Femtosecond Fluorescence Dynamics of Flavoproteins: Comparative Studies on Flavodoxin, Its Site-Directed Mutants, and Riboflavin Binding Protein Regarding Ultrafast Electron Transfer in Protein Nanospaces

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We have studied the fluorescence dynamics of "nonfluorescent" flavoproteins including flavodoxin (FD), its mutants W60F, Y98F, and W60F/Y98F, and riboflavin binding protein (RBP) with the femtosecond fluorescence up-conversion method and have observed the fluorescence quenching dynamics of FD and its mutants for the first time. The strong fluorescence quenching in these flavoproteins seems to be caused by ultrafast electron transfer (ET) from aromatic amino acid residues to the excited flavin chromophore in stacked configuration according to previous transient absorption studies. In the present work, we have made comparative studies on the dynamics of fluorescence quenching due to ET to the excited chromophore in RBP and FD. We have observed also fluorescence dynamics of FD mutants where active electron donors Trp·NH and Tyr·OH are partially (either of them) or completely replaced by inactive phenylalanine and directly demonstrated the ET mechanism of the ultrafast fluorescence quenching in PNS of FD.

Introduction

Flavoproteins with the flavin chromophore are rather ubiquitous in various biological systems, where they undergo important redox reactions.[1] In most cases, their reactions are not light-driven; however, considerable studies on photoinduced electron transfer (ET) reactions of flavins as models to facilitate the elucidation of the reaction mechanisms in those biological systems have been performed.[1]

On the other hand, although examples are rather few, some flavin enzymes seem to play also important roles in photobiological reactions. For instance, one example is the DNA photolyase that photorepairs a cyclobutane pyrimidine dimer produced by ultraviolet light in DNA. The DNA photolyase has the flavin chromophore in reduced form and split the cyclobutane ring of the dimer by photoinduced reduction.[2,3]

When the flavin chromophore is in the oxidized form, it can act as a strong electron acceptor in the photoexcited state. Therefore, if aromatic amino acid residues tryptophan (Trp·NH) and tyrosine (Tyr·OH) are placed close to the flavin chromophore in protein nanospace (PNS, protein environment of a few nanometer scale surrounding the chromophore), strong quenching of the flavin fluorescence due to the ET from the aromatic amino acid residues can take place. Actually, the bright fluorescence of the flavin chromophore, due to the common conjugate π-electronic part of isoalloxazine (ISO), in solution is strongly quenched in PNS and there are many "nonfluorescent" or only very weakly fluorescent flavoproteins. This may be the most important characteristics of flavoproteins for their functions as photoreceptors.

Experimental proof of the ET mechanism of the fluorescence quenching dynamics in PNS of flavoproteins was rather scarce.[4] We have previously tried to detect the photoinduced ET reaction by means of the picosecond (with exciting laser pulse of ∼25 ps fwhm) time-resolved transient absorption spectral measurements on some flavoproteins and also solutions of flavin chromophores with added quenchers such as indole and phenol corresponding to Trp·NH and Tyr·OH, respectively.[5,6] We have detected the transient absorption spectra that can be ascribed to the radical ions formed by the photoinduced ET reaction between the excited flavin chromophore and indole in solutions.[5] We have examined also by means of the picosecond transient absorption spectral measurements the "nonfluorescent" flavoproteins, flavodoxin (FD) from *Desulfovibrio vulgaris*, strain Miyazaki and riboflavin binding protein (RBP).[6] Both of these flavoproteins have stacked structure of PNS where the ISO of the flavin chromophore, flavin mononucleotide (FMN) for FD and riboflavin (RF) for RBP, is sandwiched between Trp·NH and Tyr·OH with very short ISO−amino acid interplanar

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distances of ca. 3.7 ~4.3 Å.[7,8] The observed transient absorption spectra could be ascribed mainly to the radical ion pair state formed by ET from the nearby Trp·NH to the excited flavin chromophore (F*) in PNS, i.e., F*···Trp·NH → F$^-$···Trp·NH$^+$, for FD.[6] Despite the stacked sandwich structure, the photoinduced ET from Trp·NH seems to be predominant due to the faster ET owing to the larger energy gap, $-\Delta G_{ET}$, for the reaction F*···Trp·NH → F$^-$···Trp·NH$^+$. The charge recombination decay of the produced ion pair state seems to be much slower (decay time due to recombination in the 100 ps regime) compared with the very fast photoinduced charge separation.[6]

On the other hand, the observed spectra of RBP immediately after excitation were very broad compared with those of FD and it was difficult to confirm the ultrafast ET to the excited riboflavin chromophore from the aromatic amino acid residues.[6] Contrary to such results of our previous ~10 ps transient absorption spectral measurements, recent fluorescence dynamics studies on RBP by means of the femtosecond fluorescence up-conversion method have indicated ultrafast fluorescence quenching in the 100 fs regime due to ET.[9,10] Very recently, however, femtosecond−picosecond transient absorption measurements on RBP have demonstrated that the ET state formation takes place in the 100 fs regime corresponding to the ultrafast fluorescence quenching dynamics, and the decay time of the ET state due to recombination is ~8 ps.[11]

Therefore, the nature of the long-lived broad transient absorption spectra observed by ~10 ps laser photolysis studies on RBP[6] is not clear. One possibility for the origin of this broad spectra might be due to the ultrafast ET from Tyr·OH to the excited riboflavin chromophore (F) followed by the proton transfer (PT) competing with the charge recombination decay; F*···Tyr·OH → F$^-$···Tyr·OH$^+$ → F·H···Tyr·O and F···Tyr·OH, in addition to the ET from Trp·NH to F*. Moreover, according to the X-ray structural analyses on RBP[7] and FD,[8] the −OH group of Ty·OH in the stacked configuration is placed very close to C═O group of ISO in RBP,[7] leading to the ultrafast ET coupled with PT easily. The closest distance between the −OH group of Tyr·OH and the heteroatom (−N═) of ISO in FD is large,[8] so that the ultrafast ET coupled with PT is not feasible, whereas the ultrafast ET can take place easily owing to the interplanar close contact between Trp·NH and ISO.[8]

In any case, femtosecond fluorescence dynamics of FD with a stacked structure of PNS very similar to that of RBP where the flavin chromophore ISO is sandwiched between Trp·NH and Tyr·OH have not yet been examined. To obtain more detailed information concerning the ultrafast ET reaction dynamics in PNS of these peculiar flavoproteins, we have performed comparative femtosecond fluorescence dynamics studies on wild-type (w-t) FD, its mutants, and RBP.

Experimental Section

RBP was prepared from egg white as apoprotein and reconstituted by adding riboflavin according to the method by Rhodes et al.[12]

Preparation and site-directed mutagenesis of FD were made as follows. *Escherichia coli* strain BL21(DE3) was used to synthesize the recombinant FD, as in the previous report.[13] Bacterial strains were cultivated in LB broth or on LB agar plates supplemented with antibiotics. Site-directed mutagenesis was done as reported previously.[13] Tryptophan and tyrosine residues at positions 60 and 98 of the FD were exchanged to phenylalanine. Plasmid pUTFLV carrying the FD gene[14] was subjected to mutagenesis with a Quick-Change site-directed mutagenesis kit obtained from Stratagene (La Jolla, CA). The primers used were designated on the basis of the DNA sequence of the FD gene and purified by high-pressure liquid chromatography before use. Mutations were confirmed by DNA sequencing of the complete FD gene subcloned into the pBluescriptII SK(+) vector. The resultant plasmids carrying the mutation, designated pUTFLV (W60F) and pUTFLV (Y98F), were introduced into the *E. coli* strain BL21(DE3) for the protein expression. For double mutation of the FD with W60F and Y98F, the *Nco*I − *Pst*I fragment of the pUTFLV (Y98F) was replaced with that of pUTFLV(W60F). The expression and the purification methods for the recombinant FD were the same as those described in the previous report,[13] except that *E. coli.* cells were disrupted by two passages through a French pressure cell (Aminco).

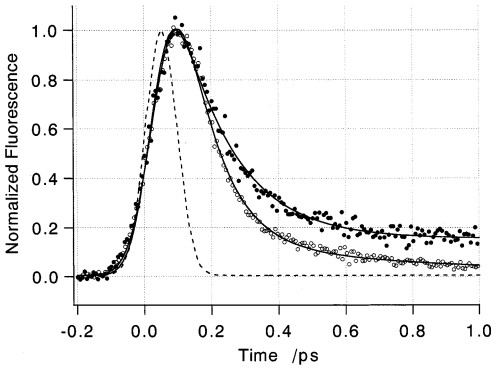

Figure 1. Fluorescence rise and decay dynamics of w-t FD (●) and RBP (○). Solid lines show the results of simulation with superposition of two exponential functions taking into consideration the instrumental response indicated by the broken line (see also Table 1). Excited at 410 nm and observed at 530 nm.

TABLE 1: Parameters for Simulations of Fluorescence Decay Curves of w-t FD, Its Three Mutants, and RBP Using the Double Exponential Model Function, $I(t) = a_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$

	$\mathbf{a}_1$	$\mathbf{a}_2$	τ_1/fs	τ_2/ps	χ^2
w-t FD	0.92	0.08	158	>500	0.27
W60F	0.83	0.17	322	5.5	0.31
Y98F	0.85	0.15	245	4.0	0.13
W60F/Y98F		1.0		~18	0.45
RBP	0.93	0.07	100	0.7	0.075

Concentrations of sample solutions used for the measurements were as follows: [w-t FD] ~ 2.5×10^{-4} M, mutant FDs' [Y98F] ~ 3.3×10^{-4} M, [W60F] ~ 4.2×10^{-4} M, [(Y98F)/(W60F)] ~ 3.6×10^{-4} M, [FMN (free)] ~ 4.2×10^{-4} M in buffer solutions with 10 mM Tris HCl, pH ~ 8.0, and [RBP] ~ 6.3×10^{-4} M in buffer solutions with 0.1 M potassium phosphate, pH ~ 6.9. The sample solutions for the measurements were made to flow through a 1 mm cell at room temperature.

Measurements of fluorescence dynamics were made by a fluorescence up-conversion apparatus similar to that described elsewhere,[12] but the fwhm of the overall instrumental response was improved to 120 fs compared with 210 fs of the previous one.

Results and Discussion

Ultrafast nonexponential fluorescence decay curves of w-t FD and RBP are shown in Figure 1 together with the instrumental response (fwhm ~120 fs). These decay curves can be reproduced satisfactorily by superposing two exponentials, as shown in Table 1. In both cases, contribution of the faster component (with lifetime $\tau_1 = 158$ fs for w-t FD and $\tau_1 = 100$

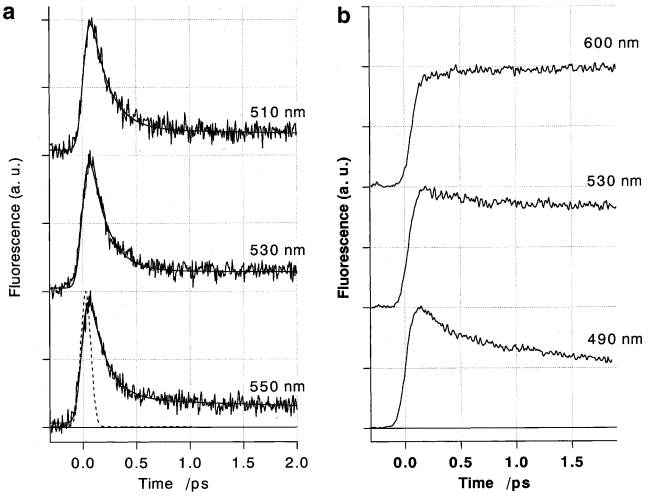

Figure 2. (a) Fluorescence rise and decay dynamics of w-t FD excited at 410 nm and observed at 510, 530, and 550 nm. Solid lines show the results of simulation taking into account the instrumental response indicated by the broken line. (b) Fluorescence rise and decay dynamics of FMN excited at 410 nm and observed at 490, 530, and 600 nm.

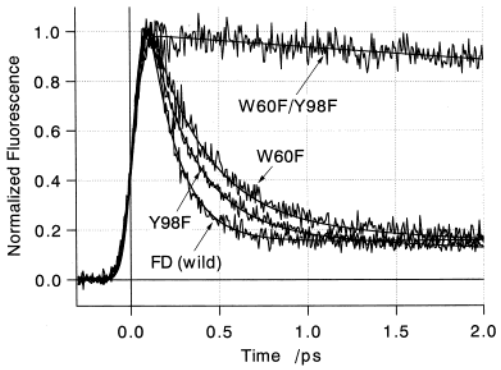

Figure 3. Fluorescence rise and decay dynamics of w-t FD and mutants Y98F, W60F, and W60F/Y98F. Solid lines are the simulations with two exponential or single-exponential functions. Excited at 410 nm and observed at 530 nm.

fs for RBP) is predominant. The lifetime τ_2 of the long-lived fluorescence component of w-t FD, as shown in Figure 1 and Table 1, is much longer than that of RBP. It seems that w-t FD is not very stable in solution but undergoes a little decomposition, producing a slight amount of free chromophore (FMN) isolated from protein, which gives a feeble fluorescence with a very long lifetime.

In our previous fluorescence dynamics studies on RBP,[9,10] we have demonstrated that the ultrafast fluorescence decay dynamics measured at various wavelengths of the fluorescence spectrum was practically the same. Namely, we could not observe any effect of the dynamic Stokes shift of the fluorescence band due to the interaction between the excited chromophore RF and the surrounding amino acid residues in PNS. In the present study, we have confirmed that the same circumstance prevails also for the w-t FD, as shown in Figure 2a. On the other hand, as shown in Figure 2b, the chromophore FMN in aqueous solution clearly shows the dynamic Stokes shift of fluorescence; a fast initial decay at the short-wavelength edge and fast rise followed by very long life fluorescence at longer wavelengths.

As discussed in the Introduction, the conjugate π-electronic part of the flavin chromophore, ISO, is sandwiched between aromatic amino acid residues Trp·NH and Tyr·OH at short distances in the approximately plane−parallel configuration in the PNS of both w-t FD and RBP. Such chromophore−amino acid residues configuration in the PNS seems to cause very rapid ET to the excited flavin chromophore F* from the amino acid residues, leading to the ultrafast fluorescence quenching reactions. Our previous transient absorption spectral measurements on FD[6] suggested that, in the competition between the ET reaction from the Trp·NH and that from the Tyr·OH to the excited flavin chromophore in PNS, the contribution of the former was the principal one for the w-t FD.

In relation to the above results of our previous studies on the photoinduced reaction products[5,6] and recent direct measurements on the ultrafast fluorescence quenching dynamics in PNS of these "nonfluorescent" flavoproteins,[9,10,11] we have examined not only the fluorescence dynamics of w-t FD but also those of site-directed mutants of FD, where either one of the aromatic amino acid residues working as strong electron donor to F* or both of them are replaced by phenylalanine residue, as indicated in Figure 3 and Table 1.

Because the electron donating ability of the phenylalanine residue is much smaller compared with those of Trp·NH and Tyr·OH residues, one may expect a large increase of the fluorescence lifetime of FD by this mutation. Actually, the fluorescence decay time of the FD mutant, W60F/Y98F (∼18 ps), where both Trp·NH and Tyr·OH adjacent to the ISO are replaced by phenylalanine, is much longer than that of the w-t FD (158 fs). Nevertheless, it is considerably shorter than that of the isolated flavin chromophore FMN in solution, which might be ascribed to the weak quenching of the FMN fluorescence by ET from more distant Trp·NH and Tyr·OH residues in protein. It should be noted here that the band shape and the peak wavelength of the absorption band responsible for the fluorescence emission is practically unchanged by mutations (∼455 nm), which indicates that no specific change of the protein environment around the flavin chromophore leading to the spectral shift of S_1 state takes place in PNS by mutations. Namely, only the electron-donating ability of the aromatic amino acid residues is changed by mutation with rather trivial effect on the other protein environment.

The decay time (322 fs) of the main component in the fluorescence decay curve of the mutant W60F, where the Trp·NH is replaced by phenylalanine, is considerably longer than that of the mutant Y98F (245 fs), where Tyr·OH is replaced by phenylalanine. This difference between the two mutants, W60F and Y98F, may be ascribed mainly to the larger energy gap $-\Delta G_{ET}$ for the ET from Trp·NH[5] to the excited flavin chromophore in the normal region than that for the ET from Tyr·OH.

If the contribution from the phenylalanine as an electron donor to the excited flavin chromophore in PNS is negligible and the arrangement (the intermolecular distances and orientations) between the flavin chromophore and the electron donor, Trp·NH or Tyr·OH, remains almost unchanged by mutation as noted above, the rate constant of the fluorescence quenching due to the ET reaction of the w-t FD may be approximately equal to the sum of the rate constants obtained for the mutants W60F and Y98F, respectively.

Because the long-lived fluorescence component of the w-t FD can be attributed to the FMN isolated from PNS into solution, the rate constant for the ET reaction in PNS of w-t FD should be given by the inverse of the decay time, $k_{ET} = \tau_1^{-1} \sim 6.3 \times 10^{12}\ s^{-1}$. On the other hand, the fast initial decays of the fluorescence for the mutants W60F and Y98F should be ascribed respectively to the ET reactions from the Tyr·OH and

Trp•NH to the excited ISO chromophore in the plane-parallel stacked configuration in PNS, whereas the slow decays may be due to the ET reaction from distant Trp•NH and/or Tyr•OH residues to excited ISO.

The fraction of the initial fast decay in single mutant is much larger compared with that of the slow decay; nevertheless, we should take into consideration also the contribution of the slow one. By adding both contributions from the fast and slow ET quenching reactions with respective fractions, the averaged ET rate constants have been estimated as follows: $k_{ET} = 2.6 \times 10^{12}$ s^{-1} for W60F and $k_{ET} = 3.5 \times 10^{12}$ s^{-1} for Y98F. Moreover, it should be noted that the sum of contributions from the initial fast decays in single mutants and k_{ET} of the double mutant gives the same result, 6.1×10^{12} s^{-1}. Therefore, the sum of the ET rate constants of these mutants is approximately the same as that of w-t FD, $k_{ET} = 6.3 \times 10^{12}$ s^{-1} within experimental error, in agreement with the above discussions that the replacement of Trp•NH or Tyr•OH with phenylalanine will only slightly disturb the other protein environment around the flavin chromophore in PNS.

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References and Notes

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