Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

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To cite this Article Isoda, Satoru and Maeda, Mitsuo(1989) 'Energy Transfer between Flavin and Cytochrome c', Journal of Macromolecular Science, Part A, 26: 2, 445 — 454
To link to this Article: DOI: 10.1080/00222338908051986
URL: http://dx.doi.org/10.1080/00222338908051986

J. MACROMOL. SCI.–CHEM., A26(2 & 3), pp. 445-454 (1989)

ENERGY TRANSFER BETWEEN FLAVIN AND CYTOCHROME c

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ABSTRACT

Energy transfer between photoexcited flavin and cytochrome c has been investigated in order to estimate intermolecular forces between flavin and cytochrome c. The quenching of the fluorescence of flavin by cytochrome c excited at 372 nm was found to be much greater than that excited at 465 nm. This dependence of the quenching on the exciting wavelength is considered to be due to the "prerelaxational" fast energy transfer. From the analysis of the quenching of the fluorescence of FMN and lumiflavin by cytochrome c excited at 465 nm, it was concluded that 1) the quenching is mainly controlled by resonance energy transfer, and 2) the heterogeneous dispersion state of molecules due to electrostatic forces makes the critical transfer distance, R_0, of the resonance process longer than the real distance. For the quenching of the fluorescence of flavodoxin by cytochrome c, it was found that complex formation is a dominant process and is controlled to a great extent by electrostatic forces. Furthermore, fluorescence decay curves were measured by a single-photon counting method in order to estimate the dynamic processes of flavin fluorescence. The results also showed that the resonance process exists in the energy transfer between flavin and cytochrome c.

INTRODUCTION

Electron-transfer reactions between reduced flavins and oxidized cytochrome c have been extensively studied by Tollin and Cusanovich et al. They showed by laser flash photolysis that the electron-transfer reactions between low molecular weight flavins such as flavin mononucleotide (FMN), riboflavin, lumiflavin, etc. and cytochrome c depend on ionic strength, and that there are correlations between the second-order rate constants and the difference in redox potential of the reactants [1, 2]. For the reduction of cytochrome c by flavodoxin semiquinones, they showed by stopped-flow spectrophotometry that the complex formation process is involved in the reaction and that the difference in redox potential of the reactants, electrostatic potential on the protein surfaces, and steric effect are all involved in controlling the specificity of the reaction [3-5].

As described above, many kinds of information have been accumulated concerning the reduction of cytochrome c by reduced flavins. Since these reactions were observed in solution, the collision of the reactants is essential for them. Accordingly, molecular interactions whose time scale is shorter than the diffusion process cannot be examined by analysis of the electron-transfer reactions in solution.

In the present work, the effect of cytochrome c on the processes of relaxation of photoexcited flavins has been investigated by fluorescence spectrophotometry in order to estimate the fast processes of molecular interaction between flavins and cytochrome c. Studies of the fluorescence of flavins have been reported by many researchers. Sun et al. analyzed the excited states of flavins by luminescence studies [6]. Holmström et al. showed that the iodide quenching of the fluorescence of riboflavin can be elucidated by the collisional process [7]. The fluorescence lifetimes of 3,7,8,9,10-pentaethylisoalloxazine in aqueous solution, lumiflavin in ethanol, and riboflavintetrabutyrate in chloroform were found to be 1.7 [8], 7.1 and 7.4 ns [9], respectively. Moore et al. reported concerning photophysical properties of flavins [10].

Although there have been many reports on the fluorescence of flavins, the energy transfer between photoexcited flavins and proteins has not been reported so far. Since flavins are involved in many photobiological processes, it is of interest to investigate the energy transfer between flavins and cytochrome c.

EXPERIMENTAL

Materials

FMN (sodium salt) and lumiflavin were purchased from Sigma. The flavodoxin from *Megasphaera elsdenii* was obtained from TNO (Netherlands). Horse-

heart cytochrome c (Type VI) was purchased from Sigma and used without further purification. The solutions used for fluorescence spectrophotometry consisted of 10 μM flavin and suitable concentrations of cytochrome c (~10-50 μM) dissolved in 100, 10, or 1 mM phosphate buffer (pH 7.0).

Methods

Fluorescence was measured with a JASCO FP-550 spectrophotofluorometer. Fluorescence-decay curves of flavins were measured by a time-correlated single-photon counting method [11]. The mode-locked Ar ion laser cavity dumped at 4.1 MHz (Spectra-physics, Inc.) was used for the exciting pulse.

THEORY

The photophysical processes of flavin and the excited energy transfer to cytochrome c can be represented as follows:

$$F \xrightarrow{h\nu_1} {}^1F_{1\pi,\pi^*} \text{ (excitation at } \sim 450 \text{ nm)}, \quad (1)$$

$$F \xrightarrow{h\nu_2} {}^1F_{2\pi,\pi^*} \text{ (excitation at } \sim 375 \text{ nm)}, \quad (2)$$

$${}^1F_{2\pi,\pi^*} \longrightarrow {}^1F_{1\pi,\pi^*} + \text{heat} \longrightarrow F + \text{heat (internal conversion)}, \quad (3)$$

$${}^1F_{1\pi,\pi^*} \longrightarrow F + h\nu_f \text{ (fluorescence)}, \quad (4)$$

$${}^1F_{1\pi,\pi^*} + C \longrightarrow F + C \text{ (thermal collision)}, \quad (5)$$

$${}^1F_{1\pi,\pi^*} + C \longrightarrow F + C^* \text{ (resonance-energy transfer)}, \quad (6)$$

$${}^1F + DH \longrightarrow ({}^1F \cdots HD)^* \text{ (formation of CT complex)}, \quad (7)$$

$$({}^1F \cdots HD)^* \longrightarrow FH\cdot + D\cdot \text{ (electron transfer)}, \quad (8)$$

$${}^1F_{2\pi,\pi^*} + C \longrightarrow F + C^* \text{ (prerelaxational energy transfer)}, \quad (9)$$

$${}^1F \longrightarrow {}^3F \text{ (intersystem crossing)}, \quad (10)$$

$${}^3F \longrightarrow F + h\nu_p \text{ (phosphorescence)}, \quad (11)$$

$${}^3F \longrightarrow F + \text{heat (nonradiative transition)}, \quad (12)$$

$${}^3F + DH \longrightarrow FH\cdot + D\cdot \text{ (electron transfer)}, \quad (13)$$

$$F + C \underset{}{\overset{K_g}{\rightleftharpoons}} (F \cdots C) \text{ (complex formation)}, \tag{14}$$

where F represents flavin, C cytochrome c, and DH the electron donor. Here, we add new processes (6, 7, 8, and 9) to the primary processes presented by Moore et al. [10]. Step (6) is a resonance process based on a long-range dipole-dipole interaction [11]. Steps (7) and (8) represent a photoreduction of flavin by way of the $^1(\pi,\pi^*)$ state [9]. Step (9) is a "fast" energy transfer taking place before the relaxation of vibrational energy had taken place [13].

According to the photophysical processes of flavin as described above, the following equation can be derived for the cytochrome c quenching of the fluorescence of flavin:

$$I_0/I = 1 + [k_t\tau_0 + (\epsilon_C/\epsilon_F)K_g + k_r + k_{ft}]C + [k_r' + k_{ft}' + k_t\tau_0(\epsilon_C/\epsilon_F)K_g]C^2, \tag{15}$$

where I_0 and τ_0 represent the fluorescence intensity and lifetime in the absence of cytochrome c as a quencher. I represents the fluorescence intensity in the presence of cytochrome c, k_t is the rate constant of the collisional process, K_g is the equilibrium constant for the complex formation, $\epsilon_C(\epsilon_F)$ is the extinction coefficient of the complex (excited molecule), and C represents the concentration of cytochrome c. k_r and k_r' represent constants due to resonance energy transfer and can be formulated by using the critical concentration (C_0) and the critical transfer distance (R_0) as follows:

$$k_r = \pi^{1/2}/C_0, \tag{16}$$

$$k_r' = (\pi - 2)/C_0^2, \tag{17}$$

$$C_0 = (7.35/R_0)^3. \tag{18}$$

k_{ft} and k_{ft}' are the constants due to prerelaxational energy transfer.

RESULTS AND DISCUSSION

Quenching of Fluorescence of FMN by Cytochrome c

The plots of $(I_0/I - 1)/C$ vs C for the FMN-cyt c solution in Fig. 1 show that the quenching is noticeably affected by the exciting wavelength. This dependence is assumed to be caused by the prerelaxational fast energy transfer for excitation at 372 nm. The quenching is increased a little by lowering

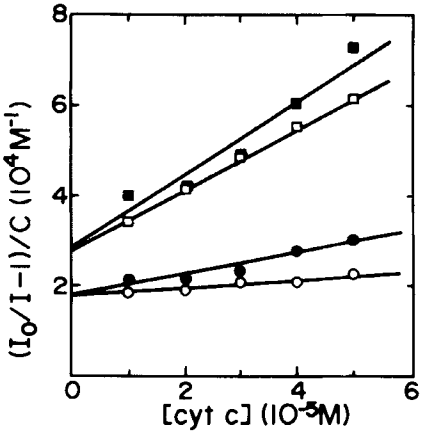

FIG. 1. Quenching of fluorescence of FMN by cytochrome c. (○): In 100 m*M* phosphate buffer with excitation at 465 nm; (●): in 1 m*M* phosphate buffer with excitation at 465 nm; (□): in 100 m*M* phosphate buffer with excitation at 372 nm; (■): in 1 m*M* phosphate buffer with excitation at 372 nm. Concentration of flavodoxin, 10 μM. Concentration of FMN, 10 μM.

the ionic strength. This result shows that the electrostatic force between FMN and cytochrome c is involved in the process of the energy transfer. It is known that the fluorescence lifetimes (τ_0) of flavins are shorter than 10 ns [8, 9] and that the rate constant of the collisional process (k_t) for the reduction of cytochrome c by FMN semiquinone in 100 m*M* phosphate buffer (pH 7.0) is about $7 \times 10^7\ M^{-1} \cdot s^{-1}$ [1]. By using these values, we can estimate $k_t\tau_0$ to be smaller than $1\ M^{-1}$. Comparison of this estimated value of $k_t\tau_0$ with the intercepts of the plots in Fig. 1 leads to the conclusion that the effect of the collisional process is too small to contribute to the quenching of the fluorescence of flavin.

On the assumption that the quenching is mainly controlled by the complex formation, the equilibrium constant (K_g) is estimated to be $1.8 \times 10^4\ M^{-1}$ for excitation at 465 nm in 100 m*M* phosphate buffer, so that $k_t\tau_0(\epsilon_C/\epsilon_F)K_g$ is estimated to be about $1.8 \times 10^4\ M^{-2}$, which is much smaller than the slope of the plot ($1.8 \times 10^8\ M^{-2}$). Accordingly, we see that the complex-formation process, if it occurs, is not a principal process for the quenching of the fluorescence of FMN by cytochrome c. In case the quenching is mainly controlled by the resonance process, the critical transfer distance, R_0, for the relaxation from the lowest singlet excited state is estimated from the slopes of the plots to be about 160 Å (100 m*M* phosphate buffer) and 180 Å (1 m*M* buffer).

Although it is known that the critical transfer distance ranges over a long distance, such as 50-100 Å, the values obtained are considered to be too long for the resonance process. From the above analysis we conclude that the quenching of the fluorescence of FMN from the lowest singlet excited state could be mainly controlled by the resonance process and that the heterogenous dispersion state of molecules due to the electrostatic forces could enhance the resonance-energy transfer.

Quenching of Fluorescence of Lumiflavin by Cytochrome c

The fluorescence quenching of FMN and lumiflavin by cytochrome c is compared in Fig. 2. The cytochrome c quenching of the fluorescence of FMN is found to be greater than that of lumiflavin. Lumiflavin is neutral whereas FMN has a negative charge, and hence this result supports the existence of the heterogeneous dispersion state in the FMN-cytochrome c solution due to electrostatic forces.

Quenching of Fluorescence of Flavodoxin by Cytochrome c

Plots of $I_0/I - 1$ vs C for the flavodoxin-cyt c solution are shown in Fig. 3. The dependence of the quenching on the exciting wavelength was also observed in this case. It was also found that the quenching is markedly con-

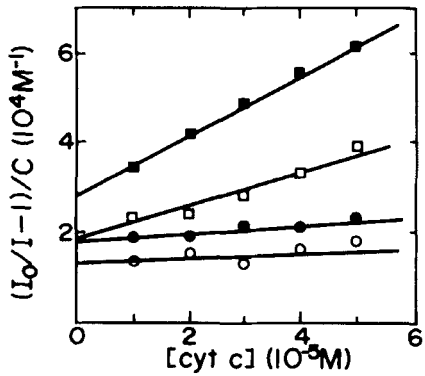

FIG. 2. Comparison of fluorescence quenching by cytochrome c between FMN and lumiflavin. (○): Lumiflavin excited at 465 nm; (●): FMN excited at 465 nm; (□): lumiflavin excited at 372 nm; (■): FMN excited at 372 nm. Concentration of flavin, 10 μM in 10 mM phosphate buffer.

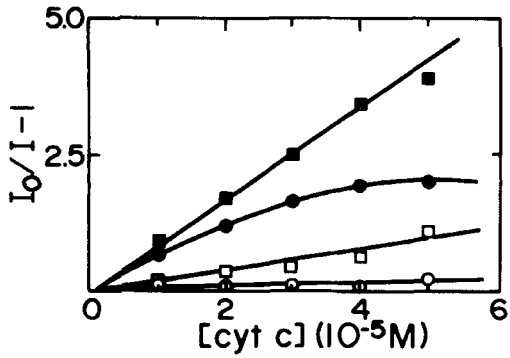

FIG. 3. Quenching of fluorescence of flavodoxin by cytochrome c. (○): In 100 m*M* phosphate buffer with excitation at 465 nm; (●): in 1 m*M* phosphate buffer with excitation at 465 nm; (□): in 100 m*M* phosphate buffer with excitation at 372 nm; (■): in 1 m*M* phosphate buffer with excitation at 372 nm. Concentration of flavodoxin, 10 μM.

trolled by ionic strength. Hence, we see that the quenching is mainly caused by the complex formation due to the electrostatic forces. That the plot of $I_0/I - 1$ vs C gives the straight line shows that the first-order constant for the concentration of the quencher is much greater than the second-order constant in Eq. (15). Accordingly, it is also found that the contribution of the complex formation for the quenching is much greater than that of the resonance or the prerelaxational process. That the plot for the quenching excited at 465 nm in 1 m*M* phosphate buffer deviates a straight line, which we cannot elucidate, is possibly due to the dynamic process in the complex state. From the slopes of the plots for the quenching of the fluorescence from the lowest singlet excited state, the equilibrium constants, K_g, are estimated to be about $4 \times 10^3\ M^{-1}$ (100 m*M* phosphate buffer) and about $7 \times 10^4\ M^{-1}$ (1 m*M* buffer). Simondsen et al. reported that K_g for 7,8-dichloro-FMN flavodoxin semiquinone oxidation by cytochrome c are $7.9 \times 10^4\ M^{-1}$ (100 m*M* NaCl) and $3.5 \times 10^5\ M^{-1}$ (20 m*M* NaCl) [4]. The difference of K_g values between our results and the results obtained by Simondsen et al is ascribed to the redox state of flavodoxin and partly to the origin of flavodoxin. The reduced form of flavodoxin is considered to be more attractive to cytochrome c than the oxidized form, which is reasonable from the view of the intermolecular electrostatic forces.

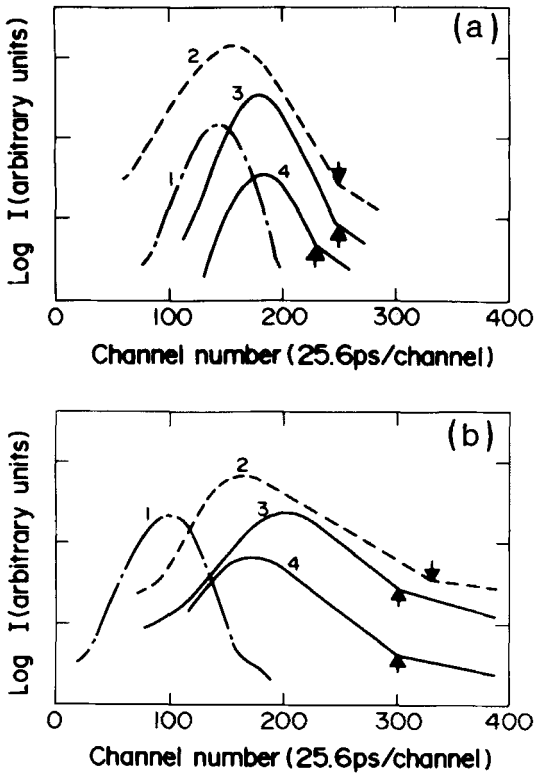

FIG. 4. Fluorescence decay curves of FMN (a) and flavodoxin (b). 1: Time course of an exciting laser pulse. 2: Flavin in 100 m*M* phosphate buffer. 3: Flavin (10 μM)-cytochrome c (40 μM) in 100 m*M* phosphate buffer. 4: Flavin (10 μM)-cytochrome c (40 μM) in 1 m*M* phosphate buffer.

Dynamic Quenching of Fluorescence of Flavins

As described above, it was found that the resonance process and the process of the complex formation are chiefly involved in the fluorescence quenching of flavins. Since the resonance process is a dynamic process as the quenching mechanism of fluorescence, whereas the process of complex formation does not contribute to the dynamic quenching of fluorescence, these two processes can be separated by observing the fluorescence decay curves. Hence, the dynamic quenching of the fluorescence of flavins was measured by a time-correlated single-photon counting method. Figure 4 shows the fluorescence decay

TABLE 1. Fluorescence Lifetimes of Flavin

Concentration of cytochrome c, μM	FMN, τ, ns[a]		Flavodoxin, τ, ns	
	1 mM PO_4	100 mM PO_4	1 mM PO_4	100 mM PO_4
0	2.7	2.7	5.7	5.7
40[b]	2.4 (2.3)	2.4 (2.7)	4.3 (4.8)	4.3 (4.8)

[a]The lifetimes were obtained from the slopes of the decay curves.
[b]Values in parentheses were obtained by the time difference between the peak of the exciting pulse and the bending points of the decay curves.

curves of flavins, and fluorescence lifetimes are summarized in Table 1. According to the theory of dynamic quenching [13-15], Eq. (15) can be transformed as follows:

$$\tau_0/\tau = 1 + (k_t C + k_R + k_{FT})\cdot\tau_0, \tag{19}$$

where τ represents the fluorescence life time in the presence of the quencher, k_R represents the constant due to the resonance process, and k_{FT} represents the constant due to the prerelaxational energy transfer. Since the value of $k_t C\tau_0$ is negligibly small, just as the static quenching, the collisional process does not contribute to the dynamic quenching. The wavelength of the exciting laser pulse is 488 nm, so that fluorescence decay curves reflect the relaxation of the lowest singlet excited state, and hence the prerelaxational process does not contribute to the dynamic quenching. Accordingly, only the resonance process leads to the dynamic quenching. Since the dynamic quenching is observed in every case, as shown in Fig. 4 and Table 1, it is confirmed that the resonance process exists in the energy transfer between flavin and cytochrome c. From analysis of the lifetimes shown in Table 1, R_0 for the FMN-cytochrome c solution is estimated to be about 150 Å, which is almost coincident with the value obtained by the static method. For the flavodoxin-cytochrome c solution, the dynamic quenching is found to be independent of ionic strength. This is an interesting contrast to the result that the quenching by the static method due to complex formation is markedly dependent on ionic strength.

ACKNOWLEDGMENT

A part of this work was performed under the management of the R & D Association for Future Electron Devices as a part of the R & D Project of

Basic Technology for Future Industries, sponsored by Agency of Industrial Science and Technology, MITI. The authors are grateful to Dr. S. Nara for helpful discussions.

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