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Measurement of Hydrogen Exchange at the Tyrosine Residues in Ribonuclease A by Stopped-Flow and Ultraviolet Spectroscopy

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Abstract: A time-dependent change in the ultraviolet absorbance at 285 nm of the phenol ring of tyrosine has been observed in a stopped-flow spectrophotometer, when tyrosine was rapidly transferred from water into deuterium oxide (the final tyrosine concentration was about 1 mM). From this experiment, the rate constant (k_e) of the hydrogen-deuterium exchange reaction of the tyrosine OH group has been determined at various pH values and at several temperatures. At pH 6.1 and 11 °C, for example, k_e was found to be as high as 70 s^{-1}. The stopped-flow ultraviolet spectroscopy has also been used for a measurement of hydrogen exchange rates at the six tyrosine residues of bovine pancreatic ribonuclease A.

Introduction

A measurement of the kinetics of the hydrogen exchange reactions of a protein in aqueous solution provides a useful piece of information on the conformation of the protein molecule. For peptide NH groups, such measurements have been made of many proteins, with various methods, by a number of investigators.[2-5] For side-chain hydrogen atoms, on the other hand, such measurements are relatively few; and in addition only tryptophan and histidine residues have so far been subjected to those measurements.[6-9] In this paper, we show that the hydrogen-deuterium exchange reaction of the tyrosine residue can be followed by stopped-flow ultraviolet spectroscopy. The stopped-flow ultraviolet method was first shown by Cross[10,11] to be applicable to a few nucleosides. We have recently shown that this method is useful for the hydrogen exchange study of the tryptophan residues of a protein.[12] The development of this method is of special significance for the tyrosine residue, because the hydrogen exchange rate at the tyrosine hydroxyl group is in general so high that no other methods seem to be easily applicable to its kinetic study. The stopped-flow ultraviolet method is useful not only because it is good for a millisecond exchange reaction but also because it is applicable to a high molecular weight protein in a relatively dilute solution.

Experimental Section

L-Tyrosine was purchased from Wako Pure Chemical Industries, Ltd., and bovine pancreatic ribonuclease A was obtained from Boehlinger Mannhein. Deuterium oxide (99.75%) was purchased from Merck.

The hydrogen and/or deuterium ion concentration of the solution were measured with a Toa Dempa pH meter and a Hitachi-Horiba F7SS pH meter. In this paper, we shall use the notation "pH" even for the deuterium ion concentration of a deuterium oxide solution, and pH-meter readings are always given without any corrections. For adjusting each pH value, HCl or NaOH was used.

Ultraviolet absorption and difference spectra were observed by the use of a Union Giken high-sensitivity spectrophotometer SM-401. The kinetics of the hydrogen-deuterium exchange reactions was examined 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, with a cell of optical path length 10 mm, and with an ultraviolet spectrophotometer of focal length 25 cm, sensitivity 0.0004 OD, and response time 0.1 ms. This was connected with a Union-Giken data processor RA-450, a monitor scope, and an *XY* plotter.

Results

Hydrogen-Deuterium Exchange in Free Tyrosine. When L-tyrosine in ^1H_2O is rapidly mixed with ^2H_2O (at pH 6.1 and 11 °C, final concentration is 0.97 mM), a time-dependent decrease of the absorbance at 285 nm is observed as shown in Figure 1(a). A replot of such data, as illustrated in Figure 1(c), shows that the absorbance decrease takes place as a single first-order process. By extrapolating the straight line in Figure 1(c) to zero time, we are able to determine the total absorbance change associated with this first-order process. The total absorbance changes measured in this way at several different wavelengths generate the kinetic difference spectrum shown in Figure 2. In this figure, the equilibrium solvent perturbation difference spectrum, which gives the total effect of ^2H_2O on the spectrum of L-tyrosine, is also shown (curve (b)). The difference between the kinetic difference spectrum and equilibrium solvent perturbation difference spectrum is attributed to a solvent perturbation which takes place during the dead time of the kinetic experiment.[10,11] The equilibrium solvent perturbation difference spectrum has two peaks at about 286 and 279 nm. The magnitude of the perturbation difference spectrum observed here is in an agreement with what was observed by Herkovits and Sorensen.[13]

The first-order process shown in Figure 1 is attributed to the hydrogen-deuterium exchange reaction of the phenol OH group of tyrosine. The rate constant is found to be 70 s^{-1} at pH

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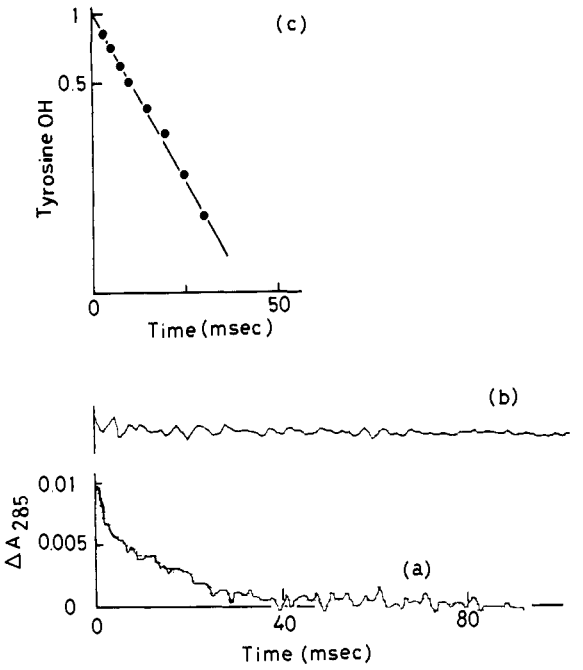

Figure 1. (a) The time dependence of the decrease in absorbance at 285 nm observed when L-tyrosine dissolved in ^1H_2O (1.94 mM, pH 6.1, 11 °C) is mixed with ^2H_2O (1:1 in volume; final concentration of L-tyrosine is 0.97 mM). This is a photographic reproduction of the curve recorded on the plotter connected with a Union Giken stopped-flow spectrophotometer RA-401 and a data processor RA-450. Here, ten scans were accumulated in the (on-line) microcomputer for averaging. (b) The curve recorded in a control experiment, in which the same tyrosine solution was mixed with ^1H_2O instead of ^2H_2O. (c) Semilogarithmic plot of the same data as that for (a).

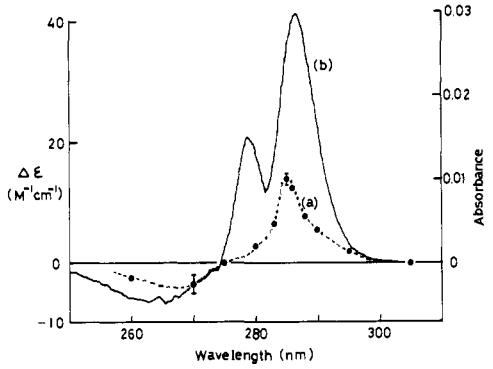

Figure 2. (a) A difference spectrum obtained from the kinetic measurement. Each point indicates the total absorbance change obtained for the first-order process observed on dilution with ^2H_2O by extrapolating to zero time. The vertical bars indicate the estimated amounts of errors. (b) The difference spectrum of L-tyrosine in ^1H_2O vs. L-tyrosine in $^2H_2O + {}^1H_2O$ (1:1 in volume), at pH 6.1 and 11 °C.

6.1 and 11 °C. In the range 0.465–1.86 mM of tyrosine concentration, this rate constant value has been found to remain constant. This is considered to be the rate constant (k_e) of a tyrosine residue which is completely exposed to the solvent, and such a value is useful as a reference in a study of tyrosine residues involved in a protein molecule.

Similar observations were made at various pH values and the observed rate constants (k_e) are given in Figure 3. The rate constants (k_e) were also determined at different temperatures (at pH 5.4). From an Arrhenius plot, shown in Figure 4, the activation energy of the hydrogen–deuterium exchange reaction of tyrosine OH is obtained as $\Delta H^{\ddagger} = 18.2$ kcal/mol.

Hydrogen–Deuterium Exchange of the Tyrosine Residues in Ribonuclease A. Bovine pancreatic ribonuclease A in ^1H_2O

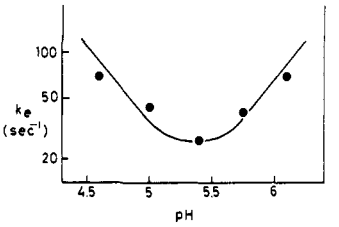

Figure 3. The rate constant k_e of the hydrogen–deuterium exchange reaction of L-tyrosine plotted on a logarithmic scale against pH of the solution at 11 °C.

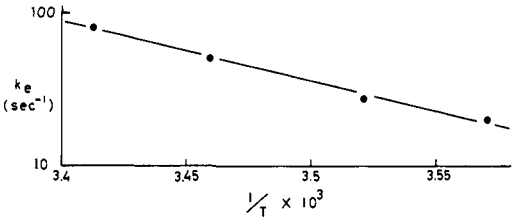

Figure 4. The rate constant k_e of the hydrogen–deuterium exchange reaction of L-tyrosine plotted on a logarithmic scale against reciprocal absolute temperature at pH 5.4 (Arrhenius plot).

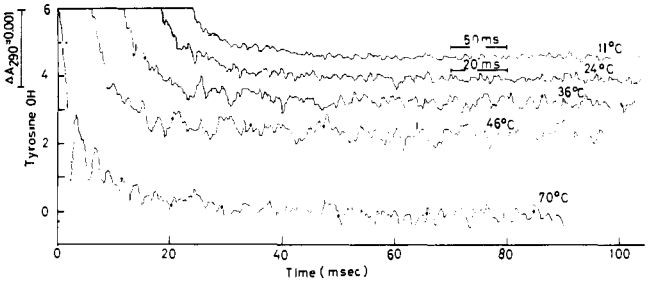

Figure 5. The time dependence of the decrease in absorbance at 290 nm observed when bovine pancreatic ribonuclease A dissolved in ^1H_2O is mixed with ^2H_2O (1:1 in volume; final concentration of the protein is 0.086 mM, pH 5.4). The ordinate scale is given also in the number of tyrosine residues per one protein molecule. This was determined on the assumption that the absorbance difference ΔA at 290 nm between the completely undeuterated and completely deuterated ribonuclease corresponds to six tyrosine residues, and that ΔA in any partially deuterated stage is proportional to the number of undeuterated tyrosine residues. The completely deuterated ribonuclease is considered to be obtained by heating the $^1H_2O + {}^2H_2O$ (1:1) solution at 70 °C for 100 ms (see the lowest curve in the figure). In the kinetic study at lower temperatures (24 and 11 °C) the abscissa scale has been changed (cf. the highest two curves).

(initial concentration 0.172 mM and final 0.086 mM) was mixed with the same volume of ^2H_2O at various temperatures and pH 5.4, and the time-dependent decrease in absorbance at 290 nm was observed. The results are shown in Figure 5. As this protein has no tryptophan, the absorbance change at 290 nm caused by deuteration should be attributed solely to the changes $O^1H \rightarrow O^2H$ at tyrosine residues. The time-dependent decrease in absorbance was also examined at 288 and at 293 nm (at 11 °C). It was confirmed that the time-dependence profile of the absorbance decrease at each of these two wavelengths is the same as that at 290 nm. The total amount of the absorbance change, however, is found to be smaller at 288 nm or at 293 nm than that at 290 nm. In any case, the observed time-dependent decrease in absorbance is taken as indicating the time course of the deuteration reaction of the tyrosine residues in the ribonuclease molecule.

Discussion

Ribonuclease A has six tyrosine residues at positions 25, 73, 76, 92, 97, and 115.[14] On the basis of a crystallographic

study[15], Tyr 76 and Tyr 92 are considered to be exposed on the surface of the molecule, Tyr 73 and Tyr 115 are halfway exposed, and Tyr 25 and Tyr 97 are buried. The last two are known not to be titrated even at high pH.[16] In parallel with this, about two tyrosine residues are found to be deuterated at 24 °C (at 60 ms, for example), about four at 46 °C, and all six at 70 °C (see Figure 5). It is therefore suggested that the two tyrosine residues deuterated at 24 °C are Tyr 76 and Tyr 92, the other two next deuterated at 46 °C are Tyr 73 and Tyr 115, and the most slowly deuterated two are Tyr 25 and Tyr 97.

Let us next attempt to obtain some idea about the environment in which the tyrosine residues in the protein molecule are placed. Such an environment may be judged by the "attenuation factor" defined by

$$\gamma_j = k_j/k_e \quad (1)$$

for each of the exposed and buried tyrosine residues (j). By replotting the kinetic data illustrated in Figure 5, it is found that the rate constant (k_j) of the two tyrosine residues deuterated at 24 °C is about 120 s^{-1}. The rate constant of free tyrosine (k_e), on the other hand, is estimated to be 125 s^{-1} (see Figure 4). Thus, the attenuation factor is nearly unity. In other words, these two tyrosine residues are considered to be fully exposed, while the attenuation factor γ_j for the most buried two tyrosine residues is as low as 10^{-2} even at 70 °C, where this protein is denatured[17,18] (k_j is estimated to be 80 s^{-1} from data given in Figure 5 and k_e at 70 °C is estimated to be 7900 s^{-1} by extrapolating the straight line obtained by the Arrhenius plot shown in Figure 4). Therefore, the "denatured" ribonuclease is suggested to have a considerable amount of a secondary structure.

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Photoelectron Spectra of Some Pyrazolthiones, 1,2,3-Triazolthiones, and 4-(1,2,3-Triazolio) Sulfides. Evidence of an Abnormal Effect of Methylation[1]

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Received September 24, 1976

Abstract: Photoelectron spectra of *N,N'*-dimethylpyrazol-3-ine-5-thione, 1,2,3-triazol-3-ine-5-thione, and 4-(1,2,3-triazolio) sulfide, together with their α-methyl derivatives, have been recorded and assigned. The *C*-methyl groups seem to stabilize the MOs in the compounds which possess a conjugated thiocarbonyl system. This unusual stabilization is discussed in terms of perturbation theory.

Previous photoelectron spectra of linear and five-membered cyclic thiocarbonyl compounds[3] revealed that the first two bands are associated with ionization of electrons from the bonding π orbital of the thiocarbonyl group and from the nonbonding atomic orbital on the sulfur atom.

The two ionization potentials at best differ only slightly within experimental accuracy in saturated thiocarbonyl compounds. When distinguishable, the first band has been assigned to ionization of the n electrons. The order is reversed and the difference in energy is larger for β,γ-unsaturated cyclic thiocarbonyl compounds.

A feature common to saturated and β,γ-unsaturated thiocarbonyl compounds is a decrease in energy of the first band by introduction of a methyl group.

The present paper deals with the effect of α,β unsaturation and subsequent methyl substitution on the photoelectron spectra of cyclic thiocarbonyl compounds using 1,2-dimethylpyrazol-3-ine-5-thione (a), 1,2-dimethyl-1,2,3-triazol-3-ine-5-thione (b), and their 4-methyl derivatives as models. In addition, the photoelectron spectra of the highly polarized thiocarbonyl group in 1,3-dimethyl-4-(1,2,3-triazolio) sulfide (c) and its 5-methyl derivative have been recorded.

Photoelectron Spectra of the Species a, b, and c. 1,2-Dimethylpyrazol-3-ine-5-thione (a). The photoelectron spectra of the pyrazolthione (a) (Figure 1) and its 4-methyl derivative exhibit an intense low-energy band corresponding to ionization of both a thiocarbonyl π electron and the sulfur n electron. This coincidence, also observed in other α,β-unsaturated thiocarbonyl compounds like the 1,2-dithiole-3-thiones[4a] and the triazolthiones (b) below, is distinct from the marked separation found in the β,γ-unsaturated compounds.

The assignment of the photoelectron signals of the pyra-

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