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# Nanosecond Pulse Fluorometry of Conformational Change in Phenylalanine Hydroxylase Associated with Activation

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ABSTRACT: Conformational change in rat liver phenylalanine hydroxylase associated with activation by phenylalanine or *N*-(1-anilinonaphth-4-yl)maleimide was investigated by measuring fluorescence spectra and fluorescence lifetimes of tryptophanyl residues as well as the probe fluorophore conjugated with SH groups of the hydroxylase. The fluorescence spectrum of tryptophan exhibited its maximum at 342 nm. It shifted by 8 nm toward longer wavelength accompanied by an increase in its intensity, by preincubation with 1 mM phenylalanine. The fluorescence intensity of tryptophan increased by 36% upon the activation. On the other hand, the binding of (6*R*)-L-*erythro*-tetrahydrobiopterin, a natural cofactor of the enzyme, induced a decrease in the fluorescence intensity by 79% without a shift of the maximum wavelength. The fluorescence lifetime of tryptophan of phenylalanine hydroxylase exhibited two components with lifetimes of 1.7 and 4.1 ns. The values of the lifetimes changed to 1.4 and 5.6 ns, respectively, upon the activation. It is considered that the change in the longer lifetime is correlated with the shift of the emission peak upon the activation. The values of both the lifetimes decreased to 0.64 and 3.6 ns upon the binding of (6*R*)-L-*erythro*-tetrahydrobiopterin, which is coincident with the decrease in the fluorescence intensity. Conjugation of *N*-(1-anilinonaphth-4-yl)maleimide with SH of phenylalanine hydroxylase brought about a decrease in both the fluorescence intensity and the value of the shorter lifetime of the tryptophanyl residues, while the longer lifetime remained unchanged. These changes could be ascribed to excitation energy transfer from tryptophan with the shorter lifetime to the anilinonaphthyl group. When *N*-(1-anilinonaphth-4-yl)maleimide-conjugated phenylalanine hydroxylase was incubated with 1 mM phenylalanine, the energy-transfer efficiency decreased. The distances between both the tryptophan residues and the probe molecule are considered to be not very short compared to the critical transfer distance (1.7-1.8 nm). The fluorescence lifetime of *N*-(1-anilinonaphth-4-yl)maleimide exhibited a single component of 4.6 ns. This suggests that the surroundings of the anilinonaphthyl group are homogeneous in phenylalanine hydroxylase. It became heterogeneous upon activation by preincubation with 1 mM phenylalanine or binding of (6*R*)-L-*erythro*-tetrahydrobiopterin, so that a hydrophobic environment appeared around the fluorophore.

Rat liver phenylalanine hydroxylase [L-phenylalanine,tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1] catalyzes the conversion of phenylalanine to tyrosine, an obligatory step in the degrdn. of phenylalanine in mammals, with tetrahydropterin as a cofactor (Milstien & Kaufman, 1975). This monooxygenase is an allosteric enzyme (Shiman & Gray, 1980). Since an absence of this enzyme in humans causes phenylketonuria, the regulatory mechanism of its catalytic activity has been of interest both enzymatically and clinically. In the presence of a natural cofactor, (6*R*)-L-*erythro*-tetrahydrobiopterin ($BH_4$)[1] (Kaufman, 1963; Matsuura et al., 1980), the enzyme is in a low-activity state (Kaufman, 1970; Hasegawa & Kaufman, 1982). The rela-

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[1] Abbreviations: ANM, *N*-(1-anilinonaphth-4-yl)maleimide; $BH_4$, (6*R*)-L-*erythro*-tetrahydrobiopterin; $6MPH_4$, 6(*RS*)-methyl-5,6,7,8-tetrahydropterin; SDS, sodium dodecyl sulfate.

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tively low activity of phenylalanine hydroxylase can be selectively stimulated to an extent of 20–30-fold by a variety of different procedures such as limited proteolysis, additions of lysolecithin and related compounds to the enzyme (Abita et al., 1984), alkylation of SH groups with *N*-ethylmaleimide (Parniak & Kaufman, 1981), and also preincubations with phenylalanine and some other aromatic amino acids (Phillips et al., 1984a).

Rat liver phenylalanine hydroxylase is a tetramer and contains four tryptophanyl residues per subunit (Shiman, 1980). Phillips et al. (1984b) have investigated a conformational change of phenylalanine hydroxylase associated with the activation by means of fluorescence emission and UV difference spectroscopy. They observed a remarkable shift of tryptophan emission upon incubation of the enzyme with phenylalanine and concluded that buried tryptophanyl residues become fully exposed upon activation. In the present work we have further examined the drastic conformational change of rat liver phenylalanine hydroxylase by means of a steady-state and nanosecond pulse fluorometry of tryptophanyl emission and also of *N*-(1-anilinonaphth-4-yl)maleimide (ANM) conjugated with the hydroxylase. ANM is a probe fluorophore which is conjugated with SH groups of the hydroxylase and is expected to activate the enzyme.

## Experimental Procedures

*Materials.* Phenylalanine hydroxylase was purified from rat liver by the method reported (Shiman et al., 1979; Koizumi et al., 1984). The purity was confirmed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). The preparation gave a single band with a molecular weight of 50 000. $BH_4$ was kindly synthesized by Dr. Sadao Matsuura of Nagoya University. ANM was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used without further purification.

*Assay of Phenylalanine Hydroxylase.* The standard reaction mixture (250 μL) contained 0.2 mM L-phenylalanine, 40 μM $BH_4$, 2 mM dithiothreithol, 60 μg of catalase, and 5 μg of intact or modified phenylalanine hydroxylase, in 0.1 M potassium phosphate buffer, pH 6.8. Concentrations of the protein were evaluated by measuring absorbance at 277 nm [$E_{1\%}$ used was 11.1, according to Iwaki et al. (1985)]. The reaction was started by the addition of enzyme into the standard reaction mixture at 25 °C and terminated after incubation for 2 min by the addition of 50 μL of 30% trichloroacetic acid. The amount of tyrosine produced was assayed by high-performance liquid chromatography (Shimadzu LC-3A) with fluorometric detection (Bailey & Ayling, 1980). A reversed-phase Unisil F3-50A column (4.6 i.d. × 50 mm, Gasukuro Kogyo Inc., Tokyo, Japan) and 0.1 M ammonium acetate buffer, pH 4.5, were used for the separation of tyrosine. The amount of tyrosine was calculated from the native fluorescence at 300 nm after excitation at 282 nm with a Shimadzu RF-500L spectrofluorophotometer.

*Modification of Phenylalanine Hydroxylase with ANM.* The reaction mixture for the modification (500 μL) contained 10 μM purified phenylalanine hydroxylase and 100 μM ANM in 0.1 M potassium phosphate buffer, pH 6.8. After incubation for 2 h at 4 °C the reaction was terminated by removal of ANM by use of a small column of Sephadex G-25. ANM combined with 0.7–0.8 mol per mole of subunit ($M_r$ 50 000). The reaction rate was much faster than those of the other reagents for SH modification (Parniak & Kaufman, 1981; Koizumi et al., 1987). When the incubation was performed at 25 °C, the activity of the modified enzyme was rather low. Intact enzyme for fluorescence measurements was obtained by the same treatment without ANM.

*Binding of L-Phenylalanine to Phenylalanine Hydroxylase.* Binding of L-phenylalanine was conducted by incubation of 1 mM L-phenylalanine with the enzyme at 25 °C for 30 min, just before fluorescence measurements, according to the method of Shiman and Gray (1980).

*Steady-State Fluorescence Measurements.* Fluorescence spectra of tryptophan and ANM were measured with a corrected spectrofluorometer (Shimadzu RF-502). Slit widths used for the measurements were 5 nm for excitation and 3 nm for emission. Temperature was controlled by circulating water of constant temperature at 25 °C. The relative quantum yield of tryptophanyl fluorescence of phenylalanine hydroxylase was determined by comparing the integrated intensity of the corrected spectrum with that of free tryptophan. The relative quantum yield of fluorescence of ANM conjugated with phenylalanine hydroxylase was determined in comparison with the integrated intensity of a standard solution (quinine bisulfate in 0.1 M $H_2SO_4$) (Tanaka et al., 1986). Molar concentrations of native and ANM-conjugated phenylalanine hydroxylases were expressed in terms of the monomer ($M_r$ 50 000).

*Measurements of Fluorescence Decay Curves.* Fluorescence decay curves were measured by an Ortec PRA single-photon-counting apparatus using a hydrogen gas charged light pulser (PRA 510, typical half-width 1.5 ns). The exciting light (excitation wavelength 285 nm for tryptophan, 350 nm for ANM) was monochromated by a PRA 1200 monochrometer, and the emitted light was passed through Toshiba cutoff filters, UVD-35 for tryptophan and UV-39 for ANM, and was collected with a photomultiplier (Hamamatsu Photonics, R212UH) (Kano et al., 1984).

*Analysis of Fluorescence Decay Curve.* The fluorescence decay curves were analyzed by assuming a multiexponential decay function. The lifetime was obtained from the decay curve through a deconvolution procedure by the method of moments (Isenberg et al., 1973; Isenberg, 1973). A decay curve observed, $I_0(t)$, is related to a decay function of fluorescence, $F(t)$, and exciting pulse, $E(t)$, according to the equation

$$I_0(t) = \int_0^t E(t)\, F(t - t')\, dt' \qquad (1)$$

where $F(t)$ is a single- or two-exponential function. We have also attempted to analyze observed decay curves with a three-exponential decay function as in the case of previous works (Kido et al., 1980; Kaneda et al., 1983, 1985). However, it was found that a two-exponential decay function was adequate for the present analyses. Because decay parameters were dependent on an index, λ, introduced as an exponential depression by Isenberg et al. (1973) for improvement of convergency in calculating time moments, they were determined so as to obtain the minimum value of a $\chi^2(\lambda)$ distribution (Kido et al., 1980; Kaneda et al., 1983, 1985; Tanaka et al., 1986)

$$\chi^2(\lambda) = \frac{1}{n}\sum_{i=1}^{n}[I_0(t_i) - I_c(t_i)]^2/I_0(t_i) \qquad (2)$$

where $I_c(t_i)$ represents the intensity at time $t_i$ of a calculated decay curve, which was obtained by a convolution of the decay function determined by the method of moments with the observed time profile of the pulsed lamp.

## Results

*Enzyme Activity of Phenylalanine Hydroxylase.* Specific activity of native phenylalanine hydroxylase was 0.18 μmol/(min·mg of protein). When the native enzyme was

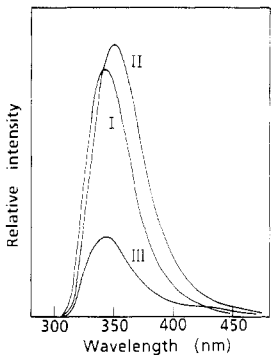


FIGURE 1: Fluorescence spectra of tryptophanyl residues of phenylalanine hydroxylase. Corrected fluorescence spectra were obtained at 25 °C by exciting at 290 nm. Concentration of the enzyme was 1.2 μM in 0.1 M phosphate buffer, pH 6.8. (I) Native phenylalanine hydroxylase; (II) native phenylalanine hydroxylase incubated with 1 mM phenylalanine for 30 min at 25 °C; (III) native phenylalanine hydroxylase + 4 μM $BH_4$.

incubated with 1 mM phenylalanine, the activity increased to 3.4 μmol/(min·mg of protein). Phenylalanine hydroxylase contains a single SH per subunit to which *N*-alkylmaleimide binds, and consequently the enzyme is activated 20–30-fold (Parniak & Kaufman, 1981). ANM is considered to bind to this SH. Upon conjugation with 0.7–0.8 mol of ANM per mole of subunit, ANM-conjugated phenylalanine hydroxylase exhibited a specific activity of 0.33 μmol/(min·mg of protein). Thus, the activity increased only about 2-fold, and the activation by ANM conjugation was relatively low compared with the case of *N*-alkylmaleimide. This low activation is considered to be due to quite different structures of ANM from those of the other SH reagents; ANM combines a large aromatic ring while the other SH reagents contain only aliphatic chains (Patniak & Kaufman, 1981; Koizumi et al., 1987). When 1 mM phenylalanine was added to ANM-conjugated phenylalanine hydroxylase, the specific activity increased to 3.4 μmol/(min·mg of protein). Thus, the activity was further elevated about 10-fold.

*Fluorescence Spectra of Tryptophanyl Residues of Phenylalanine Hydroxylase.* Corrected fluorescence spectra of tryptophanyl residues of phenylalanine hydroxylase are shown in Figure 1. The emission maximum of phenylalanine hydroxylase was at 342 nm, the wavelength of which is considerably longer than the one reported by Phillips et al. (1984b) and Marota and Shiman (1984). When 1 mM phenylalanine was added to the native enzyme, the maximum wavelength shifted toward longer wavelength by 8 nm, accompanied by an increase in the fluorescence intensity. On the other hand, the fluorescence intensity decreased by 79% without any shift of the maximum wavelength, upon addition of 4 μM $BH_4$ (Table I). The features of the spectrum are in accord with one of the previous works (Phillips et al., 1984b). Excitation spectra of tryptophan monitored at various emission wavelengths were also examined (Figure 2). The wavelengths of the excitation spectra at the maximum intensity were 280–283 nm, which are longer than the wavelength of the maximum absorption, 278 nm. The wavelength of the maximum absorption is probably due to tyrosinyl residues, since the enzyme contains 20 tyrosinyl residues per subunit. These facts indicate that the emission band comes from tryptophanyl residues rather than tyrosine. The intensity ratio of the excitation spectrum at 280 nm to that at 300 nm varied and decreased as the emission wavelength monitored was changed from a shorter to a longer one (Figure 3), which suggests that some of the tryptophans are in different environments. The values of the intensity ratio decreased when phenylalanine hydroxylase is activated by preincubation with 1 mM phenylalanine, which implies that the absorption band with longer emission peak also shifted to longer wavelength upon activation.

Table I: Relative Fluorescence Quantum Yields[a]

| systems | Trp fluorescence ex[b] (nm) | Trp fluorescence em peak[c] (nm) | Trp fluorescence rel yield[d] | ANM fluorescence ex[b] (nm) | ANM fluorescence em peak[c] (nm) | ANM fluorescence rel yield[e] |
|---|---|---|---|---|---|---|
| PAH[f] | 290 | 342 | 1.00 | | | |
| PAH + 0.1 mM Phe | 290 | 348 | 1.29 | | | |
| PAH + 1 mM Phe | 290 | 350 | 1.36 | | | |
| PAH + 4 μM $BH_4$ | 290 | 342 | 0.35 | | | |
| PAH + 40 μM $BH_4$ | 290 | 342 | 0.21 | | | |
| ANM-PAH[g] | 290 | 342 | 0.31 | 290 | 430 | 0.34 |
| ANM-PAH + 1 mM Phe | 290 | 350 | 0.34 | 290 | 431 | 0.33 |
| | | | | 330 | 433 | 0.97 |
| ANM-PAH + 4 μM $BH_4$ | 290 | 342 | 0.18 | 290 | 433 | 0.31 |
| | | | | 330 | 433 | 0.98 |

[a] Relative quantum yield of phenylalanine hydroxylase to free L-tryptophan was 0.14 in 0.1 M phosphate buffer, pH 6.8. Temperature was 25 °C. [b] Excitation wavelength. [c] Wavelength of emission peak. [d] Quantum yields relative to fluorescence of tryptophan of native phenylalanine hydroxylase. [e] Quantum yields relative to fluorescence of ANM of ANM-conjugated phenylalanine hydroxylase. [f] PAH denotes phenylalanine hydroxylase. [g] ANM-PAH denotes ANM-conjugated phenylalanine hydroxylase.

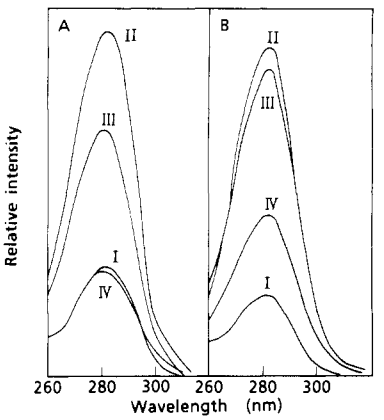


FIGURE 2: Fluorescence excitation spectra of tryptophanyl residues of phenylalanine hydroxylase. Corrected excitation spectra were measured by changing the emission wavelength monitored. (A) Phenylalanine hydroxylase in the absence of phenylalanine; (B) phenylalanine hydroxylase with 1 mM phenylalanine added. Emission wavelength monitored was (I) 320, (II) 340, (III) 360, or (IV) 380 nm. Temperature was 25 °C in 0.1 M phosphate buffer, pH 6.8.

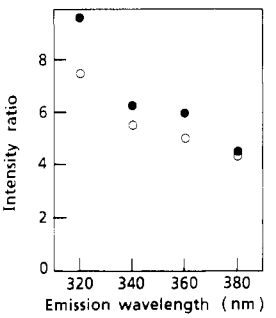


FIGURE 3: Intensity ratios of excitation spectra at 280 nm to those at 300 nm. Intensity ratios of excitation spectra at 280 nm of excitation wavelength to those at 300 nm were obtained from Figure 2. Closed circles indicate the values of the ratio of phenylalanine hydroxylase in the absence of phenylalanine. Open circles indicate those in the presence of 1 mM phenylalanine.

*Fluorescence Spectra of ANM-Conjugated Phenylalanine Hydroxylase.* Fluorescence spectra of ANM-conjugated phenylalanine hydroxylase upon exciting at 290 nm are shown

Table II: Fluorescence Lifetimes

| systems | Trp fluorescence[a] | | | | ANM fluorescence[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | $\tau_1$ (ns) | $\alpha 1$ | $\tau_2$ (ns) | $\alpha 2$ | $\tau_1$ (ns) | $\alpha 1$ | $\tau_2$ (ns) | $\alpha 2$ |
| PAH[c] | 1.7 | 0.73 | 4.1 | 0.27 | | | | |
| PAH + 1 mM Phe | 1.4 | 0.75 | 5.6 | 0.25 | | | | |
| PAH + 10 $\mu$M $BH_4$ | 0.64 | 0.88 | 3.6 | 0.12 | | | | |
| ANM-PAH[d] | 1.2 | 0.80 | 4.1 | 0.20 | 4.6 | 1.00 | | |
| ANM-PAH + 1 mM Phe | 1.5 | 0.71 | 4.8 | 0.30 | 4.1 | 0.79 | 8.7 | 0.22 |
| ANM-PAH + 10 $\mu$M $BH_4$ | 0.88 | 0.94 | 4.6 | 0.06 | 3.1 | 0.65 | 7.9 | 0.35 |

[a] Excitation wavelength was 285 nm, and emission was monitored by a band-pass filter. [b] Excitation wavelength was 350 nm, and emission was monitored by a band-pass filter. [c] PAH denotes phenylalanine hydroxylase. [d] ANM-PAH denotes ANM-conjugated phenylalanine hydroxylase.

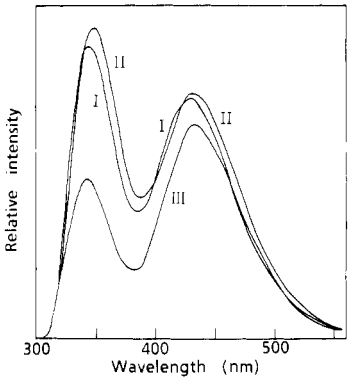


FIGURE 4: Fluorescence emission spectra of ANM-conjugated phenylalanine hydroxylase. ANM was conjugated with the SH group of the hydroxylase. Excitation wavelength was 290 nm. Emission bands around 350 nm were from tryptophan, and those around 430 nm were from ANM. (I) ANM-conjugated phenylalanine hydroxylase in the absence of ligands; (II) ANM-conjugated phenylalanine hydroxylase with 1 mM phenylalanine added; (III) ANM-conjugated phenylalanine hydroxylase with 4 $\mu$M $BH_4$ added. Temperature was 25 °C in 0.1 M phosphate buffer, pH 6.8.

in Figure 4. The emission bands at about 350 nm are from tryptophan, and those at about 430 nm are from ANM. The emission maximum of tryptophanyl residues of ANM-conjugated phenylalanine hydroxylase was 342 nm and was coincident with that of the native enzyme. However, relative quantum yield of tryptophan decreased by 69% upon the modification, which should be due to excitation energy transfer from tryptophan to ANM in the protein (see Table I). The maximum wavelength also shifted toward red by 8 nm upon activation, accompanied by a slight increase in the relative quantum yield (see Table I). Addition of 4 $\mu$M $BH_4$ induced the decrease in the relative quantum yield by about 40%. The extent of the change in the relative quantum yield upon addition of phenylalanine or $BH_4$ was less in the case of ANM-conjugated phenylalanine hydroxylase than in the case of native enzyme. The reason may be complicated because the energy-transfer phenomenon involves in the quenching of the tryptophan fluorescence. Fluorescence excitation spectra of ANM are shown in Figure 5. The intensity of ANM emission increased in the absence or presence of phenylalanine (curves I and II in Figure 5) when the absorption band of tryptophan (280–290 nm) was excited, where ANM-conjugated acetylcystine in ethanol solution does not have any absorbance (Dr. Okamoto of Juntendo University, personal communication; Okamoto & Sekine, 1980). It decreased when $BH_4$ was added to ANM-conjugated phenylalanine hydroxylase. These observations suggest that the energy-transfer phenomenon from tryptophan to ANM is suppressed when $BH_4$ binds to ANM-conjugated phenylalanine hydroxylase.

Fluorescence spectra of ANM in ANM-conjugated phenylalanine hydroxylase upon exciting ANM directly at 330 nm are shown in Figure 6. The spectra were little affected upon activation by phenylalanine or binding of $BH_4$.

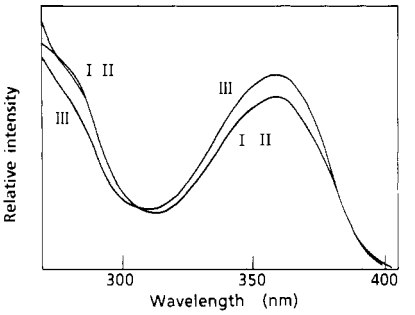


FIGURE 5: Fluorescence excitation spectra of ANM-conjugated phenylalanine hydroxylase. The fluorescence excitation spectra were measured by monitoring ANM emission (440 nm). (I) ANM-conjugated phenylalanine hydroxylase; (II) ANM-conjugated phenylalanine hydroxylase with 1 mM phenylalanine hydroxylase added; (III) phenylalanine hydroxylase with 4 $\mu$M $BH_4$ added. Temperature was 25 °C in 0.1 M phosphate buffer, pH 6.8.

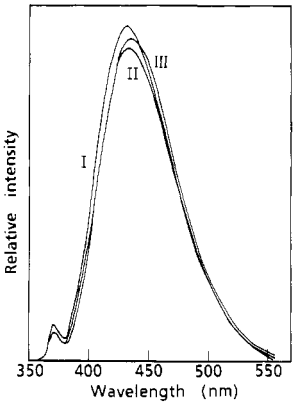


FIGURE 6: Fluorescence spectra of ANM of ANM-conjugated phenylalanine hydroxylase. Fluorescence spectra of ANM of ANM-conjugated phenylalanine hydroxylase was obtained by exciting the ANM fluorophore directly at 350 nm. (I) ANM–phenylalanine hydroxylase in the absence of ligands; (II) ANM-conjugated phenylalanine hydroxylase with 1 mM phenylalanine added; (III) ANM-conjugated phenylalanine hydroxylase with 4 $\mu$M $BH_4$ added. Temperature was 25 °C in 0.1 M phosphate buffer, pH 6.8.

*Fluorescence Lifetimes of Tryptophan and ANM.* The fluorescence decay curve of tryptophan in the native enzyme is shown in Figure 7. Observed decay curves of tryptophan and ANM were analyzed with single-, two-, and three-exponential decay functions. It was found that the most of the decay curves werre able to be analyzed with two-exponential decay functions. Obtained lifetimes are listed in Table II. The lifetimes of tryptophan in phenylalanine hydroxylase were 1.7 and 4.1 ns. These changed to 1.4 and 5.6 ns upon activation by phenylalanine. It is noted that the longer lifetime appeared to become longer by 1.5 ns upon activation. This is reasonable because the fluorescence quantum yield increased, accompanied by the shift in the emission peak toward longer wavelength. The results support the interpretation presented above and also that given by Phillips et al. (1984b) that the sur-

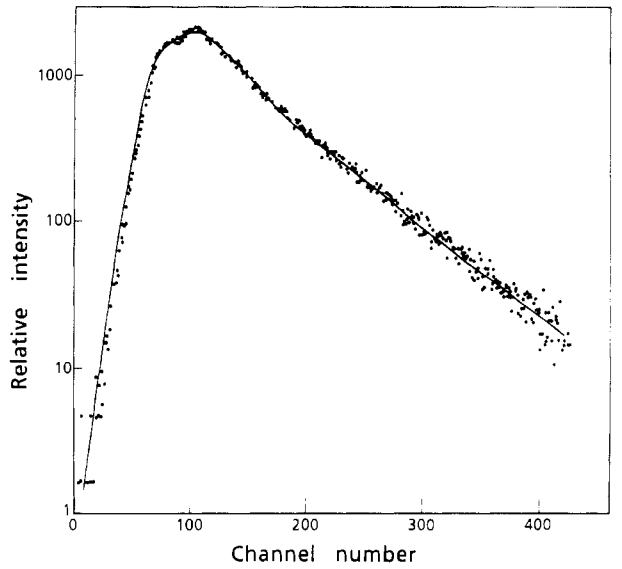


FIGURE 7: Fluorescence decay curve of tryptophan of phenylalanine hydroxylase. The fluorescence decay curve of tryptophan of phenylalanine hydroxylase was measured by exciting at 285 nm with a nanosecond pulsed lamp charged with hydrogen gas (typical half-width 1.5 ns). Observed fluorescence intensities are indicated with dots. Calculated intensities with a double-exponential decay function were obtained by the method of moments (Isenberg et al., 1973; Isenberg, 1973) and are shown by the solid curve. The time interval was 58.8 ps/channel. Obtained decay parameters are listed in Table II.

roundings of the tryptophanyl residue become more hydrophilic upon activation. However, only tryptophanyl residues with longer lifetime seemed to be placed in a more hydrophilic microenvironment. Both fluorescence lifetimes were drastically reduced when $BH_4$ was bound to the enzyme, which is coincident with the decrease in relative quantum yield.

Fluorescence lifetimes of tryptophan in ANM-conjugated phenylalanine hydroxylase were also obtained and are listed in Table II. Only the shorter lifetime became modified and further shortened by the binding of ANM. The change in the shorter lifetime should be due to energy transfer from tryptophan with shorter lifetime to ANM conjugated with an SH group of the enzyme, as observed in the steady-state fluorescence measurements. When ANM-conjugated phenylalanine hydroxylase was activated with phenylalanine, the longer lifetime of the two components again became longer, which was also coincident with the result of relative quantum yield. When we compared the values of lifetime of activated, ANM-conjugated phenylalanine hydroxylase with those of activated native enzyme, only the longer lifetime was shorter in the ANM-conjugated enzyme than in the native enzyme. The fact suggests that energy transfer takes place from tryptophan with the longer lifetime to ANM in the activated ANM-conjugated phenylalanine hydroxylase, which is in contrast with the case of ANM-conjugated enzyme before full activation by phenylalanine. When 10 $\mu$M $BH_4$ was added to ANM-conjugated enzyme, the shorter lifetime of tryptophan became shorter and the longer lifetime became longer. However, if we compare these values with the case of the native enzyme, both lifetimes were longer in ANM-conjugated enzyme than in the native enzyme, which indicates that no energy transfer takes place in the complex of ANM-conjugated phenylalanine hydroxylase and $BH_4$. These observations are in good agreement with those obtained by the measurements of the fluorescence excitation spectra of ANM-conjugated enzyme (see Figure 5, curves I and II).

Fluorescence of ANM conjugated with the enzyme exhibited a single lifetime component of 4.6 ns. This implies that the environment surrounding ANM is homogeneous. When ANM-conjugated hydroxylase was fully activated by the binding of phenylalanine, or when it formed a complex with $BH_4$, the lifetime of ANM became two components, 4.1 and 8.7 ns or 3.1 and 7.9 ns, respectively. These results suggest that the microenvironment around ANM becomes heterogeneous upon binding of phenylalanine or $BH_4$.

*Critical Energy-Transfer Distance and Apparent Distance between Tryptophan and ANM.* Probability of excitation energy transfer is expressed as (Forster, 1951; Stryer, 1978)

$$k_t = \frac{1}{\tau_D}\left(\frac{R_0}{R}\right)^6 \tag{3}$$

where $\tau_D$ is the fluorescence lifetime of a donor in the absence of an acceptor, $R$ is the distance between the donor and the acceptor, and $R_0$ is the critical transfer distance, where $k_t$ becomes equal to $\tau_D^{-1}$. $R_0$ can be obtained from experimental data as (Stryer, 1978)

$$R_0 \text{ (nm)} = (9.7 \times 10^2)(J_\lambda \kappa^2 Q_D n^{-4})^{1/6} \tag{4}$$

where $Q_D$ is the quantum yield of the donor in the absence of the acceptor, $n$ is the refractive index, $\kappa^2$ is the orientation factor between donor and acceptor molecules, and $J_\lambda$ is the spectral overlap integral. $J_\lambda$ (in units of $cm^3$ $M^{-1}$) may be evaluated from the corrected fluorescence spectrum of the donor and absorption spectrum of the acceptor as follows:

$$J_\lambda = \frac{\int F(\lambda)\, \epsilon(\lambda)\, \lambda^4\, d\lambda}{\int F(\lambda)\, d\lambda} \tag{5}$$

where $F(\lambda)$ is the spectrum distribution of the donor fluorescence in the absence of acceptor and $\epsilon(\lambda)$ is the extinction coefficient of the acceptor (in units of $cm^{-1}$ $M^{-1}$).

$\kappa^2$ depends on an individual system and varies from 0 to 4. When both the donor and acceptor rapidly rotate, it is $^2/_3$. We may define $R$ as an apparent distance between donor and acceptor, obtained from $R_0$ by assuming $\kappa^2 = {}^2/_3$. Although the value of $R$ does not always mean a correct distance between the donor and acceptor, it should be the most probable distance (Stryer, 1978). $R$ was evaluated from the energy-transfer efficiency $E_t$ according to

$$R = R_0[(1 - E_t)/E_t]^{1/6} \tag{6}$$

where $E_t$ is equal to $(1/\tau - 1/\tau_D)\tau$ and $\tau$ is the fluorescence lifetime of the donor in the presence of the acceptor.

From the results of spectral measurements and fluorescence lifetimes of tryptophan, $R_0$, $E_t$, and $R$ were calculated and are listed in Table III. It was assumed that energy transfer takes place between tryptophan with the shorter lifetime and ANM in the system of ANM-conjugated phenylalanine hydroxylase, and between tryptophan with the longer lifetime and ANM in the system of ANM-conjugated phenylalanine hydroxylase activated by phenylalanine, on the basis of the results of fluorescence lifetime of tryptophan. In both cases the values of $R$ should be close to $R_0$. It is interesting that $E_t$ between tryptophan with the shorter lifetime and ANM became negligibly small upon activation by phenylalanine, and instead that between tryptophan with the longer lifetime and ANM became dominant. This may be due to the decrease of $R_0$ between tryptophan with the longer lifetime and ANM by the change of $\kappa^2$, or due to the elongation of the distance between them, upon activation. In the case of ANM-conjugated phenylalanine hydroxylase and $BH_4$, the energy transfer was not observed. Disappearance of the energy-transfer phenomenon

Table III: Calculations of Critical Transfer Distance and Apparent Distance between Tryptophan and ANM[a]

| systems | $Q_D$[b] | $J_\lambda$[c] ($\times 10^{-14}$ cm$^3$ M$^{-1}$) | $R_0$ (nm) | $E_t$ | $R$ (nm) |
|---|---|---|---|---|---|
| ANM–PAH[d] | 0.0182 | 1.22 | 1.7 | 0.31 | 1.9 |
| ANM–PAH + 1 mM Phe[e] | 0.0248 | 1.30 | 1.8 | 0.16 | 2.4 |
| ANM–PAH + 10 $\mu$M $BH_4$ | 0.0038 | 1.19 | 1.3 | | |

[a] ANM–PAH denotes ANM-conjugated phenylalanine hydroxylase. The value of the refractive index of the solution used was 1.5, according to Lehrer (1969). [b] Quantum yields of tryptophan were obtained from Table I, and quantum yield of free tryptophan, 0.13, was obtained according to Tatischeff and Klein (1976). [c] Fluorescence spectra of tryptophan were obtained from Figure 1. [d] It is assumed that energy transfer takes place from tryptophan(s) with the shorter lifetime to ANM. [e] It is assumed that energy transfer takes place from tryptophan(s) with the longer lifetime to ANM.

in this system is considered to be due to a drastic decrease in $R_0$, which is caused by a remarkable quenching of tryptophan fluorescence by the binding of $BH_4$. It is reasonable not to see an energy-transfer phenomenon in the present system, since $E_t$ becomes less than 10% of those in the other cases, even though the real distance between tryptophan and ANM was not changed appreciably by the binding of $BH_4$.

## Discussion

The conformational change of phenylalanine hydroxylase upon activation by phenylalanine or upon the binding of $BH_4$ was studied by observing fluorescence of tryptophan as well as ANM conjugated with the enzyme by means of steady-state and nanosecond pulse fluorometry. The results obtained by steady-state measurements were essentially coincident with those by Phillips et al. (1984b). Measurements of fluorescence excitation spectra revealed that at least two kinds of tryptophan residues exists, one residue in a relatively hydrophobic microenvironment and the other residue in the more hydrophilic microenvironment, which are also in accord with the results of UV difference spectra (Phillips et al., 1984b). The fluorescence lifetime measurements of tryptophan residues exhibited two lifetime components, even in the absence of any ligands. Normally, the emission peak of free tryptophan shifts toward longer wavelength than that of tryptophanyl residues in proteins. It has been demonstrated with lysozyme derivatives that the tryptophan lifetime appeared to increase with exposure of tryptophan to water (Formoso & Forster, 1975). Therefore, the tryptophanyl residue placed near the surface of the enzyme could become exposed to water molecules upon activation by phenylalanine.

There are still several disagreements between the previous reports (Phillips et al., 1984b; Marota & Shiman, 1984) and the present work about the shape of tryptophan fluorescence of phenylalanine hydroxylase. Phillips et al. (1984b) and Marota and Shiman (1984) showed the wavelength of the maximum peak to be from 310 to 320 nm, while it was 342 nm in the present work. The spectrum reported by Phillips et al. (1984b) exhibited a shoulder at about 340 nm, which was not observed by Marota and Shiman (1984). Marota and Shiman (1984) obtained the spectrum by exciting at 275 nm, where tyrosine residues should have considerable absorbance since rat liver phenylalanine hydroxylase contains 20 tyrosine residues in contrast to only 4 tryptophan residues per subunit (Shiman, 1980). Therefore, the fluorescence spectrum given by Marota and Shiman (1984) is considered to be composed of spectra of both tyrosine and tryptophan residues. We have confirmed that fluorescence in the present work is from tryptophan residues by measuring fluorescence excitation spectra. When 1 mol of a cofactor, 6-methyl-5,6,7,8-tetrahydropterin ($6MPH_4$), binds to 1 mol of the subunit of phenylalanine hydroxylase, the cofactor is oxidized in the presence of oxygen (Marota and Shiman, 1984). The oxidation of $6MPH_4$ is required for catalytic hydroxylation of phenylalanine independent of activation by phenylalanine (Marota & Shiman, 1984) and is induced by the reduction of non-heme iron (Wallick et al., 1984). Fluorescence intensity of phenylalanine hydroxylase, which displays a peak at 310–315 nm, increased upon binding of $6MPH_4$ in the presence of oxygen (Marota & Shiman, 1984) but decreased upon binding of $BH_4$ in the absence of oxygen (Phillips et al., 1984b) and in the presence of oxygen in the present work. These contradictory observations may be partly related to the phosphorylation state of the prepared enzyme (Abita et al., 1976) and could be also caused by the change in the association state of the tetrameric enzyme, which was determined by gel filtration at concentrations of 17–34 $\mu$M monomer ($M_r$ 50 000) (Shiman, 1980). The enzyme may partly dissociate into dimer, since fluorescence measurements were made at rather low concentrations (less than 1.5 $\mu$M monomer). In addition, effects of the partial binding of phenylalanine to the substrate binding site on the conformational change are not well characterized.

Modification of SH groups in rat liver phenylalanine hydroxylase by *N*-alkylmaleimide induced a remarkable increase of its catalytic activity (Parniak & Kaufman, 1981). The hydroxylase has two reactive SH groups; one of the SH groups is selectively modified by an asymmetric thiol–disulfide exchange reagent, dinitrophenyl alkyl disulfides with a neutral or hydrophilic alkyl chain, accompanied by marked activation, and the other SH group is modified preferentially by dinitrophenyl alkyl disulfides with an anionic group without appreciable activation (Koizumi et al., 1987). ANM possesses a bulky hydrophobic aromatic ring and induced a partial activation (about 2 times) upon the conjugation. Therefore, ANM is considered to conjugate with the former SH group. Fluorescence lifetime measurement indicates that the microenvironment surrounding ANM is uniform in the enzyme, since the fluorescence decay exhibited a single-exponential function with a lifetime of 4.6 ns. The fluorescence lifetime of ANM changed to 4.1 and 8.7 ns upon the binding of phenylalanine, and to 3.1 and 7.9 ns upon the binding of $BH_4$, whereas the fluorescence spectrum of ANM was not modified significantly. The binding of phenylalanine as an activator (Nielsen, 1969; Kaufman, 1970) or that of $BH_4$, which is considered to reduce non-heme iron as in the case of $6MPH_4$ (Wallick et al., 1984) and activates the enzyme (Marota & Shiman, 1984), may bring about a nonuniform microenvironment around ANM. It is interesting that the surroundings of some ANM, which is considered to locate on the enzyme surface, became hydrophobic upon the binding of phenylalanine or $BH_4$, since the longer lifetime component appeared as a result of conformational change. The present result is coincident with the observation by Shiman and Gray (1980) that phenylalanine hydroxylase becomes hydrophobic upon activation by phenylalanine.

The quenching of the fluorescence of tryptophan upon conjugation of ANM was due to excitation energy transfer from tryptophan with the shorter lifetime to ANM. The energy transfer could be efficient when the distance between the tryptophan with shorter lifetime and ANM is shorter than $R_0$. The real distance between them should not be much different from $R_0$, since energy-transfer efficiency was not very large (see Table III). When phenylalanine hydroxylase was activated by the substrate, energy transfer did not occur any

more between tryptophan with the shorter lifetime and ANM and, alternatively, seemed to occur between tryptophan with the longer lifetime and ANM. The disappearance of energy transfer between tryptophan with the shorter lifetime upon the activation may be due to elongation of the distance between them, or to the decrease in the value of $R_0$, which could be taking place by the reduction of $\kappa^2$. On the other hand, the energy transfer did not occur at all when $BH_4$ was added to the enzyme solution. The reason is that the remarkable quenching of fluorescence of tryptophan upon the binding of $BH_4$ made the value of $R_0$ reduced (see Table III).

It is noteworthy to discuss the remarkable quenching of tryptophan in the both native and ANM-conjugated phenylalanine hydroxylase upon binding of $BH_4$. $BH_4$ is considered to be oxidized by the hydroxylase, which is necessary for the catalytic hydroxylation of the substrate (Marota & Shiman, 1984). The oxidized biopterin has appreciable absorbance at the wavelength region of tryptophan fluorescence (320–380 nm). Accordingly, it is probable that the fluorescence of tryptophan is quenched by the oxidized biopterin due to energy transfer from tryptophan residues to the oxydized cofactor. If this is the case, the binding site of $BH_4$ should be close to fluorescent tryptophan residues within critical transfer distance between them.

In the present work we assumed that every tryptophan has a single lifetime component in phenylalanine hydroxylase. Although the assumption may not be valid if we take into account that fluorescence of single tryptophan in proteins did not always decay with a single lifetime component (Beechem & Brand, 1985), the results of steady-state fluorescence measurements and analysis of the excitation energy transfer from tryptophanyl residues to ANM were reasonably interpreted from the values of the fluorescence lifetime of tryptophanyl residues.

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