THE JOURNAL OF PHYSICAL CHEMISTRY B LETTERS

2009, *113,* 8439–8442
Published on Web 06/01/2009

Ultrafast Solvation Dynamics of Flavin Mononucleotide in the Reductase Component of *p*-Hydroxyphenylacetate Hydroxylase

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Received: February 7, 2009; Revised Manuscript Received: April 4, 2009

The reductase unit of *p*-hydroxyphenylacetate hydroxylase contains flavin mononucleotide (FMN) as a cofactor. Fluorescence decay curves measured by fluorescence up-conversion method were remarkably dependent on monitored emission wavelength. The fluorescence lifetime was shorter at the shorter emission wavelengths and longer at the longer wavelengths. Spectral shift correlation function of *p*-coumaric acid in water and FMN in C_1 protein in buffer solution were expressed by two-exponential functions. Correlation times, ϕ_1 and ϕ_2, of *p*-coumaric acid were 0.053 and 0.650 ps, respectively, which was similar to previous works. ϕ_1 and ϕ_2 of C_1 were 0.455 and 250 ps, respectively. The Stokes shift from $t = 0$ to $t = \infty$ was 2200 cm^{-1}, while it is 500 cm^{-1} in the static Stokes shift obtained by the solvent effect of the fluorescence spectrum under static excitation. This suggests that the isoalloxazine ring of FMN in C_1 is exposed in hydrophilic environment. Such large Stokes shift was unusual among flavoproteins. The biphasic decay of the spectral correlation function in C_1 was discussed and compared to the biphasic decay of tryptophan in proteins.

Introduction

p-Hydroxyphenylacetate hydroxylase (HPAH) from *Acinetobactor baumannii* catalyzes the hydroxylation of *p*-hydroxyphenylacetate (HPA) to yield 3,4-dihydroxyphenylacetate.[1−3] HPAH is composed of two proteins, a reductase unit (C_1 protein)[1,3] and an oxygenase unit (C_2 protein).[1,2] C_1 contains flavin mononucleotide (FMN) as a cofactor which can be reduced by NADH. After the reduction, the reduced FMN dissociates from the C_1 protein and rapidly binds to the C_2 active site.[4,5] Three-dimensional structures of the C_2 protein were reported[6] but not yet for the structure of C_1.

In most flavoproteins, fluorescence of flavins is almost completely quenched upon binding to the protein moiety. In these flavoproteins, tryptophan (Trp) and/or tyrosine exist near an isoalloxazine moiety (Iso). Ultrafast fluorescence dynamics of various flavoproteins have been investigated by fluorescence up-conversion technique.[7−14]

Recently, a number of investigations have been focused on theoretical elucidation of ultrafast solvation dynamics of Trp in proteins.[15−18] Spectral correlation function of Trp near the protein interface exhibited bimodal decays. The longer correlation time was not observed in free Trp. In the present work using ultrafast fluorescence up-conversion method, we have found that the spectral correlation function of FMN in C_1 decayed with a two-exponential function similar Trp in the proteins. To our knowledge, this is the first observation of pronounced dynamic Stokes shift and the presence of slowly decaying component in the spectral correlation function in flavoproteins.

Materials and Methods

Materials. ***Reagents.*** Reduced form of nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), and snake venom from *Crotalus adamanteus* were purchased from Sigma. 4-Hydroxyphenylacetic acid (HPA) and dithionite were purchased from Fluka. Pure FMN was conversed from FAD using snake venom and purified by a C18 Sep-Pak cartridge (Waters) as previously described.[3] Free FMN, C_1, and the reaction mixture were dissolved in 50 mM Na phosphate buffer at pH 7.0. The reaction mixture contained 20 μM of C_1, 100 μM of HPA, and 1 mM of NADH in 50 mM Na/Pi at pH 7.0. Dithionite (ca. 30 mg) was added for the flavin reduction.

Measurements. Femtosecond time-resolved fluorescence decays were measured using a homemade fluorescence up-conversion apparatus as described elsewhere.[7−9,13,14] In the experiments, the optical density per 1 cm path-length was ∼3 at 410 nm. Measurement of free reduced FMN and C_1 were performed under N_2 gas flow into sample cuvette after adding appropriate amount of dithionite. Pure C_1 protein and free CA were used for the measurements of time-dependent Stokes shift.

Time-Resolved Fluorescence Spectra. We constructed the fluorescence spectra from the fluorescence decays at several emission wavelengths according to Peon et al.[15] and Jarzęba et

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10.1021/jp901136y CCC: $40.75

al.[21] It was assumed that the fluorescence spectrum at $t = \infty$ is the same with one under steady-state excitation in wavenumber scale, and the spectral shape does not change with time. $\nu(0)$ was obtained as wavenumber of the spectral peak at $t = 0$. We also assume that effect of the spectral broadening on $\nu(t)$ (at emission peaks) is negligible.

Results and Discussion

Fluorescence Spectra of C_1 Protein. Fluorescence spectra of free FMN, C_1 protein, and a reaction mixture C_1 and NADH are shown in Figure S1 as Supporting Information. Relative intensities evaluated by ratios of the spectrum area of C_1 and the reaction mixture to that of free FMN were 0.0247 and 0.0101, respectively. Fluorescence intensity of C_1 was 2.5% of free FMN, which was not very weak compared to other flavoproteins with very short lifetimes.

Fluorescence Lifetimes of C_1. Fluorescence decay curves of C_1 are illustrated in Figure 1. The decay curves were markedly dependent on emission wavelength monitored. At shorter wavelengths, an instantaneous rise and very fast decay were observed, while at longer wavelengths the intensity rise and decay were very slow. Fluorescence lifetimes were 3 ps (25%), 42 ps (50%), and ca. 150 ps (25%) (average 59 ps) at 490 nm. The lifetimes were 9.65 ps (14%) and 146 ps (86%) (average 126 ps) at 520 nm, and 6.3 ps (4.7%) and 156 ps (95.3%) (average 148 ps) at 550 nm. The lifetimes were much longer than those of other nonfluorescent flavoproteins.[7−14]

Spectral Shift Correlation Function. Figure 2 shows time-resolved fluorescence spectra, which were constructed from the decay curves at the wavelengths, 470, 480, 490, 510, 520, 550, 570, and 600 nm. The spectrum shifted toward longer wavelength with time. $\nu(0)$ and $\nu(\infty)$ denote wavenumbers of emission peaks at $t = 0$ and $t = \infty$ in Figure 2. A spectral shift correlation function, $C(t)$, is defined by eq 1[19,20]

$$C(t) = \frac{\nu(t) - \nu(\infty)}{\nu(0) - \nu(\infty)} \quad (1)$$

Here, $\nu(t)$ represents the wavenumber of the transient spectral peak at time t. $C(t)$ was analyzed with a double exponential function as described in eq 2

$$C(t) = \alpha_1 \exp(-t/\varphi_1) + \alpha_2 \exp(-t/\varphi_2) \quad (2)$$

ϕ_1 and ϕ_2 are correlation times of component 1 and component 2, respectively, and α_1 and α_2 are corresponding fractions. These parameters determined give the minimum value of χ^2 between the observed and calculated $C(t)$ by a nonlinear least-squares method. Figure 3 shows the $C(t)$ functions of *p*-coumaric acid (CA) in water, which was extracted from photoactive yellow protein, and of C_1 in buffer solution. Although free FMN in water might be suitable to be used for the measurements of $C(t)$ in water as a reference to C_1, the measurement was not possible, because photodegradation took place during the measurements of $C(t)$. ϕ_1 and ϕ_2 of CA were 0.053 and 0.650 ps, respectively. ϕ_1 and ϕ_2 of C_1 were 0.455 and 250 ps, respectively. α_1 and α_2 were 0.14 and 0.86 in CA, and 0.816 and 0.184 in C_1, respectively. The value of ϕ_1 in CA may not be very accurate based on time-resolution of the instruments.

Ultrafast solvation dynamics depends mostly on solvent, but not much on solute probe.[21−24] When vibrational−rotational motions of solvent molecules are faster, the correlation time becomes shorter.[19,20] Jarzęba et al.[21] obtained $\phi_1 = 0.25$ ps ($\alpha_1 = 0.50$) and $\phi_2 = 0.96$ ps ($\alpha_2 = 0.50$) for coumarin 343 in water by means of ultrafast fluorescence spectroscopy. Jimenez et al.[22] introduced a Gaussian component into eq 2 in addition to two exponential terms. Average correlation time, $\alpha_1\phi_1 + \alpha_2\phi_2$, was 0.57 ps in the present work, and 0.61 ps by Jarzęba et al.[21] In case of C_1 protein, the shorter correlation time, $\phi_1 = 0.46$ ps, was close to the average correlation time of CA dye in water, and also to the one by Jarzęba et al.[21] This result suggests that the shorter correlation time of C_1 protein is due to water molecules near Iso in the protein. Although the estimated value of 250 ps may not be very accurate due to a much shorter dynamic range of the measurements relative to 250 ps, the existence of a slower component in $C(t)$ for C_1 protein was clearly observed and made clear difference from the free dye.

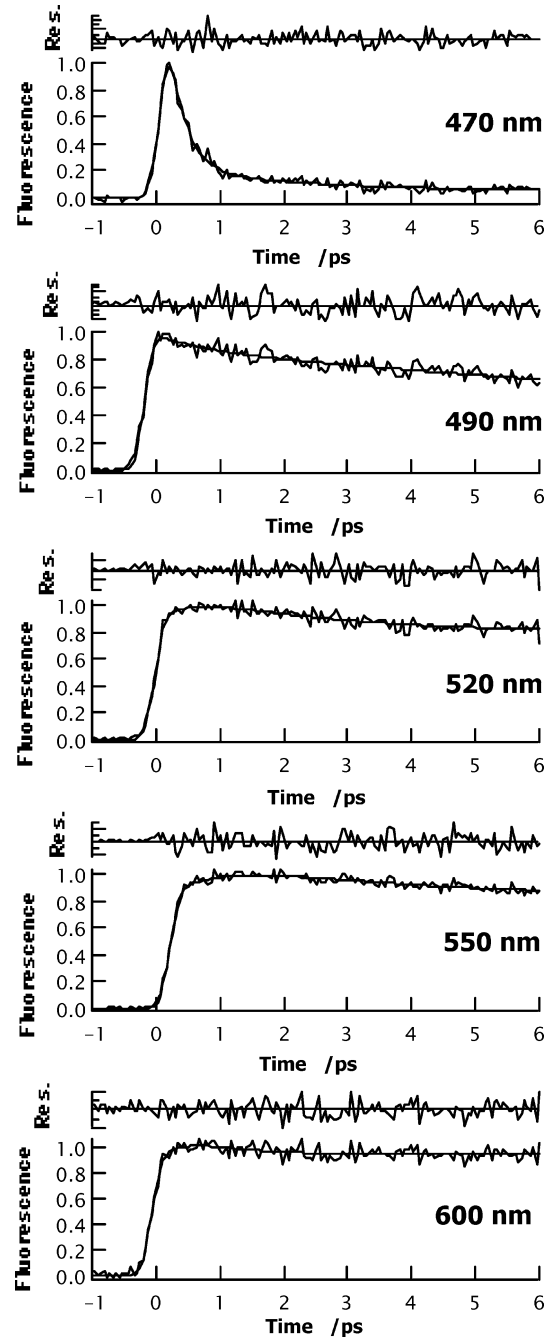

Figure 1. Normalized fluorescence decay curves of C_1 protein at different emission wavelengths as indicated at each panel. Res shown at each panel indicates residuals between the observed and calculated intensities with three-exponential functions.

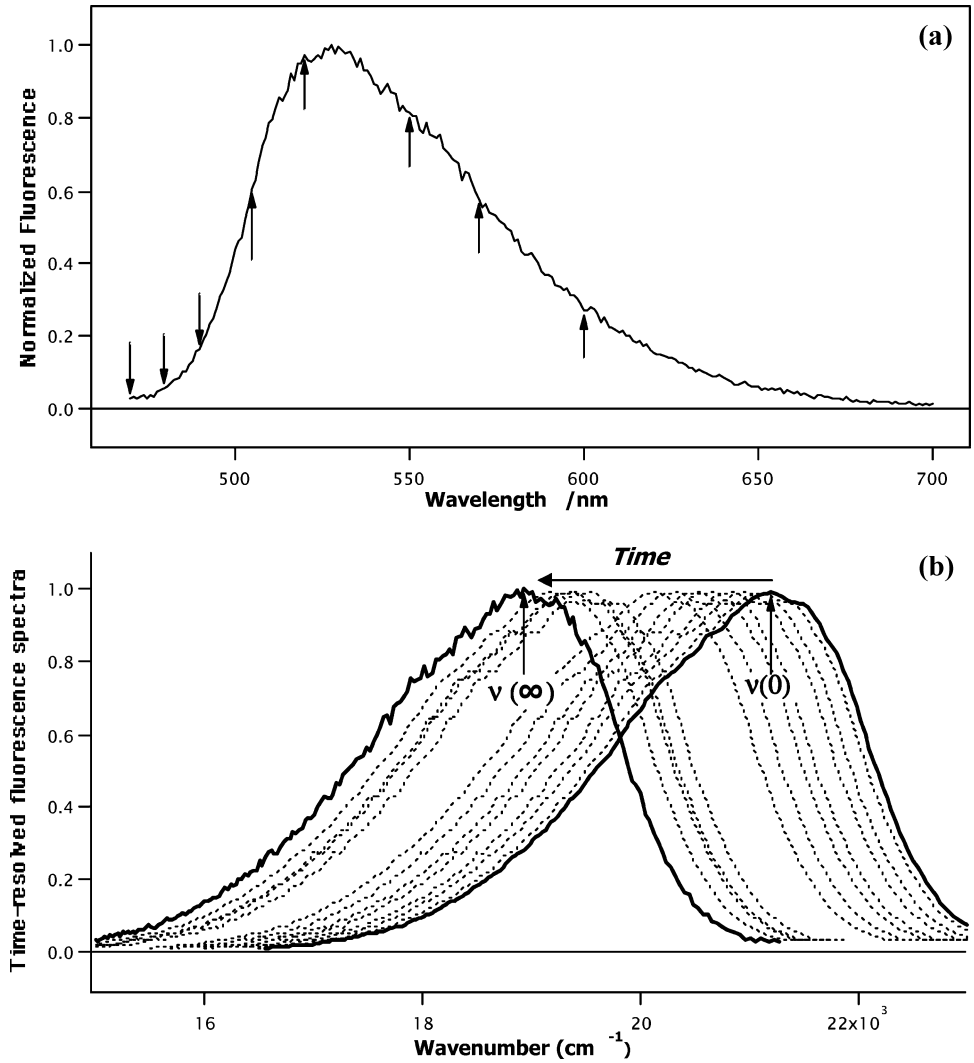

Figure 2. Time-resolved fluorescence spectra of C_1. (a) Steady-state fluorescence spectrum of C_1 protein. Arrows shown in this spectrum indicate monitored decay wavelengths. (b) Time-resolved spectral shift of C_1 fluorescence. $\nu(0)$ and $\nu(\infty)$ denote wavenumbers of emission peaks at $t = 0$ and $t = \infty$, respectively. $\Delta\nu$ was 2300 cm^{-1}.

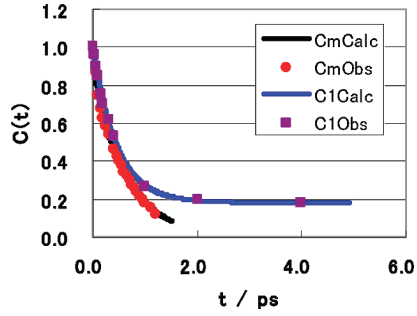

Figure 3. Spectral shift correlation function of *p*-coumaric acid and C_1. CmCalc and CmObs denote the calculated and observed correlation functions of *p*-coumaric acid, respectively. C_1Calc and C_1Obs denote the calculated and observed correlation functions of C_1, respectively. Correlation times, ϕ_1 and ϕ_2 in eq 1, of *p*-coumaric acid were 0.053 and 0.650 ps^{-1}, respectively. ϕ_1 and ϕ_2 of C_1 were 0.455 and 250 ps^{-1}, respectively. α_1 and α_2 were 0.14 and 0.86 in *p*-coumaric acid, and 0.816 and 0.184 in C_1, respectively.

Fluorescence Dynamics of Reduced FMN and Reduced C_1. Figure 4 shows fluorescence dynamics of reduced free FMN. Fluorescence lifetimes were $\tau_1 = 94$ fs (82%), $\tau_2 = 650$ fs (17%), $\tau_3 = 4.7$ ns (1%). The value of χ^2 was 0.915. τ_3 was assumed to be equal to the lifetime of free FMN and kept constant during the deconvolution procedure. The result

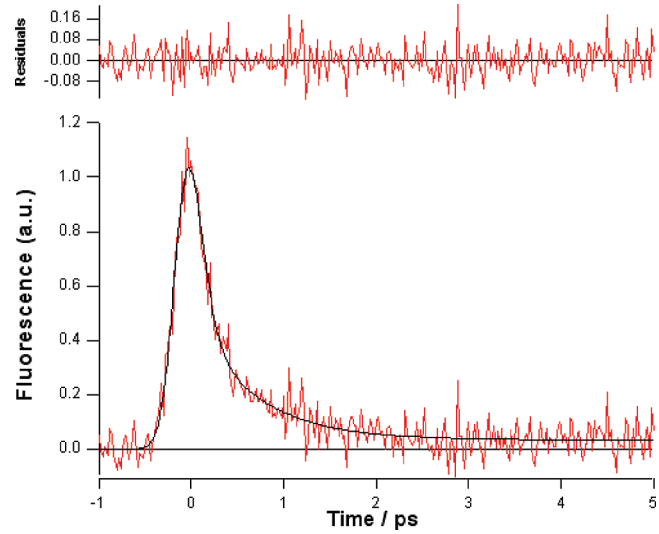

Figure 4. Fluorescence dynamics of reduced FMN. Fluorescence lifetimes were $\tau_1 = 94$ fs (82%), $\tau_2 = 650$ fs (17%), and $\tau_3 = 4.7$ ns (1%). The value of χ^2 was 0.915. τ_3 was assumed to be equal to the lifetime of free FMN and was kept constant in the deconvolution procedure. Concentration of FMN was ca. 0.5 mM. FMN was reduced with about 27 mg of dithionite. Emission monitored was 520 nm.

on the lifetimes of reduced FMN was obtained first time in femtoseconds−picoseconds time domain. Dynamics of reduced C_1 with dithionite did not differ at all from the oxidized C_1 protein, despite the fluorescence intensity decreasing markedly, suggesting that fluorescence lifetime of reduced C_1 was too fast to be detected by the present measurement systems with 100 fs time resolution. The observed decay was responsible for the decay of trace amount of the oxidized C_1.

Remarks on a Probable Protein Structure at FMN Binding Site. The longer correlation time (16 ps) in addition to the shorter correlation time (1.3 ps) was also found in Trp3 of monellin.[15] Trp3 locates at the surface of the protein. Average correlation time of free Trp was 0.92 ps.[15] Origin of the longer correlation time in the protein was elucidated in terms of the dynamical exchange of water molecules between free and immobilized water molecules at the protein surface.[15,16] Li et al.[17] obtained 5 and 87 ps correlation times of Trp7 in apomyoglobin. They interpreted the slower correlation time to be the result of slower rotational−translational motions of hydration water molecules coupled with protein dynamics.[17] Golosov and Karplus also supported the concept of coupling of the hydration with protein conformational dynamics for the longer correlation time by the method of molecular dynamics simulation.[18]

In some flavoproteins, Iso ring is near the interface. However, we have never observed time-dependent Stokes shift such as C_1, which is different from Trp. In the flavoproteins,[7−14] the fluorescence decays are very fast within a few picoseconds, whereas Trp in proteins normally decays within a few nanoseconds. This seems to make quite different behavior of the dynamic Stokes shift between Iso and Trp. Iso in C_1-bound FMN has quite long lifetimes compared to other flavoproteins.[7−14] Accordingly, Iso in C_1 is considered to be in quite polar environment both with freely librating water molecules and with hydrated water molecules coupled with the conformational dynamics at the protein surface.[17,18] Dynamics of polar amino acid groups with charges near Iso may also contribute to the dynamic Stokes shift. In DNA-photolyase, the solvation dynamics was reported in reduced flavin ($FMNH^-$) with 2 ps correlation time obtained by a transient absorption spectroscopy,[23] though the methodology may not be straightforward for investigation of the dynamic Stokes shift as in fluorescence dynamics. In this protein, water molecules may easily diffuse from the active site according to the X-ray structure.[24] Most of the 14 amino acid residues near Iso are polar and charged.

The spectral shift, $\Delta\nu = \nu(0) - \nu(\infty)$, was 2200 cm^{-1} in C_1, while 500 cm^{-1} in the Stokes shift of riboflavin tetrabutylate (soluble in organic solvents) under steady-state excitation was observed.[25] The dynamic Stokes shift in C_1 was much greater than the Stokes shift obtained by solvent effect under steady-state excitation. This also suggests that polar groups with charges near Iso play important role for the dynamic Stokes shift.

Acknowledgment. This work was supported by a grant from Mahasarakham University, the Thailand Research Fund Grant BRG5180002 and a Grant from the Faculty of Science of Mahidol University (P.C.). T.P. is a recipient of a CHE-PhD-SW scholarship from the Commission on Higher Education, Thailand. We thank Worapoj Oonanant for valuable discussion.

Supporting Information Available: Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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JP901136Y