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TYROSYL RESIDUES OF TWO MOLECULAR FORMS OF HUMAN URINARY UROKINASE: THEIR IONIZATION MODE CHARACTERIZED BY SPECTROPHOTOMETRIC AND CD TITRATIONS AND REACTIVITY WITH CYANURIC FLUORIDE IN RELATION TO ENZYMIC ACTIVITY§

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The spectrophotometric titration curves (λ_{max}: 295 nm) of the tyrosyl residues (Tyr) of MW 55000 (H-UK) and 36000 (L-UK) forms of human urinary urokinase and their pH-esterase activity curves with N-acetyl glycyl-L-lysine methyl ester as a substrate showed that most of the activity was lost by alkali with the ionization of only 2 and 1 Tyr of all the 19 and 12 Tyr, respectively. The alkalized H- and L-UK showed difference CD spectra (λ_{max}: 250 nm) attributed to ionized Tyr. H-UK had the non-ionized Tyr (4.1) much more than those of L-UK (0.4), and the rapidly (11.8) and slowly (3.1) ionized Tyr as many as those of L-UK (7.7 and 3.9). Most of the activity decreased gradually as 14 and 10 Tyr of H- and L-UK reacted with cyanuric fluoride (CyF), respectively. For H-UK, the activity drastically decreased after 14 Tyr reacted with CyF, suggesting that some of 5 CyF-non-reactive Tyr including 4 Tyr of the non-ionized type stabilized the conformation near the active site.

Human urinary urokinase [EC3.4.21.31] is classified into two molecular forms. H-UK* (MW 5.4-5.5x10^4) has two polypeptide chains, while L-UK (MW 3.3-3.6x10^4) has a single chain involving the active site (1, 2). We have highly purified H- and L-UK, whose homogeneity is certified in view of molecular weight and immunogenicity (2), and investigated their electrophoretical properties (3), stability of plasminogen activator activity (4) and conformational change in the protein denaturation (5).

Any physico-chemical properties of individual amino acid residues of UK are unknown. In the case of trypsin, which is one of serine proteases (6) as UK (7), a specific Tyr is inferred to be hydrogen-bonded to one of the active site amino acid residues (8, 9). It is of interest whether the Tyr in H- and

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* ABBREVIATIONS: UK, human urinary urokinase; H-UK (L-UK), the heavy (light) form of UK; Tyr, tyrosyl residue(s); AGLME, N-acetyl glycyl-L-lysine methyl ester; CyF, cyanuric fluoride

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L-UK participate in the activity exhibition or stabilize the conformation near the active site. In this paper, we will show that alkaline inactivation of H- and L-UK is followed by ionization of only 2 and 1 Tyr of all the 19 and 12 Tyr, respectively. It will be suggested that any Tyr of the rapidly ionized type including these 1-2 Tyr and most of the slowly types do not participate in the conformational stabilization near the active site. Further, it will be indicated that there are few Tyr of the non-ionized type in L-UK, whereas H-UK has a considerable number of the non-ionized types, some of which possibly contribute to the conformational stabilization near the active site.

MATERIALS AND METHODS

Materials---UK purified from human urine by serial column chromatography (2) was used, whose homogeneity was examined by SDS-polyacrylamide gel electrophoresis, isotachoelectrophoresis, density-gradient isoelectric focusing and Ouchterlony's double immunodiffusion (2, 3). H-UK (MW 5.5×10^4) and L-UK (3.6×10^4) exhibited specific activities of 1.20×10^5 and 1.52×10^5 IU/mg protein, respectively (2). AGLME was purchased from Peptide Institute Inc., Osaka. CyF was prepared from cyanuric chloride by the method of Maxwell et al. (10). The solvents used for dissolving UK were (μ=0.30; KCl): pH 7-9, Tris-HCl buffer; pH 9-12, borate-HCl buffer; >pH 12, KOH solution. The pH's were readjusted with 1N HCl and KOH after dissolving UK.

CD Titration---The CD spectra were measured at 25°C under constant nitrogen flush by a JASCO spectropolarimeter, J-40, equipped with a data processor after UK solutions were left at 25°C and various pH's for 18 hr. The CD data were expressed in terms of mean residue ellipticity, [Θ], taking 111 and 112 for average residue weights of H- and L-UK, respectively (2).

Spectrophotometric Titration---The difference spectra were read at 25°C on a HITACHI spectrophotometer, 124, after UK solutions kept at a given pH and pH 7.0 for 10 min or 18 hr at 25°C were separately placed in the two compartments.

pH-Esterase Activity Curves---The activity was measured essentially by the method of Walton (11). To 4.8 ml of UK solution left at 25°C and various pH's for 10 min and 18 hr, 0.2 ml of AGLME solution was added. The carboxyl groups produced by hydrolysis of AGLME were determined by a Radiometer Inc. pH-stat apparatus. The carboxyl groups of AGLME solution alone were determined under the same conditions without UK, and this value was used for the correction.

Time Course of Activity and the Number of Ionized Tyr---Borate-HCl buffer, pH 10 and 12 (2 ml). was added to 0.5 ml of UK solution in distilled water at 25°C. The UK solution was subject to measurements of the number of ionized Tyr and esterase activity toward AGLME as described above.

Reaction of UK with Cyanuric Fluoride---To UK solutions (2.5 ml) left at pH 10 for 10 min or at pH 12 for 10 min and 18 hr (25°C), 0.3 ml of CyF solutions in dioxane was added. The mixtures were kept at 5°C for 2 hr. The number of CyF-reacting Tyr was spectrophotometrically determined by Kurihara et al. (12). The esterase activity toward AGLME was assayed after the by-products such as cyanuric acid were promptly removed from the CyF-reacting UK through Sephadex G-25 columns (1.2x10 cm) equilibrated with the same buffer as used for CyF-reaction. UK solutions were treated similarly without CyF, and their activity values were used for the correction.

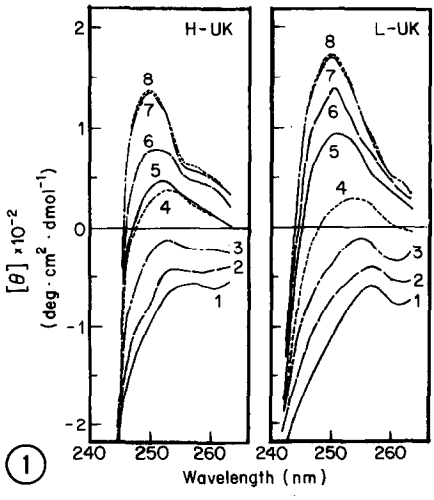
The Number of Ionized Tyrosyl Residues---The number was determined with the use of the $\Delta\varepsilon_{295}$ values per ionized Tyr (H-UK, 2410; L-UK, 2340 $M^{-1} \cdot cm^{-1}$) which were given when the maximal $\Delta\varepsilon_{295}$ measured with 6 M guanidine was divided by the number of all the Tyr per molecule (H-UK, 19; L-UK, 12) determined by the amino acid analysis (2).

Concentration of Protein---The protein concentrations of H- and L-UK were spectrophotometrically determined with $E^{1\%}_{1cm}$=12.6 and 12.8 at pH 7, respectively.

RESULTS

The CD spectra of H- and L-UK showed the increase in the ellipticity near 250 nm along with pH enhancement (Fig. 1). The difference CD spectra were obtained by subtracting the CD spectrum at pH 7.0 from those at alkaline pH's (Fig. 2). The maximal wavelength of the difference CD spectra was fixed almost to 250 nm. Sigmoid curves were produced by the plot of the maximal difference ellipticity at 250 nm against pH (Fig. 3).

The difference absorption spectra of alkalized UK had two maxima of 245 and 295 nm with or without 6 M guanidine (Fig. 4). The ratio of OD_{245}: OD_{295} was retained to be 4.6 below pH 12.6, which roughly accorded with the ratios in L-tyrosine (4.5) (13) and its derivative (4.8) (14). The spectrophotometric titration curves of H- and L-UK were obtained after alkalizing periods of 10 min and 18 hr at diverse pH's (Fig. 5). The Tyr could be categorized into three types according to their ionization modes without guanidine (Table 1). "Rapidly ionized type" was defined to be titrated at an alkalizing period



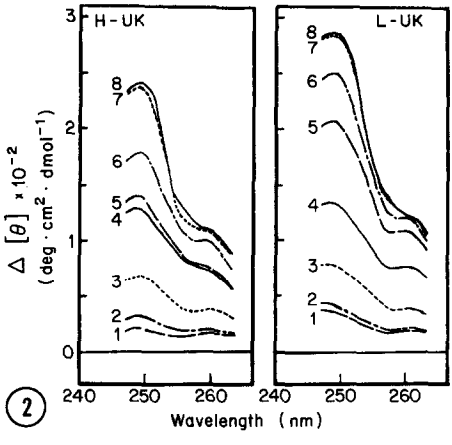


Fig. 1. Dependency on pH of CD spectra of H- and L-UK near 250 nm. pH: 1) 7.0; 2) 10.0; 3) 10.5; 4) 11.0; 5) 11.5; 6) 12.0; 7) 12.5; 8) 13.5

Fig. 2. Dependency on pH of difference CD spectra of H- and L-UK near 250 nm. The difference spectra were obtained by subtracting the spectrum at pH 7.0 from those at various pH's: 1) 9.5; 2-8) the same as in Fig. 1.

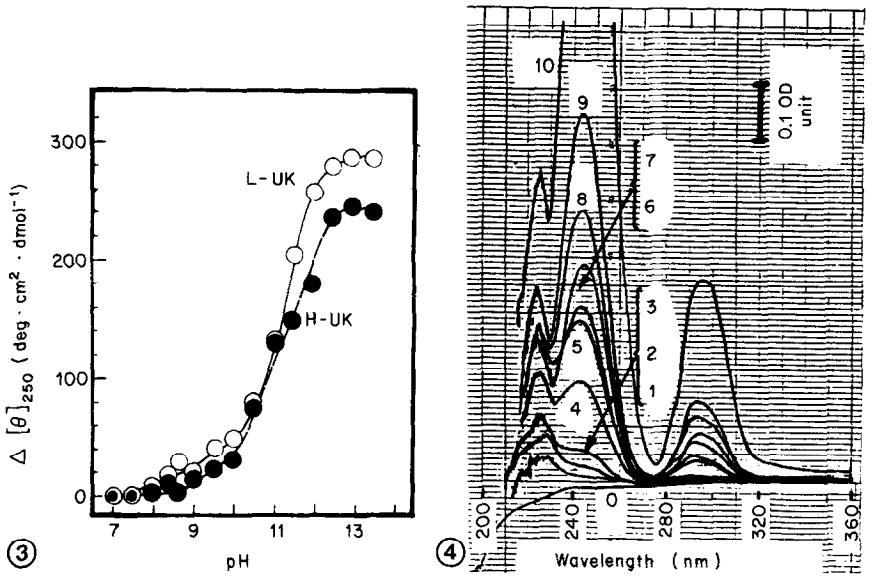


Fig. 3. CD titration curves of alkalized H- and L-UK at 250 nm. The data were obtained from the difference CD spectra as shown by Fig. 2.

Fig. 4. Difference UV absorption spectra of alkalized UK. The difference spectra of 2.49×10^{-5} M H-UK solutions kept at a given pH and pH 7.0 were obtained. pH: 0) 7.0; 1) 7.5; 2) 8.0; 3) 8.5; 4) 9.2; 5) 9.7; 6) 10.2; 7) 10.1; 8) 10.4; 9) 10.4; 10) 11.7. Shapes of the spectra were unaltered whether H- or L-UK was left at alkaline pH's for 10 min or 18 hr.

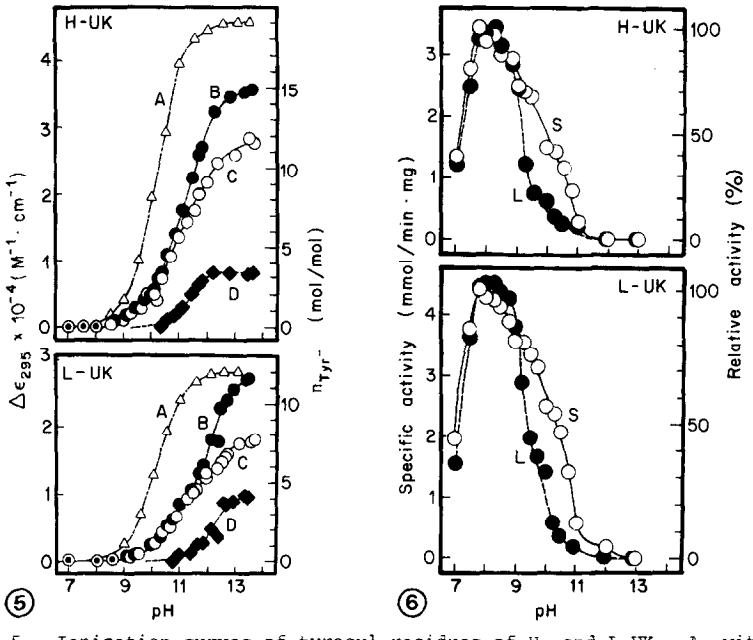


Fig. 5. Ionization curves of tyrosyl residues of H- and L-UK. A, with 6 M guanidine for 18 hr; B and C, without guanidine 18 hr and 10 min after pH adjustment, respectively; D, theoretical curve obtained by calculating for the Tyr of H- and L-UK with pK=11.3 and 12.1 (m=2), respectively; ◆, the values obtained by subtracting Curve C from the experimental values on Curve B; n_{Tyr^-}, the number of ionized Tyr per UK molecule

Fig. 6. The pH dependency of the activity of H- and L-UK. The esterase activity toward AGLME was determined at 25°C after H- and L-UK were kept at indicated pH's and 25°C for 10 min (S) and 18 hr (L).

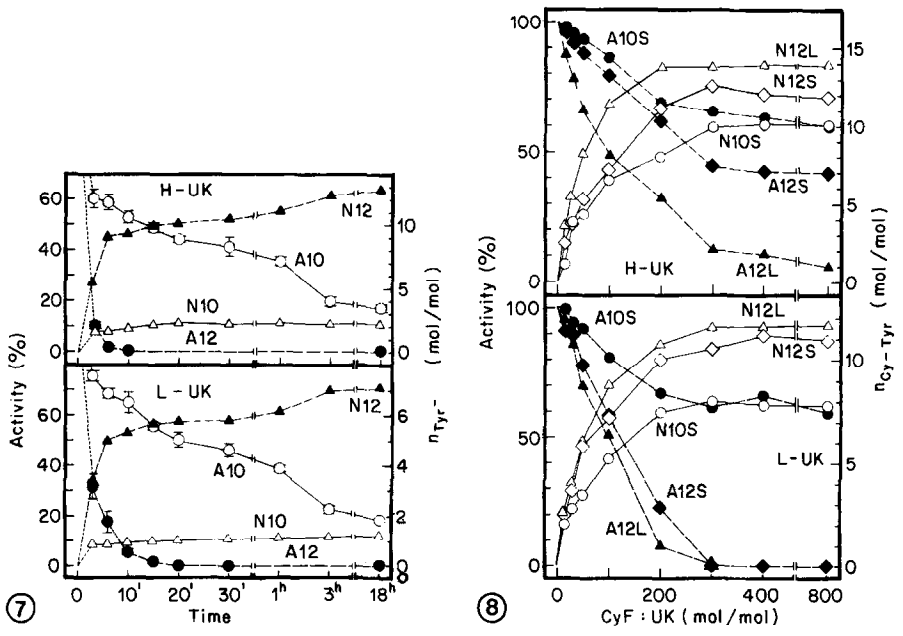


Fig. 7. Time course of the activity and the number of ionized tyrosyl residues of H and L-UK left at alkaline pH's. H- and L-UK were left at pH 9.98±0.04 (10) and 11.89±0.05 (12) for indicated periods, respectively. Then, the esterase activity toward AGLME (A) and the number of ionized Tyr (N) were determined.

Fig. 8. Reaction curves of UK and cyanuric fluoride in relation to the activity. H- and L-UK (2.49×10^{-5} M) were incubated at pH 9.94 for 10 min (10S) or at pH 11.92 for 10 min (12S) and 18 hr (12L), and then mixed with CyF at the indicated molar ratios. The number of CyF-reacting Tyr (N; $n_{Cy\text{-}Tyr}$) was spectrophotometrically determined. The esterase activity (A) toward AGLME was assayed after column chromatography on Sephadex G-25.

of 10 min. "Slowly ionized type" was titrated after 18 hr but not after 10 min. "Non-ionized type" was not titrated even after 18 hr.

The pH-esterase activity curves with AGLME as a substrate were determined at alkalizing periods of 10 min and 18 hr (Fig. 6). H- and L-UK were inactivated above pH 9.0 from 10 min to 18 hr. L-UK was slightly activated at pH 7.8-9.0 with the optimal pH shift of 7.8 to 8.0, while H-UK was scarcely activated. The esterase activity toward AGLME decreased in parallel with the increase in the number of ionized Tyr in the lapse of alkalizing period at pH 10 and 12 (Fig. 7).

The esterase activity toward AGLME and the number of CyF-reacting Tyr were measured, after UK was left at pH 10 for 10 min or at pH 12 for 10 min and 18 hr and then reacted with various amount of CyF (Fig. 8). The activity decreased in parallel with the increase in the number of CyF-reacting Tyr.

DISCUSSION

The ratio of the rapidly ionized Tyr to all the Tyr in H-UK (11.8/19.0) is as high as that in L-UK (7.7/12.0) as shown in the ionization curves determined by the spectrophotometric titration at 295 nm (Fig. 5), indicating that most of Tyr are situated at the outer portion of the protein molecules. The slowly ionized types (H-UK, 3.1/19.0; L-UK, 3.9/12.0) need 2 hydroxyl ions per Tyr in the ionization process (Table 1), which indicates that one hydroxyl ion splits a hydrogen bond between the Tyr of the bound form and another residue, while another hydroxyl ion transforms a phenolic group of the free Tyr into the phenoxide ion. The pK's of the rapidly and slowly ionized types are higher than that of L-tyrosine (10.0) (15). On the other hand, H-UK has a considerable number of non-ionized Tyr (4.1/19.0), whereas L-UK has a much smaller number (0.4/12.0). The results indicate that 4 Tyr are deeply embedded inside hydrophobic regions of H-UK as reported for ribonuclease (16, 17), and/or hydrogen-bonded to CO groups of the polypeptide backbone or to COOH groups of aspartyl or glutamyl residues of anomalously high pK's as observed for myoglobin and egg white albumin (18, 19).

Alkalized H- and L-UK show positive difference CD spectra at 250 nm (Fig. 2), which are attributed to the ionized Tyr as indicated by the parallelism between the CD titration curves (Fig. 3) and the ionization curves of the Tyr (Fig. 5). Any ionized Tyr of H- and L-UK show similar $\Delta[\theta]_{250}$ per ionized Tyr (8400 and 7700 $deg \cdot cm^2 \cdot dmol^{-1}$, respectively) obtained by dividing the maximum of $\Delta[\theta]_{250}$ (Fig. 3) by the ratio of ionized Tyr (Table 1) to all the amino acid residues (2). Any ionized Tyr do not differ in the CD property.

The correlation between the number of ionized Tyr and the esterase activity toward AGLME is clarified (Fig. 9 I&II) by comparing the ionization curves

Table 1. Characterization of ionization of the tyrosyl residues in H- and L-UK

	Rapidly ionized type			Slowly ionized type			Non-ionized type	Total
	n	pK	m	n	pK	m	n	n
H-UK	11.8	11.0	1	3.1	11.3	2	4.1	19.0
L-UK	7.7	11.1	1	3.9	12.1	2	0.4	12.0

n represents the number of each type per UK molecule. m represents the number of hydroxyl ions to ionize a tyrosyl residue, and corresponds to the order of the sigmoid ionization curve. The total number of tyrosyl residues was determined by the amino acid analysis (2). The other data were obtained from Fig. 5.

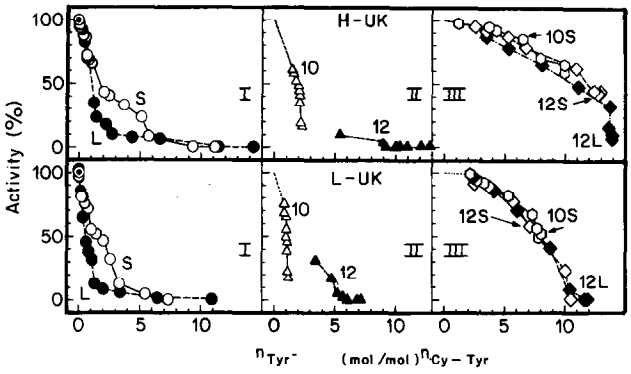


Fig. 9. Correlation between the activity and the number of ionized or cyanuric fluoride-reacting tyrosyl residues of H- and L-UK. I, II) The correlation between esterase activity toward AGLME and the number of ionized Tyr (n_{Tyr}-) was clarified by comparing Fig. 5 with Fig. 6 at alkalizing periods of 10 min (S) and 18 hr (L), and also by combining Curves A and N at pH 10 and 12 in Fig. 7, respectively. III) The correlation between the activity and the number of CyF-reacting Tyr (n_{Cy-Tyr}) was elucidated by combining Curves A and N at pH 10 for 10 min (10S), or at pH 12 for 10 min (12S) and 18 hr (12L) in Fig. 8.

(Fig. 5) with the pH-activity curves (Fig. 6) and also by combining the time course curves of the activity and the number of ionized Tyr (Fig. 7). The first phase of loss of most of the activity can be expressed as a linear function of increase in the number of ionized Tyr. This linear portion corresponds to 2 and 1 ionized Tyr for H- (Fig. 9 I-S, 2.0; I-L, 1.4; II, 2.0) and L-UK (I-S, 1.0; I-L, 0.9; II, 1.0), indicating that the conformational disruption near the active site in H- and L-UK is followed by ionization of 2 and 1 Tyr, respectively. These Tyr appear not to contribute to the conformational stabilization near the active site, since they belong to the rapidly ionized types m=1 (Table 1). In contrast, one specific Tyr of the slowly ionized type is inferred to be hydrogen-bonded to the aspartyl residue of the active site for trypsin (8, 9), which is one of serine proteases (6) as UK (7).

The correlation between the number of CyF-reacting Tyr and the remaining activity is elucidated (Fig. 9 III) for the reaction at various molar ratios of CyF to UK (Fig. 8). Most of the activity gradually decreases as 14 and 10 Tyr of all the 19 and 12 Tyr in H- and L-UK react with CyF, respectively, indicating that these Tyr are not essential for stabilizing conformation near the active site. Moreover, these CyF-reacting Tyr are suggested to include all of the rapidly ionized types and most of the slowly ionized types, but not

the non-ionized types, since the reactivity of a Tyr with CyF is closely associated with its ionization degree despite some deviations due to the differences in the molecular size and hydrophobicity of CyF and hydroxyl ion (12, 17, 20, 21). On the other hand, the 5 and 2 Tyr other than these 14 and 10 CyF-reacting Tyr appear to be occupied mainly by the non-ionized types for H-UK and by the slowly ionized types for L-UK, respectively. In the case of H-UK, some of the non-ionized types, which show a considerable number in contrast to L-UK (Table 1) and are CyF-non-reactive (Fig. 8), possibly contribute to the conformational stability near the active site. This is also suggested by a drastic decrease from 32 to 0% in the activity corresponding to 14 CyF-reacting Tyr for H-UK (Fig. 9 III).

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