Ultrafast Fluorescence Quenching Dynamics of Flavin Chromophores in Protein Nanospace

Noboru Mataga,* Haik Chosrowjan, and Yutaka Shibata

Institute for Laser Technology, Utsubo-Hommachi 1-8-4, Nishi-ku, Osaka 550-0004, Japan

Fumio Tanaka

Mie Prefectural College of Nursing, Yumegaoka 1-1-1, Tsu 514-0166, Japan

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We have studied excited-state dynamics of "nonfluorescent" flavoproteins by means of the femtosecond fluorescence up-conversion method. We have interpreted the ultrafast fluorescence quenching mechanisms of these flavoproteins as due to the ultrafast electron transfer or electron transfer followed by proton-transfer interactions between excited flavin chromophore and nearby tryptophan and tyrosine residues placed in the protein nanospace, on the basis of their X-ray structures. Comparisons of fluorescence time profiles and spectral characteristics of flavin chromophores in solutions with those in protein environments have suggested the existence of extremely fast Franck−Condon → fluorescent state relaxations specific to the protein environments. These results of the ultrafast fluorescence dynamics studies on the nonfluorescent flavoproteins have many features in common with other photobiologically important proteins.

Introduction

Flavoproteins with flavin chromophore are rather ubiquitous in various biological systems, where they undergo important redox reactions.[1] Although, in most cases, those reactions of flavins in biological systems are not light-driven, many studies on photoinduced reactions of flavins have been performed as models to facilitate the elucidations of the reaction mechanisms in those biological systems.[1]

On the other hand, although examples are rather few, flavins and flavoproteins seem to play also some important roles in photobiological reactions. One example is the DNA photolyase which photorepairs cyclobutane pyrimidine dimer produced by ultraviolet light in DNA. The DNA photolyases have the flavin chromophore in the reduced form, 1,5-dihydroflavin adenine dinucleotide ($FADH_2$), and bind to damaged DNA and split the cyclobutane ring of the dimer. It has been directly proved by means of the picosecond laser photolyses and time-resolved spectral measurements that splitting takes place with very high efficiency owing to the electron transfer (ET) from the photoexcited $FADH_2$ to the dimer.[2]

Another example is the blue light effect in the photosynthesis of plants. Namely, the CO_2 absorption through the stoma of plant leaf during the photosynthesis by red light seems to be regulated by flavoproteins excited with blue light,[3] where flavoproteins are in the oxidized form (yellow protein) and working as photosensor systems. Moreover, there seems to be a considerable amount of photobiological systems in which flavoproteins function as blue light photoreceptors, although direct experimental demonstrations of the relevant photoreactions by means of the time-resolved transient spectroscopy are rather scarce.[4]

When the flavin chromophore is in the oxidized form, it can act as a rather strong electron acceptor as well as proton acceptor in the photoexcited state, in view of its molecular and electronic structures. Therefore, if such amino acid residues as tryptophan (Trp·NH) and tyrosine (Tyr·OH) are placed close to the flavin chromophore in protein, strong quenching of the flavin fluorescence due to electron transfer (ET) or ET followed by proton transfer (PT) from the amino acid residues may be possible. Actually, although the flavin chromophores in solutions show strong fluorescence, there are many "nonfluorescent" or very weakly fluorescenct flavoproteins. The latter facts suggest the strong quenching of the flavin fluorescence in protein due to the above mechanisms. This is the most important characteristic of flavoproteins for their functions as photoreceptors, and furthermore, they are also very interesting and important model systems for elucidating the ultrafast reaction dynamics in protein nanospace as in the case of the ultrafast primary photoreactions in photosynthetic reaction centers and photosensory proteins studied extensively up to now.

In this respect, we have previously made picosecond (∼10 ps) time-resolved transient absorption spectral studies and fluorescence decay time measurements on some flavoproteins and also solutions of flavin chromophores with and without added quenchers such as indole and phenol corresponding to the amino acid residues Trp·NH and Tyr·OH, respectively.[5,6] These studies on nonfluorescent or very weakly fluorescent flavoproteins indicated that the flavin chromophore (F) actually underwent ultrafast quenching owing to ET immediately after picosecond pulsed excitation

$$\mathrm{F\cdots Trp{\cdot}NH} \xrightarrow{h\nu} \mathrm{F^*\cdots Trp{\cdot}NH} \xrightarrow{\mathrm{ET}} \mathrm{F^-\cdots Trp{\cdot}NH^+}$$

or electron transfer followed by PT

$$\mathrm{F\cdots Tyr{\cdot}OH} \xrightarrow{h\nu} \mathrm{F^*\cdots Tyr{\cdot}OH} \xrightarrow{\mathrm{ET}} \mathrm{F^-\cdots Tyr{\cdot}OH^+} \xrightarrow{\mathrm{PT}} \mathrm{F{\cdot}H\cdots Tyr{\cdot}O}$$ [5,6]

Namely, by means of the usual picosecond (∼10 ps) laser photolyses, the product ($F^-\cdots Trp{\cdot}NH^+$) or ($F{\cdot}H\cdots Tyr{\cdot}O$) was detected. Nevertheless, the observation of the reaction dynamics

* To whom correspondence should be addressed.

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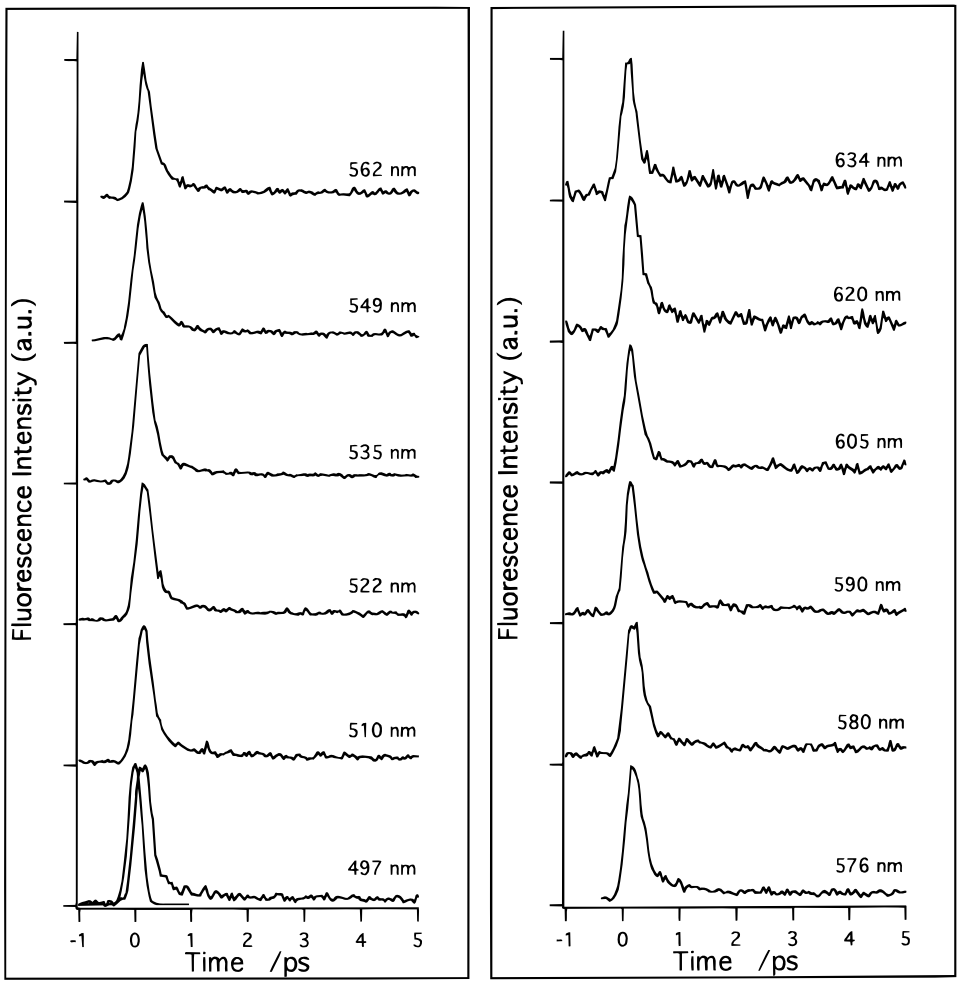

Figure 1. Fluorescence dynamics of RBP excited at 410 nm and observed at various wavelengths. The instrumental response (fwhm ~ 210 fs) is also indicated in the figure.

leading to this product states formation was not possible.[5,6] To examine directly the ultrafast reaction dynamics and to elucidate the nature of this photoinduced reaction in these nonfluorescent proteins, we have measured fluorescence dynamics by means of the femtosecond fluorescence up-conversion method as demonstrated and discussed in the following.

Experimental Section

Riboflavin binding protein (RBP) was prepared from egg white as apoprotein and reconstituted by adding riboflavin according to the method by Rhodes et al.[7] Glucose oxidase from *Aspergillus niger* (GOD) was purchased from Nacalai Tesque (Kyoto, Japan) and purified according to Tsuge et al.[8]

Buffer solution of flavoprotein sample was made to flow through a 1 mm cell, and a femtosecond up-conversion apparatus for the measurement of fluorescence dynamics was similar to that described elsewhere.[9] The fwhm of the instrumental response was 210 fs. Measurements were made at room temperature (23 °C), and observed fluorescence rise and decay curves were deconvoluted taking into account the instrumental response function and reproduced by superposing exponential functions.

Results and Discussion

Among the nonfluorescent or very weakly fluorescent flavoproteins, we have tried hitherto to observe the femtosecond to picosecond fluorescence dynamics of RBP, GOD, MCAD (medium chain acyl CoA dehydrogenase), and DAOB (D-amino acid oxidase−benzoate complex) for which information on the high-resolution X-ray structures is already available. This information on the structures of the protein nanospace of the amino acid residues surrounding the flavin chromophore is very useful for the investigations on the quenching mechanisms of the chromophore fluorescence. In this short report, we show our experimental results and give some discussions on typical examples of ultrafast fluorescence dynamics of flavoproteins, RBP and GOD.

In Figure 1, we show fluorescence rise and decay profiles of RBP excited at 410 nm and observed at various wavelengths from 497 to 634 nm and normalized at peak position. The rise and decay curves were practically identical with each other. The fluorescence rise and decay profiles of RBP observed by exciting at 440 and 428 nm were practically the same as those in Figure 1. The decay curves were nonexponential and could be reproduced well by superposing three exponential model functions, $I(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + a_3 \exp(-t/\tau_3)$, as follows, RBP (at 620 nm): $a_1 = 0.87$, $\tau_1 = 98$ fs, $a_2 = 0.11$, $\tau_2 = 0.41$ps, $a_3 = 0.02$, $\tau_3 = 6.2$ ps ($\chi^{1/2} = 3.16$), where the contribution from the fastest component with decay time of ~100 fs was overwhelming. This is an extremely short decay time compared with the decay time of several nanoseconds of the flavin chromophore in solutions. RBP is a globular monomeric protein of approximate dimensions 5 nm × 4 nm × 3.5 nm, and the riboflavin chromophore is accommodated in a cleft of 2 nm wide and 1.5 nm deep (protein nanospace), where

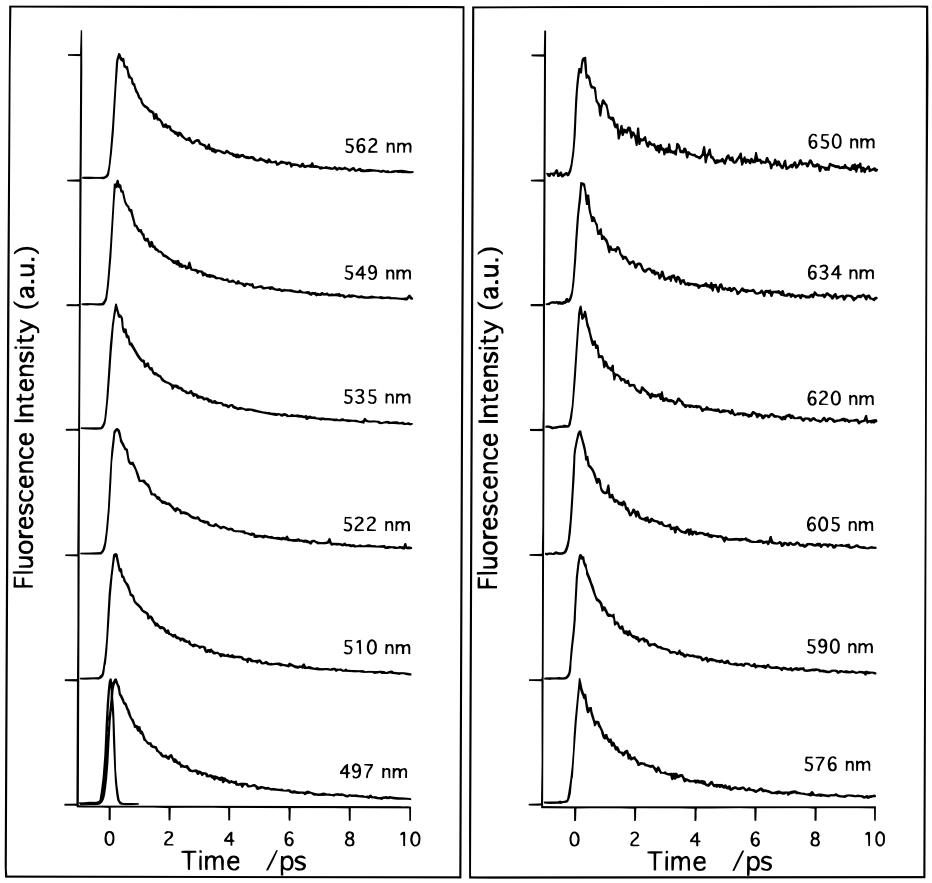

Figure 2. Fluorescence dynamics of GOD excited at 410 nm and observed at various wavelengths. The instrumental response is also indicated in the figure.

the isoalloxazine ring of riboflavin is stacked between Tyr·OH and Trp·NH placed in contact with the chromophore.[10] This stacked system is further surrounded by several Trp·NH residues.[10] This arrangement of the chromophore and Tyr·OH as well as Trp·NH amino acid residues in protein nanospace is supremely favorable for the ultrafast fluorescence quenching due to the ET and ET followed by PT from the amino acid residues placed close to the excited chromophore.

Our previous comparative studies on solutions of riboflavin chromophore added with quenchers (indole and phenol, respectively) and RBP, with picosecond laser photolysis and time-resolved transient absorption spectral measurements, suggested that the main process of the quenching was the ET followed by PT from the Tyr·OH to excited riboflavin chromophore, F*···Tyr·OH → F^-···Tyr·OH^+ → F·H···Tyr·O, rather than the ET from the Trp·NH.[6] This suggestion might not be unreasonable because the Tyr·OH group in contact with the flavin chromophore in the protein nanospace seems to be directly interacting with the heteroaromatic ring of isoalloxazine including the hydrogen-bonding interactions,[10] while the Trp·NH group seems to be mainly interacting with the benzene ring of the isoalloxazine,[10] and the transient absorption spectrum obtained by picosecond laser photolyses on RBP was similar to that of the solution of the riboflavin chromophore−phenol system.[6] Nevertheless, it is necessary to confirm such conclusions by means of more detailed femtosecond pump−probe time-resolved absorption spectral studies on RBP. At any rate, the ultrafast fluorescence quenching dynamics due to ET or ET followed by PT taking place in 100 fs presumably involves coherent processes. Investigations on this problem by means of the fluorescence dynamics measurements with higher time resolution are now going on in our laboratory.

In Figure 2, we show time profiles of fluorescence of GOD excited at 410 nm and observed at various wavelengths from 497 to 650 nm and normalized at peak position. These decay curves were practically in agreement with each other. Similar results were obtained also when excited at 430 nm. The nonexponential decay curves were reproduced by superposing three exponential functions as in the case of RBP, GOD (at 620 nm): $a_1 = 0.45$, $\tau_1 = 413$ fs, $a_2 = 0.38$, $\tau_2 = 1.9$ ps, $a_3 = 0.17$, $\tau_3 = 6.0$ ps ($\chi^{1/2} = 1.52$). Thus, the fluorescence decay of GOD is considerably slower compared with that of RBP. Nevertheless, this fluorescence decay of the flavin chromophore (flavin adenine dinucleotide, FAD) in the protein nanospace is still much faster than that in solutions. According to the X-ray crystallographic studies on GOD,[11] the isoalloxazine chromophore of FAD is also surrounded by several Tyr·OH and Trp·NH amino acid residues in the protein nanospace. However, these amino acid residues are placed neither so closely to the isoalloxazine ring nor in stacked form sandwiching the isoalloxazine ring as in the case of the RBP.[10] Two Tyr·OH residues and one Trp·NH residue, which are placed somewhat closer to the chromophore ring, are on the aromatic hydrocarbon (benzene ring) part of the isoalloxazine ring. Therefore, the chromophore−aromatic amino acid residue interactions responsible for the photoinduced ET or ET followed by PT may be considerably weaker for GOD compared with RBP, resulting in the much slower fluorescence decay of GOD.

Although we examined also GOD in our previous picosecond laser photolysis and transient absorption spectral studies on flavoproteins, it was not possible to detect the transient absorption spectra owing to the products of the quenching reaction. This result indicates that the transient ion radical pair or neutral radical pair produced by photoinduced ET or ET

followed by PT undergoes rapid recombination leading to the ground state within a few picoseconds. According to the above discussions based on the crystal structure of GOD, ET followed by PT from Tyr·OH to the excited chromophore may be difficult because amino acid residues are interacting with the benzene part of the isoalloxazine ring. Photoinduced ET from Trp·NH and rapid recombination to give the ground state, F*···Trp·NH → F$^-$···Trp·NH$^+$ → F···Trp·NH, may be more probable. It should be noted here that these flavoproteins are very stable for the irradiation with laser pulse, which means that the radical pairs produced in protein nanospace by irradiation rapidly return almost completely to the ground state in both RBP and GOD. They can act as ultrafast photosensors as in the case of the photoactive yellow protein undergoing the ultrafast photoisomerization and reverse process in the dark.[9] At any rate, we should confirm the reaction mechanism in the fluorescence quenching by means of detailed femtosecond time-resolved absorption spectral measurements also in the case of the GOD.

We should point out here that there is another interesting and important problem concerning the fluorescence dynamics of these nonfluorescent proteins. It is well-known that flavin chromophores in solutions show an absorption peak at 450−460 nm and a fluorescence band with a peak at ca. 530 nm. Most flavoproteins show absorption peaks at the same wavelength as that of the free chromophore solution. Although the fluorescence band maxima of nonfluorescent flavoproteins are not very clear, our results of fluorescence decay curve measurements at various wavelengths by fluorescence up-conversion with femtosecond lasers indicate that the fluorescence wavelength ranges and peak positions of those flavoproteins are rather similar to those of the chromophore solutions.

We have measured time profiles of fluorescence at various wavelengths between 480 and 610 nm for FMN and FAD in aqueous solutions and detected the indications of faster decay at the shortest wavelengths and a little rise at the longest wavelengths owing to the time-dependent Stokes shift of the fluorescence caused by solvation dynamics. However, we cannot recognize such an effect of the dynamic Stokes shift of fluorescence in both RBP and GOD as indicated in Figures 1 and 2, respectively. This result means that the relaxation processes from excited FC (Franck−Condon) to Fl (fluorescence) state coupled with vibrational modes of chromophore as well as protein environment are presumably too fast to time resolve with the present measurements by means of the 100 fs laser pulses. As discussed above, the magnitude of the fluorescence Stokes shift of the flavin chromophore in protein nanospace seems to be rather close to that in solution (ca. 3500−4000 cm^{-1}). Similar ultrafast FC → Fl relaxation processes are working also in photobiologically important proteins such as visual rhodopsins (Rh),[12] bacteriorhodopsin (bR),[12] photoactive yellow protein (PYP),[13] etc., where such ultrafast relaxations seem to play important roles in facilitating the very fast and efficient photoreactions. Namely, ultrafast FC → Fl relaxation processes are coupled with many vibrational modes of chromophore and protein environments including coherent processes in 10 fs regimes.[12] In this respect, systematic studies including measurements of fluorescence dynamics with higher time resolution are now going on in this laboratory.

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