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FLUORESCENCE ENERGY TRANSFER IN TRYPTOPHANASE

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Excitation of apotryptophanase from *Escherichia coli* B/lt7-A at 290 nm yielded a fluorescence emission centered at 340 nm. Binding of pyridoxal phosphate to apoenzyme induced quenching of protein fluorescence concomitant with an appearance of another peak at 510 nm by way of energy transfer from tryptophan. Based on the results, an approximate distance between the coenzyme and tryptophan was estimated to be 18-24 Å according to the Förster's theory. The ozone-inactivated enzyme yielded only the 340 nm-peak upon excitation at 290 nm following reconstitution with the coenzyme. The fluorescence decay time of the tryptophyl residue was somewhat increased by ozone-inactivation. These results suggest that the tryptophyl residue essential for the activity is involved in a direct interaction with the coenzyme.

Tryptophanase [EC 4.1.99.1] purified and crystallized from *Escherichia coli* B/lt7-A has a molecular weight of 220,000 and is composed of four identical subunits (1,2). Extensive studies by Snell and his coworkers also revealed that the enzyme reaction proceeds through α- and β-elimination reactions to yield indole, pyruvate and ammonia (3). As reported recently from this laboratory (4), the enzyme was inactivated by ozonization following pseudo-first-order kinetics. As the ozonization proceeded, the absorption peak of the enzyme at 280 nm gradually decreased concomitant with an appearance of new peaks at 260- and 320 nm reflecting a conversion of the tryptophyl residue to N'-formylkynurenine. Although the enzyme has two tryptophyl residues per subunit, one essential for the activity was found to be more susceptible to ozonization than the other. The spectrophotometric titration of the coenzyme binding to the enzyme protein at 430 nm indicated that the dissociation constant for the

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coenzyme was almost 100 times increased upon ozonization possibly by weakening the interaction between the coenzyme and the indole moiety of the tryptophyl residue. In this communication we wish to report that the tryptophyl residue essential for the activity is involved in a direct interaction with the coenzyme.

MATERIALS AND METHODS

Escherichia coli B/lt7-A was kindly provided from Dr. E.E. Snell. Crystalline tryptophanase was prepared from the organism by a modification of the procedure described by Morino and Snell (3). The apoenzyme was prepared according to the method of Morino and Snell using DL-penicillamine (2). All amino acids were products of Kyowa Hakko Kogyo (Tokyo). All other chemicals were of reagent grade commercially available.

The activity of tryptophanase was routinely determined as described by Newton *et al.* based on the colorimetric measurement of indole with *p*-dimethylaminobenzaldehyde (1). One unit of the enzyme was defined as the amount which produced one μmol of indole per min at 37°C.

Ozonization was carried out according to the method of Kuroda *et al.* (5). Prior to ozonization, apotryptophanase was gel-filtered through a Sephadex G-50 column (1.0 x 20 cm), which had been equilibrated with 0.1 M potassium phosphate buffer, pH 7.7. The desalted enzyme preparation (0.6 mg of protein per ml) was treated at 0-4°C with a slow stream of ozone (0.1-0.2 μmol/min, 0.001-0.002% O_3/O_2 ; Ozonizer, Nihon Ozone Co., Ltd. model O-T-2). The concentration of ozone was estimated by titration with KI-$Na_2S_2O_3$.

Fluorescence spectra were measured in a Shimadzu model RF 502 spectrofluorometer which gives corrected quantum spectra. Fluorescence decays were measured by the single photon counting method (half-width of the light pulse ≃ 2 ns) using an Ortec, Inc. system No. 9200 equipped with a constant temperature cell housing, with a Hitachi multichannel analyzer, model 505. Fluorescence quantum yields were determined relative to that of quinine bisulfate at a concentration of 0.1 mM in 1 N sulfuric acid whose reported quantum yield is 0.546 at 25°C (6).

The distance between the energy donor, tryptophan and the acceptor, pyridoxal phosphate was estimated according to the Förster's theory (7) both by the fluorescence quenching of the donor and by the increased fluorescence intensity of the acceptor associated with complex formation. The efficiency of this energy transfer process can be determined by Method 1, in which the fluorescence quantum yield of the protein is measured in the presence (Φ_D) and absence (Φ_D^o) of pyridoxal phosphate:

$$E = 1 - \frac{\Phi_D}{\Phi_D^o}$$

and by Method 2, in which the increase in the fluorescence intensity of the acceptor is measured:

$$E = \frac{A_A(\lambda_D)}{A_D(\lambda_D)} \left\{ \frac{I(\lambda_D)}{I_A(\lambda_D)} - 1 \right\}$$

where $A_D(\lambda_D)$ and $A_A(\lambda_D)$ are the absorbance of donor and acceptor at the wavelength in absorption range of donor λ_D; $I(\lambda_D)$ and $I_A(\lambda_D)$ are intensities of acceptor emission in the presence and absence of donor with excitation at wavelength λ_D, respectively. The distance r between the donor and acceptor is given by the equation:

$$r = (E^{-1} - 1)^{1/6} R_o$$

where R_o is the critical distance in which the transfer efficiency is 50% and is given by the equation:

$$R_o = [8.79 \times 10^{-25} \frac{\kappa^2}{n^4} \Phi_D^o J_{AD}]^{1/6}$$

where κ is the orientation factor, n the refractive index, Φ_D^o the quantum yield of the donor, and J_{AD} the spectral overlap integral of donor emission and acceptor absorption. R_o was determined as follows: The integral J_{AD} and Φ_D^o were obtained from the spectral data to be 4.21×10^{-15} cm^6/mol and 0.123, respectively. The refractive index n was taken as that of water (n = 1.333). Then, R_o was obtained to be 21.7 Å. Assuming that the tryptophyl and pyridoxal phosphate residues orient randomly each other (κ^2 = 2/3), it is possible to estimate the distance of energy transfer r. Since two tryptophyl residues are present per one pyridoxal phosphate, calculation was made for the following two models: In Model 1, the energy transfer occurs from two identical tryptophan molecules to single pyridoxal phosphate, while in Model 2, from one tryptophan to pyridoxal phosphate, leaving another tryptophan without interaction.

All spectrophotometric determinations were carried out either in a Hitachi 124 or 323 automatic recording spectrophotometer, both of which were equipped with a constant temperature cell housing. Protein concentration was determined by measuring the optical density at 278 nm, taking $E^{1\%}_{1cm}$ as 7.95 (2) or by the method of Lowry *et al.* (8) using bovine serum albumin as a standard.

RESULTS

Fluorescence Spectroscopy ——— When apotryptophanase was excited at 290 nm, a fluorescence emission spectrum with a maximum at 340 nm was observed which coincided with the fluorescence signal of tryptophan. Binding of pyridoxal phos phate to apoenzyme induced a marked quenching in the protein fluorescence at 340 nm. In addition, a new fluorescence peak appeared at 510 nm, which coincided with the emission band due to pyridoxal phosphate excited at 330 nm and arose through dipole-dipole energy transfer from the tryptophyl residue to pyridoxal phosphate as predicted by Förster (7). These results are shown in Fig. 1. Based on the results, estimation of the distance between the energy donor and acceptor was attempted according to the Förster's theory. The results of the calculation are shown in Table I. The estimated distance was in a range from 18.0 to 24.5 Å.

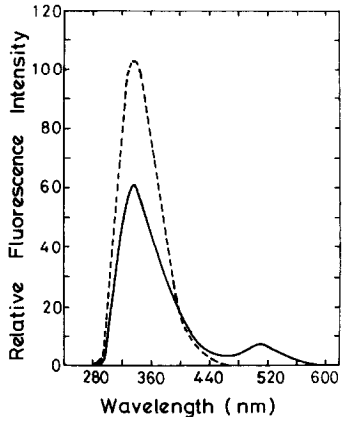

Fig. 1. Fluorescence spectra. Fluorescence emission spectra of apo (---) and holo (——) tryptophanase preparations were measured as described in "MATERIALS AND METHODS". Excitation, 290 nm. Protein concentration, 0.22 mg/ml of 0.1 M potassium phosphate, pH 7.7.

When the ozone-inactivated tryptophanase was excited at 290 nm following reconstitution treatment with a large excess of pyridoxal phosphate, only the 340 nm-peak was observed as shown in Fig. 2. The small peak centered at 430 nm was found to have an excitation maximum at 320 nm, which coincided with that of kynurenine. These results indicate that no appreciable energy transfer occurs, when the tryptophyl residue essential for the activity is oxidized to N'-formylkynurenine.

Fluorescence Decay Measurements ——— Native and ozone-inactivated apo-tryptophanase preparations were separately excited at 293 nm and the fluores-

TABLE I. Determination of the distance between the tryptophyl residue and pyridoxal phosphate

Procedures		Distance (r) (Å)
Model 1	Method 1	22.9
	Method 2	24.5
Model 2	Method 1	18.0
	Method 2	19.8

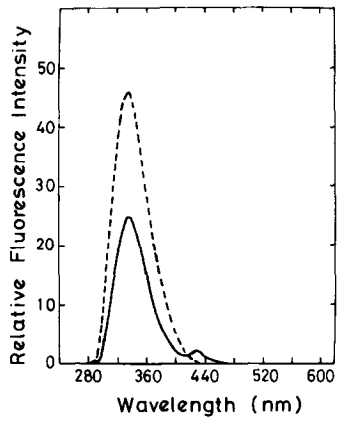

Fig. 2. Fluorescence spectra of ozone-inactivated tryptophanase. The experimental conditions were the same as in Fig. 1, except that the ozone-inactivated enzyme was used. (---), apoenzyme with a residual enzyme activity of 10%; (——), reconstituted enzyme in the presence of 1 mM pyridoxal phosphate.

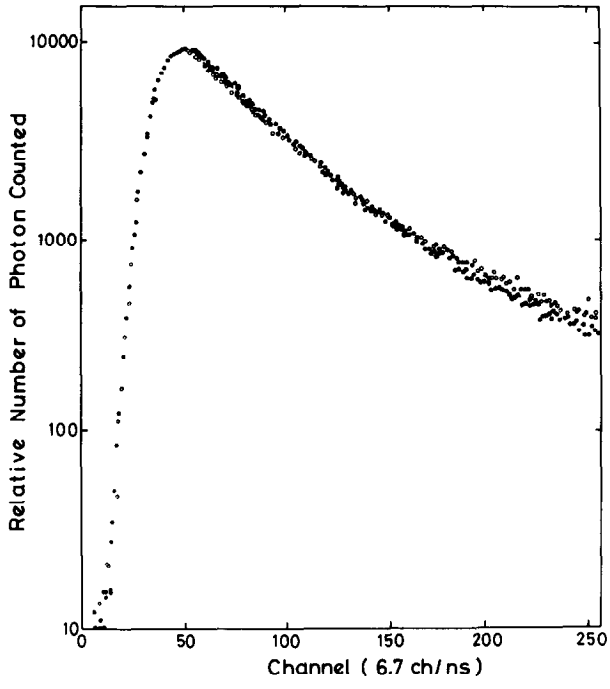

Fig. 3. Fluorescence decay measurements. The experimental conditions were described in "MATERIALS AND METHODS". Protein concentration was adjusted to 0.8 mg/ml using 0.1 M potassium phosphate, pH 7.7. Excitation, 293 nm; fluorescence emission, 370 nm. (●), native enzyme; (O), ozone-inactivated enzyme with a residual enzyme activity of 20.3%.

cence decay of the tryptophyl residue was measured at 370 nm. Although quantitative treatment of the data by deconvolution technique was not made yet, the results in Fig. 3 show that the ozone-modified enzyme has somewhat longer lifetime than the native enzyme. These results indicate that the tryptophyl residue essential for the activity locates rather outside of the enzyme molecule.

DISCUSSION

Our recent work indicated that the tryptophyl residue is essential for the activity of tryptophanase possibly by contributing to the binding of pyridoxal phosphate to the enzyme protein (4). The results obtained in the present investigation support the idea that the tryptophyl residue in the enzyme locates fairly close to the coenzyme and is involved in a direct interaction with it. Although the nature of the interaction remains to be elucidated, it is expected to be quite different from those of the lysyl (9), sulfhydryl (10), arginyl (11) and histidyl (12) residues, which were reported to be essential for the enzyme activity.

A similar fluorescence energy transfer was reported by Isom and DeMoss that holotryptophanase of *Bacillus alvei* yielded two distinct peaks, one at 350 nm and one at 500 nm, when excited at 280 nm (13). Oh and Churchich estimated the distance beteen the tryptophyl residue and pyridoxal phosphate in cystathionase from rat liver and obtained a value of 21 Å based on Model 1 and Method 1 (14). The estimated distance in the present work is, however, expected to be substantially shortened provided that the orientation factor is actually determined or its influence can be excluded.

In view of the fact that a large number of other coenzymes, in addition to pyridoxal phosphate, are of aromatic nature, it seems quite plausible that the tryptophyl residue is ubiquitously important for binding of various coenzymes. In fact, O'Brien and Gennins recently reported that the tryptophyl residue in pyruvate oxidase is essential for binding of thiamine pyrophosphate, as revealed by chemical modification studies with *N*-bromosuccinimide (15).

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