By measuring the temperature dependence of this process and using Arrhenius plots, we are able to deter-

Table 1
^{1}H NMR assignments for *cis* and *trans* isomers of HBDI[a]

Proton	Cation	Neutral	Anion	Assignment
H_a	6.86 (6.78) d[b]	6.82 (6.82) d	6.30 (6.1 8) d	phenol
H_b	7.28 (7.12) s[b]	7.00 (7.13) s	6.60 (6.75) s	bridge
H_c	7.50 (8.00) d	7.98 (8.23) d	7.40 (7.76) d	phenol

[a] Values for *trans* isomers appear in parentheses.

[b] Singlets, s; doublets, d.

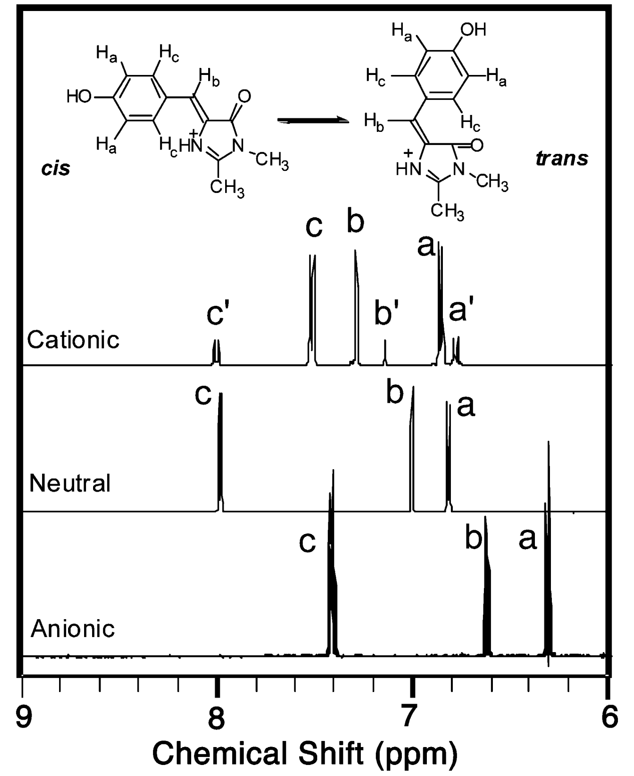

Fig. 2. ^{1}H NMR spectra of cationic, neutral, and anionic HBDI in D_2O.

mine the activation energy for *cis–trans* isomerization in the three protonation states. Specifically, we irradiated the cationic, neutral, and anionic forms of HBDI in D_2O with 405, 365, and 437 nm light, respectively, to boost the population of the *trans* isomer and then obtained kinetic data for the dark regeneration of the *cis* isomer. Using this approach the energy barriers to ground state isomerization are determined to be 48.9, 54.8, and 54.8 kJ/mol for cationic, neutral, and anionic forms, respectively. Combining these results on activation energies with the data on the free energy differences for the *cis* and *trans* isomers of HBDI in their different protonation states, the ground state energy profiles are shown in Fig. 3.

We also examined the solvent dependence of the activation energy for *cis–trans* isomerization for the neutral form of HBDI. It is well known that the viscosity and polarity of solvents can alter the activation energy for isomerization [16]. With this in mind we measured recovery rates in water, methanol, and isopropanol. The polarity in this series of solvents decreases in the order water > methanol > isopropanol whereas the viscosity order is isopropanol > water > methanol. Interestingly, although the *cis* isomer is regenerated within 3–5 min at room temperature in D_2O, the same process takes about 48 h in methanol and is only slightly faster in isopropanol. These data indicate that the transition state for isomerization is somehow stabilized by D_2O as compared to either methanol or isopropanol. This would be consistent with a transition state for ground state isomerization that has diradical character and could thus be stabilized by polar water molecules.

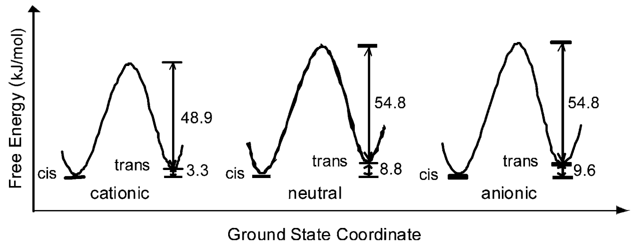

Fig. 3. The energy profiles for ground state *cis–trans* isomerization of cationic, neutral, and anionic forms of HBDI.

4. Discussion

It is expected that both the protonation state and *cis–trans* isomerization of the chromophore within GFP are important in determining the photophysical properties of this valuable marker protein. While the protonation state of the GFP chromophore has been extensively studied, little is known about the possible role of *cis–trans* isomerization in controlling the properties of GFP chromophores. We chose to study a model chromophore in solution where, unlike the protein, we can control both isomerization and protonation state and examine their interplay. Overall our studies reveal only a weak dependence of the ground state isomerization of HBDI on protonation state in aqueous solution. However, it is striking that the activation energies for isomerization of HBDI are much lower than those predicted by quantum chemical calculations [17–19] and are small compared to similar systems such as stilbene and retinal which have values of 41–46 kcal/mol and approximately 50 kcal/mol, respectively [21,22]. This independence on protonation state and the unusually low activation energy suggests a common pathway for *cis–trans* isomerization involving a sub-population that is equally accessible to all three protonation states but has a small barrier for isomerization. Two obvious candidates for the identity of this sub-population are forms of the chromophore that are twisted away from planarity around the bridging methylene group or the zwitterionic form of the chromophore that has the N3 imidazolinone ring nitrogen protonated and the phenolic group deprotonated. Given the similar accessibility of this sub-population to all three protonation states we believe it less likely that the zwitterionic form of the chromophore is responsible for the ease of *cis–trans* isomerization in HBDI.

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