Hydrogen-Deuterium Exchange Rate between a Peptide Group and an Aqueous Solvent as Determined by a Stopped-Flow Ultraviolet Spectrophotometry

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When an ^1H_2O solution of *N*-methylacetamide was rapidly mixed with ^2H_2O, a time-dependent decrease of the ultraviolet absorbance at 227 nm was observed. This was not the case with *N,N*-dimethylacetamide. Hence, the time-dependent decrease in absorbance was attributed to be caused by the deuteration (NH→ND) reaction of *N*-methylacetamide. This provides an appropriate method for determining deuteration rate of the peptide group in a polypeptide or in a protein. By this method the deuteration rate of poly-D,L-alanine has been examined in the pH=5—8 and temperature=10—40 °C range. In this range, the deuteration was found to proceed always as a single first-order reaction, and the rate constant k_e is expressed within an experimental error as $k_e=6.4\times 10^{(6+pH-3850/T)}$. This is to be used as a standard rate-constant value in estimating the attenuation factor $\gamma_j=k_j/k_e$ of a given peptide group (j) in a protein molecule whose rate constant value is k_j.

When a protein or a polypeptide is placed in an aqueous medium, a proton exchange reaction takes place between the peptide NH group and the solvent H_2O. The rate of this exchange reaction depends upon the intramolecular environment of the peptide group in question, and a kinetic study of such an exchange reaction provides a useful piece of information of the protein or the polypeptide structure.[1-5] The kinetics has so far been examined mostly by an infrared spectrophotometric tracing of the deuteration process of the peptide groups of a protein molecule thrown into ^2H_2O.[6] The kinetics was also examined by tracing hydrogen-tritium exchange reaction by means of a radioactivity measurement in combination with the Sephadex column chromatography.[7] By these methods, however, the reactions faster than 0.1 s^{-1} are not easily traced.[7,8]

The scope of this paper is two-fold. First we report that the hydrogen-deuterium exchange reaction of the peptide NH group can be followed by a stopped-flow ultraviolet spectrophotometry.[9-11] It will be shown that, by this method, the exchange reaction as fast as 30 s^{-1} can readily be traced. Secondly, rate constant values (k_e) for a peptide group fully exposed to the solvent are given in the pH=5—8 and temperature=10—40 °C range. Each of these values is serviceable as a standard in estimating an attenuation factor γ_j for a given peptide group (j) in a given protein molecule. Thus,

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

where k_j is the hydrogen exchange rate constant of the j-th peptide group now in question at a given pH and temperature. The attenuation factor γ_j is a measure of how deeply the j-th peptide group is buried in the protein molecule, and $\ln \gamma_j$ is often considered to be proportional to the free energy required for bringing this j-th peptide group into a state fully exposed to the solvent. In the most useful range of pH (5—8) and temperature (10—40 °C), however, the k_e values are so high (or the exchange reaction of the peptide NH is so fast) that only the new method is applicable. By the former methods, the measurements were so far made only in the lower pH region and/or lower temperature region, and the k_e value in the range now in question was estimated only by an extrapolation.

Experimental

N-Methylacetamide was obtained from Aldrich Chemical Company, and *N,N*-dimethylacetamide from Wako Pure Chemical Industries, Ltd. Poly-D,L-alanine with an average molecular weight 3800 (average degree of polymerization=53) was purchased from Miles Chemical Company and purified by a Sphadex G25 column chromatography. ^2H_2O (deuterium atom content 99.8%) was purchased from CEA-CEN-SACLAY.

The fast deuterations were examined by the use of a Union Giken Stopped-Flow Spectrophotometer RA-401. This is equipped with a rapid mixing device of a dead time 0.5 ms, with two syringes operated with nitrogen gas pressure. A fast flow of the mixture goes through a cell of optical path-length 10 mm. At about 17 millisecond after the pressure is applied, the flow is stopped, and a time-dependent absorbance change starts in the cell. This is detected by an ultraviolet spectrophotometer of focal length 25 cm, and of sensitivity 0.0001 OD. The temperature of the solution was controlled by circulating ethylene glycol through a mantle from a Haake model KT41 circulator. The actual temperature of the solution was monitored by a thermocouple.

Slower exchange reactions were traced with another stopped-flow equipment and a Hitachi EPS-3T Recording Spectrophotometer.

The hydrogen ion concentration of the solution was determined with a Hitachi-Horiba F7SS pH meter. The pH values given in this paper were always those of the solutions after the mixing (*i.e.*, in 50% ^1H_2O+50% ^2H_2O). Here, the pH-metric readings are given without any correction.

Hydrogen-Deuterium Exchange in *N*-Methylacetamide

When *N*-methylacetamide is suddenly brought into a ^2H_2O medium, a time-dependent decrease in absorbance at 227 nm is observed as illustrated in Fig. 1(a). Such an absorbance change is not observed at all when this molecule is brought by the same equipment into pure ^1H_2O (see Fig. 1(b)). This is not observed also when *N,N*-dimethylacetamide, where no NH group remains, is brought into the same ^2H_2O medium (Fig. 1(c)). Therefore, the time-dependent absorbance decrease is attributable to the NH→ND

reaction in *N*-methylacetamide. As is shown in Fig. 2(a), *N*-methylacetamide has a strong absorption band at about 190 nm. It is probable that this band is shifted very slightly towards shorter wavelength on deuteration, such a shift is considered to cause a slight but appreciable absorbance drop at 227 nm.

A replot of the kinetic data such as that given in Fig. 1(a) shows that the absorbance decrease takes place as a single first-order process. By extrapolating this first-order process to zero time, we are able to determine the total absorbance change associated with this process (NH→ND). The absorbance changes

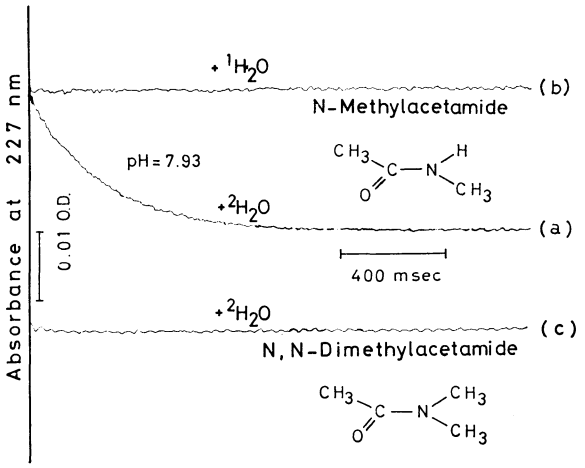

Fig. 1. (a) The time dependence of the decrease in absorbance at 227 nm observed when *N*-methylacetamide dissolved in ^1H_2O is mixed with ^2H_2O, so that final concentration of *N*-methylacetamide is 0.14 M and the solvent is 50% ^1H_2O+50% ^2H_2O of pH 7.93 at 22 °C. This is a photographic reproduction of the curve record on the plotter connected with a Union Giken stopped-flow spectrophotometer RA-401 and a data processor RA-450. This is a one scan datum; no accumulation was made. (b) The curve recorded in a control experiment, in which the same *N*-methylacetamide solution was mixed with pure ^1H_2O instead of ^2H_2O. (c) The curve recorded in another control experiment, in which a ^1H_2O solution of *N*,*N*-dimethylacetamide (instead of *N*-methylacetamide) was mixed with ^2H_2O.

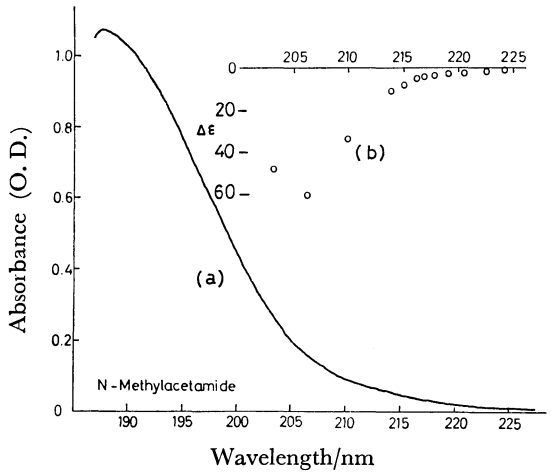

Fig. 2. (a) Ultraviolet absorption spectrum of 1.22×10^{-4} M *N*-methylacetamide in ^1H_2O at pH 5 and 25.2 °C. (b) A difference spectrum of *N*-methylacetamide *versus* *N*-methylacetamide-*d*, obtained from the kinetic measurement. Each point indicates the total absorbance change obtained for the first-order process observed on dilution with ^2H_2O to zero time. Ordinate scale $\Delta\varepsilon$ is the difference in the molar extinction coefficient ε.

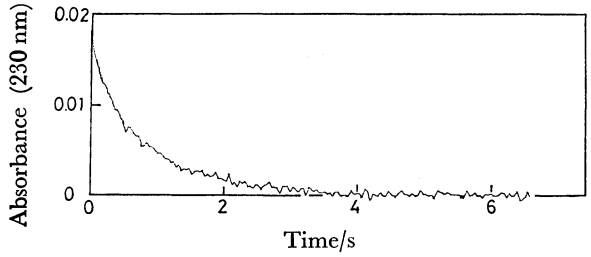

Fig. 3. The time dependence of the decrease in absorbance at 230 nm observed when poly-D,L-alanine dissolved in ^1H_2O is mixed with ^2H_2O, at pH 6.67 and at 13.5 °C. Final concentration of poly-D,L-alanine is 5.5 mM (for alanine residue).

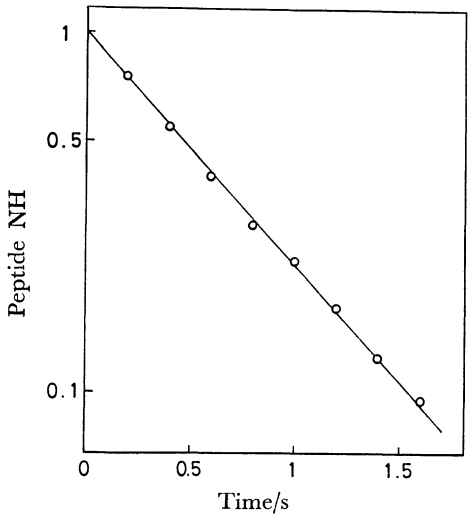

Fig. 4. Semilogarithmic plot of the data shown in Fig. 3.

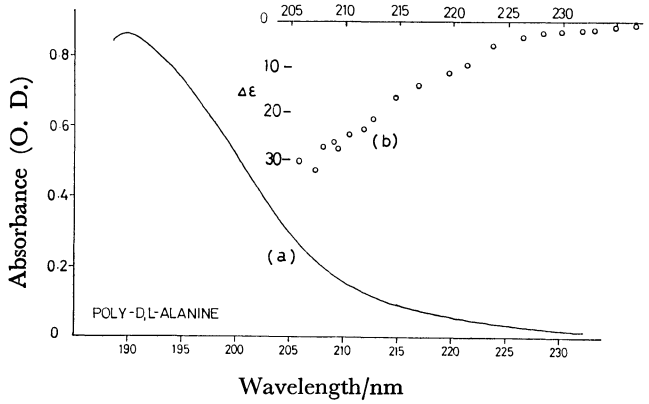

Fig. 5. (a) Ultraviolet absorption spectrum of poly-D,L-alanine in H_2O at pH 4 and 25.2 °C. (b) A difference spectrum of poly-D,L-alanine *versus* poly-D,L-alanine-*d*, obtained from the kinetic measurement, similar to what was described for Fig. 2(b).

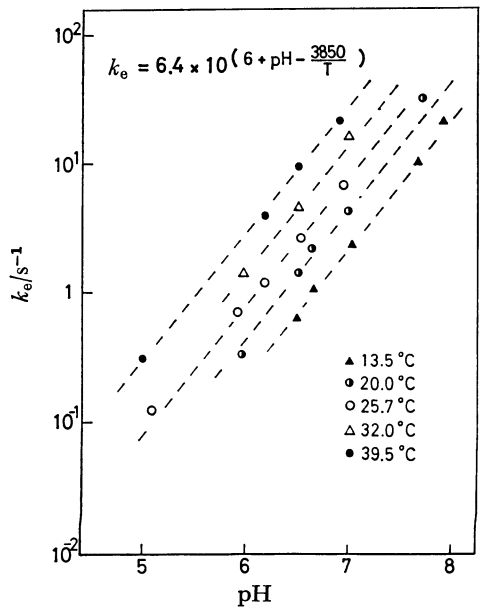

Fig. 6. The first-order rate constants k_e of the hydrogen-deuterium exchange reaction of poly-D,L-alanine, determined at various pHs and temperatures.

masured in this way at several different wavelengths generate a difference spectrum shown in Fig. 2(b). The rate constant of the NH→ND reaction is found to be 4.17 s^{-1} at pH 7.93 and at 22 °C and 0.31 s^{-1} at pH 6.83 and at 21 °C.

Hydrogen-Deuterium Exchange in Poly-D,L-alanine

Poly-D,L-alanine is an unfolded, non-helical polypeptide, and is soluble in H_2O. Every peptide group in this polypeptide is supposed to be "fully" exposed to the solvent at every pH and temperature in a proper range. This has been selected as a material, whose deuteration (NH→ND) rate (k_e) is used as a standard in estimating an attenuation factor γ_j with Eq. 1.

In Fig. 3, an example of the observed curve is given, which shows a time course of the absorbance change at 230 nm caused by the deuteration of poly-D,L-alanine. A replot of such a kinetic data on a logarithmic ordinate scale, as illustrated in Fig. 4, shows that the absorbance change takes place as a single first order process. The total absorbance change in such a process has been examined at various wavelengths. A difference spectrum of poly-D,L-alanine *versus* poly-D,L-alanine-*d*, thus obtained, is shown in Fig. 5.

The first-order rate constant k_e of the hydrogen-deuterium exchange reaction of poly-D,L-alanine has been determined in the pH range of 5—8 and temperature range of 10—40 °C. The results are graphically shown in Fig. 6. As may be seen in the figure, $\log_{10} k$ values fall on a single straight line at every pH, when they are plotted against reciprocal absolute temperature T. From the slope of this straight line the activation energy is estimated to be $\Delta H^* = 17.6 \pm 0.3$ kcal/mol, at every pH in the 5—8 range. When $\log_{10} k_e$ is plotted against pH, on the other hand, the points fall on a straight line of solpe=1, at every temperature. This fact indicates that in this pH range the reaction is catalyzed by OH^-. The empirical formula for the k_e value of poly-D,L-alanine is now given as

$$k_e = 6.4 \times 10^{(6+pH-3850/T)}. \quad (2)$$

It should be pointed out that what were previously given by an extrapolation[12-14] are slightly but appreciably different from the k_e values directly determined in our present work.

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