Deuteration Kinetics of Tyrosine by Means of Fluorescence Measurement

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When tyrosine was rapidly transferred from water into deuterium oxide medium by means of a stopped-flow device, a time-dependent change in the fluorescence intensity at 305 nm (excited at 275 nm) has been observed. The rate constant (k_e) was found to be 116 s^{-1} at pH 5.8 and 14 °C. This k_e value has been attributed to the rate constant of the $O^1H \rightarrow O^2H$ reaction of the phenolic OH group, on the basis of similar fluorescence examinations on phenol, anisole, glycyl-L-tyrosine, and N^{α}-acetyl-*N*-methyl-L-tyrosinamide as well as on the basis of a stopped-flow ultraviolet absorption study of tyrosine. The stopped-flow fluorometry has been applied to a study of tyrosine residues in yeast 3-phosphoglycerate kinase.

Deuteration kinetics can, in general, be a sensitive means of detecting differences in the intramolecular environment of a given functional group in a biological macromolecular system. We have recently shown that an ultraviolet absorption measurement in combination with a stopped-flow device provides a useful method for tracing a rapid deuteration process of tryptophan or tyrosine residues in a protein in an aqueous solution.[1,2] It is known, however, that a fluorescence measurement often provides a greater sensitivity and selectivity than an ultraviolet absorption measurement for an examination of the same chromophore. We, therefore, started a development of "stopped-flow fluorometry," with a possibility in mind that this may be a more useful method for deuteration kinetics.

In general, the isotope effect on fluorescence intensity is not a simple problem.[3,4] There can be a number of possible mechanisms for the occurrence of such an effect, and the intensity does not always reflect the extent of deuteration of the chromophore (or the fluorophore) in question. Indeed, the fluorescence increase of tryptophan on bringing it from ^1H_2O into ^2H_2O medium is attributable to the deuteration of the α-ammonium ($-NH_3^+$) group, but not to that of the imino group of the indole ring.[5,6] For tyrosine, however, the fluorescence increase on bringing it from ^1H_2O into ^2H_2O medium seems to be caused simply by the $O^1H \rightarrow O^2H$ change at its phenolic hydroxyl group. This is shown in this paper, with an example which suggests a possible application to a protein study.

Experimental

Phenol, anisole, L-tyrosine, and glycyl-L-tyrosine were obtained from commercial sources, and were used without further purification. Acetyl-L-tyrosine-*N*-methylamide (N^{α}-acetyl-*N*-methyl-L-tyrosinamide) was prepared by the Protein Research Foundation, Osaka, Japan, and was placed at our disposal by the courtesy of Dr. Y. Koyama, Kansei Gakuin University. Its purity was confirmed by an examination of its proton magnetic resonance. The sample of 3-phosphoglycerate kinase of yeast was purchased from Boeringer, Mannheim. The solvents ^1H_2O and ^2H_2O were used after being distilled. Emission spectra were observed by the use of a Hitachi MPF-4 fluorometer.

The time dependence of the fluorescence intensity or of the ultraviolet absorbance was observed by the use of a Union Giken stopped-flow spectrophotometer RA-401. This is equipped with a rapid-mixing device of dead time 500 μs, and with an ultraviolet monochromator of focal length 25 cm. For stopped-flow fluorometry, a 200 W deuterium discharge lamp was placed at the entrance of the monochromator, and at its exit a cell with optical path length of 2 mm was placed. The solution, after rapid mixing, is led into this cell, and when the flow is stopped the time dependent fluorescence change (if any) should start. The fluorescence is excited by monochromatic light, and is observed through a Hoya UV 30 filter, which allows the emitted light of wavelength longer than 300 nm to come into the detector. For a stopped-flow ultraviolet absorption study, the same deuterium discharge lamp and the same cell were used with the same monochromator as above. Only the location of the photomultiplier was changed. The fluorescence intensity *versus* time curve or the absorbance *versus* time curve was obtained by the use of a Union Giken data processor RA-450, a monitorscope, and an XY recorder.

Results and Discussion

Fluorescence of Tyrosine and Its Change on Deuteration. The observed emission spectra are reproduced in Fig. 1. The fluorescence peaks are found at 296 nm for phenol and at 295 nm for anisole. On transfering from ^1H_2O to $^2H_2O+^1H_2O$, the peak intensity increases by 22% for phenol, but only 2% for anisole. L-Tyrosine, glycyl-L-tyrosine, and N^{α}-acetyl-*N*-methyl-L-tyrosinamide show their fluorescence maxima at 303 nm. On changing the solvent from ^1H_2O to $^2H_2O+^1H_2O$, the intensity increases of 18, 13, and 16% are found, respectively, for these three compounds. Of the total 13% fluorescence increase for L-tyrosine, 9% takes place rapidly within 1 ms, while the remaining 4% increase takes place more slowly, in a period of about 20 ms (see Fig. 2(c)). A re-plot of such a fluorescence intensity on a logarithmic scale against time indicates that the process is apparently a single first order process. The rate constant is found to be 116 s^{-1} at pH 5.8 and 14 °C. This is exactly equal to the rate constant value obtained by a stopped-flow ultraviolet absorption measurement at 285 nm as described in our previous paper[2] (see also the curve given at the bottom of Fig. 2, (c)). This rate constant was assigned to that of the deuteration reaction $O^1H \rightarrow O^2H$ of the phenolic hydroxyl group of tyrosine.[2]

A similar fluorescence increase takes place with nearly equal rate for phenol (Fig. 2, (a)), but not at all for anisole (Fig. 2, (b)). In contrast with glycyl-L-tryptophan, which shows no time-dependent fluorescence increase when it is transferred from ^1H_2O into ^2H_2O medium,[6] glycyl-L-tyrosine shows an increase with nearly an equal rate constant to that for tyrosine (Fig. 2, (d)). All of these facts support the idea that

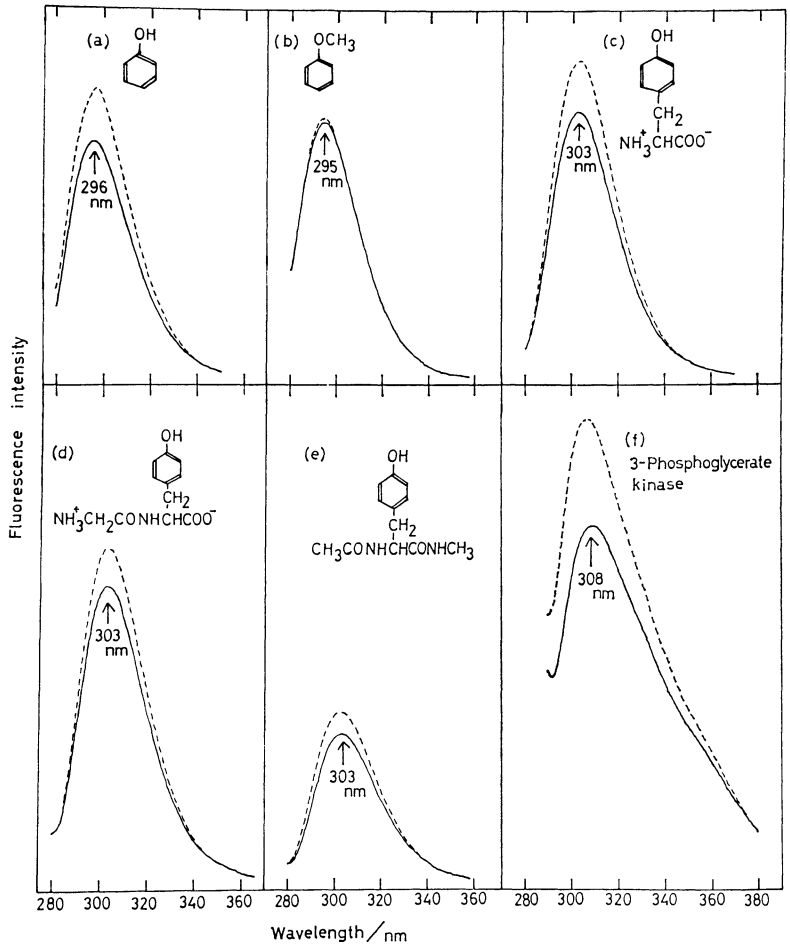

Fig. 1. Emission spectra in ^1H_2O (full line) and in $^2H_2O+^1H_2O$ (broken line), at pH 5.8 and at 14 °C.
(a) Phenol, excited at 270 nm. (b) Anisole, excited at 270 nm. (c) L-Tyrosine (0.15 mM), excited at 275 nm. (d) Glycyl-L-tyrosine, excited at 275 nm. (e) N^{α}-Acetyl-N-methyl-L-tyrosinamide, excited at 275 nm. (f) Yeast 3-phosphoglycerate kinase (0.01%), excited at 280 nm.

the time-dependent fluorescence increase in question is caused by the $O^1H \rightarrow O^2H$ process of the phenolic hydroxyl group.

The amount and the rate of fluorescence increase for N^{α}-acetyl-N-methyl-L-tyrosinamide is also nearly equal (Fig. 2(e)) to those of L-tyrosine or to phenol. Such a rate constant value is to be used as a standard (k_e) value in estimating the attenuation factor $\gamma_j = k_j/k_e$ of a given tyrosine group (j) in a protein molecule whose rate constant value is k_j.

Application to a Protein Study. 3-Phosphoglycerate kinase (EC 2.7.2.3) from yeast contains two tryptophan and eight tyrosine residues per molecule.[7] Nojima *et al.*[8] has found that, in its emission spectrum, a fluorescence maximum is present at 308 nm if excited at 280 nm, while at 329 nm if excited at 295 nm. The former (308 nm) is considered to be caused by the tyrosine residues, and the latter (329 nm) by the tryptophan residues. The emission spectrum of this protein excited at 280 nm is shown in Fig. 1, (f). As may be seen here the fluorescence intensity increases by 28% on bringing it from ^1H_2O to $^2H_2O+^1H_2O$. The same amount (27.5%) of total fluorescence increase was observed when an ^1H_2O solution of this protein is rapidly mixed with the same volume of ^2H_2O by means of the stopped-flow fluorometry technique. Of this total 27.5% increase, 22% takes place within the dead time of the stopped-flow equipment, and the remaining 5% increase takes place very slowly (see Fig. 3). The rate of this slow process was found to be 3.9 s^{-1} at pH 5.9 and at 29 °C. At this pH and at this temperature, k_e of tyrosine is estimated to be 350 s^{-1}, and therefore the attenuation factor $\gamma_i = 3.9/350 = 1/90$. It may be interpreted that the tyrosine residue (or residues) now in question is rather rigidly fixed inside of the protein molecule, and that the chance of its being exposed to the solvent is only 1/90.

In a protein molecule an energy transfer takes place often from tyrosine to tryptophan, and even when a tyrosine residue is excited the fluorescence comes often from the tryptophan residue instead of the tyrosine residue itself.[9] If a protein containing both tyrosine and tryptophan residues gives a tyrosine fluorescence, such a tyrosine residue is considered to be firmly fixed at a particular orientation in the protein molecule, so that no energy transfer takes place between the tyrosine residue now in question and any of the tryptophan residues in the same molecule. Because 3-

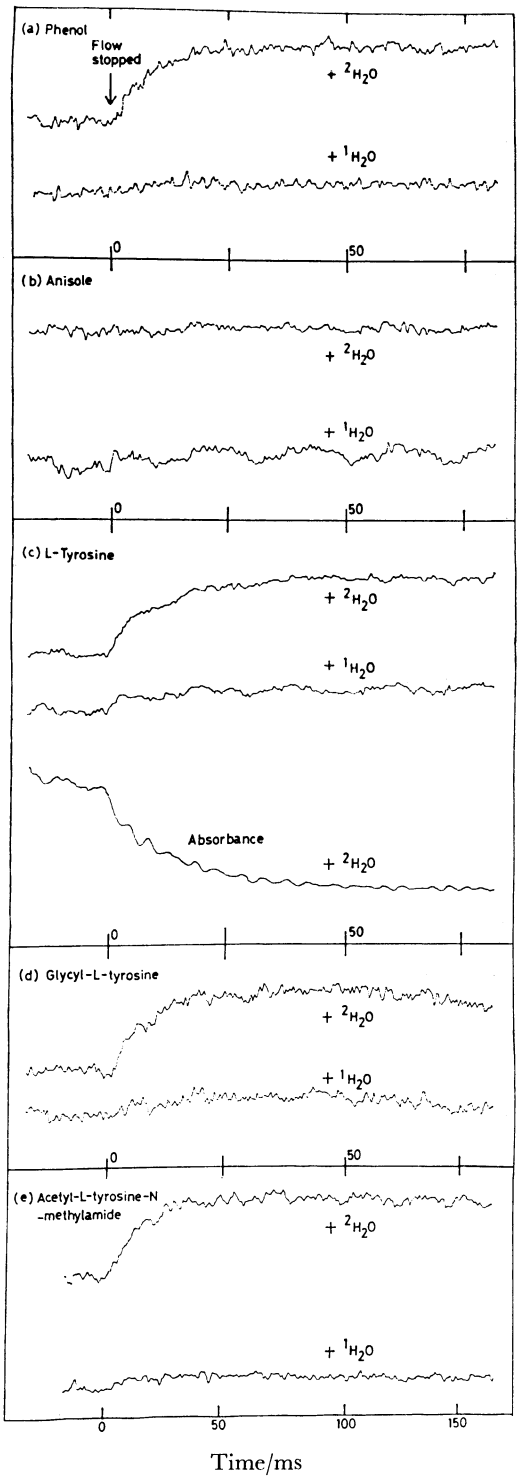

Fig. 2. The time dependence of the fluorescence intensity observed when an ^1H_2O solution of each compound indicated is rapidly mixed with the same volume of ^2H_2O, so that the solvent of the final solution is 1 : 1 $^1H_2O+^2H_2O$. For each compound, a curve recorded in a control experiment in which the same ^1H_2O solution was mixed with ^1H_2O instead of ^2H_2O is also shown.

(a) Phenol (pH 5.3, at 15 °C), excited at 255 nm. (b) Anisole, (pH 5.5, at 15 °C), excited at 255 nm. (c) L-Tyrosine (final concentration 1.2 mM, pH 5.8, at 12.5 °C), excited at 275 nm. The curve at the bottom is the time dependence of the ultraviolet absorbance recorded at 285 nm when an L-tyrosine in ^1H_2O solution was mixed with the same volume of ^2H_2O, so that the final concentration was 1.2 mM, pH 5.8, at 12.5 °C. (d) Glycyl-L-tyrosine, pH 5.5, at 17 °C, excited at 275 nm. (e) N^{α}-Acetyl-N-methyl-L-tyrosinamide pH 5.5, at 17 °C, excited at 275 nm.

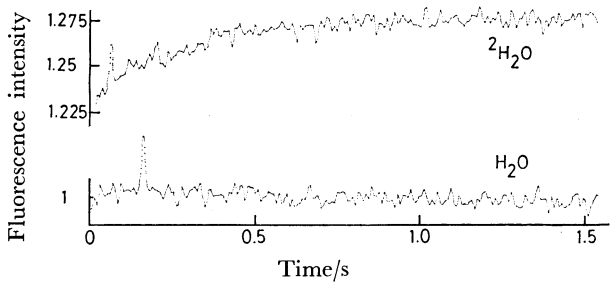

Fig. 3. Change in time of fluorescence intensity at 308 nm (excited at 280 nm) is observed for yeast 3-phosphoglycerate kinase in ^1H_2O upon mixture with ^2H_2O (1 : 1 in volume; final concentration of the protein is 0.05%, pH 5.9) at 29 °C.

phosphoglycerate kinase has both the tyrosine and tryptophan residues, the tyrosine fluorescence (at 309 nm) we have observed is attributed to such a tyrosine residue (or tyrosine residues) rigidly fixed in a special orientation. The fact that the hydrogen-deuterium exchange reaction between this tyrosine residue and the solvent ^2H_2O is 90 times as slow as the tyrosine exposed to the solvent is therefore understandable.

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