

[Chem. Pharm. Bull.]
30(10)3669—3678(1982)

Alteration of Polypeptide Backbone Structure as determined by Circular Dichroic Spectral Analysis in Relation to Change of Plasminogen Activator Activity of Two Forms of Human Urinary Urokinase denatured with Guanidine Hydrochloride¹⁾

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(Received February 18, 1982)

Alterations of polypeptide backbone structure and plasminogen activator activity of human urinary urokinase (UK) denatured with 0–6 M guanidine hydrochloride (Gdn-HCl) were investigated. The fractions of helix, β -form, β -turn and unordered structure were determined by the best-curve-fitting method based on the circular dichroic (CD) spectra at 200–240 nm to be 0.14, 0.23, 0.26 and 0.37 for M. W. 55000 form (H-UK) and 0.12, 0.17, 0.32 and 0.40 for M.W. 36000 form (L-UK), respectively.

In view of the variation with Gdn-HCl concentration of ellipticity at the negative extrema (204 nm for H-UK; 202 nm for L-UK), the denaturation process was concluded not to be a two-state transition: at 2.0–3.0 M Gdn-HCl, the helical and β -form fractions were immobilized within a narrow range of 0.00–0.01 for both UK forms, corresponding to almost unchanged minimal ellipticities even with the increase of Gdn-HCl concentration. This indicates that an intermediate state (I) exists besides the native (N) and denatured (D) states.

At 0.75–1.25 M Gdn-HCl, an activated state (A) intervened between N and I for H-UK but not for L-UK. The N→A transition of H-UK was an exceptional transition in that the helicity steadily increased with the enhanced minimal ellipticity. This corresponded to the observation that the activity of H-UK was potentiated by 18% during this transition. At the same Gdn-HCl concentration, L-UK reduced both its activity by 27–38% and its minimal ellipticity in contrast to H-UK.

These findings show that the denaturation processes consist of “four states-three transitions” for H-UK and “three states-two transitions” for L-UK.

Keywords—urokinase; circular dichroism of protein; polypeptide backbone structure; protein denaturation; guanidine hydrochloride; plasminogen activator activity; best-curve-fitting method for CD spectrum

The secondary structure and conformation of human urinary urokinase (UK) [EC 3.4.21.31] have been investigated only to a limited extent by means of circular dichroic (CD) techniques. This is mainly because UK has not been purified sufficiently highly to exclude traces of optical-rotatory contaminants from preparations of the heavy form (H-UK; M.W. $5.4\text{--}5.5 \times 10^4$) and the light form (L-UK; M.W. $3.3\text{--}3.6 \times 10^4$) from human urine.²⁾

H- and L-UK were shown to be composed of 429 and 279 amino acid residues per molecule, respectively.²⁾ H- and L-UK were suggested to consist of two and a single polypeptide chains, respectively,^{3,4)} and they were demonstrated to be glycoproteins.^{5,6)} H-UK appeared to activate glutamyl-plasminogen by splitting a single peptide bond, whereas L-UK split 2 peptide bonds of glutamyl-plasminogen.⁷⁾

In this work, the secondary structure in native UK was determined by CD analysis. The changes of CD spectra in the far UV region with alteration of pH and with increasing denaturant concentration were investigated, using highly purified²⁾ H- and L-UK preparations whose enzymological⁸⁾ and electrophoretic^{2,9)} properties have already been examined. Furthermore, we tried to determine, by comparison of the two UK forms, how the alteration of the polypeptide backbone structure corresponded to the change of the enzymic activity during protein denaturation with guanidine hydrochloride.

Experimental

Materials—Highly purified UK's were obtained by serial column chromatography²⁾ and their homogeneity was examined by SDS-polyacrylamide gel electrophoresis, isotachoelectrophoresis, isoelectric focusing and Ouchterlony's double immunodiffusion method.^{2,9)} H- and L-UK have molecular weights of 5.5×10^4 and 3.6×10^4 , respectively.²⁾ The specific activities of H- and L-UK were 1.20×10^5 and 1.52×10^5 IU/mg protein, respectively.²⁾ Guanidine hydrochloride (Gdn-HCl) was purchased from Nakarai Pure Chemicals Co., Kyoto, and was recrystallized once from ethanol-benzene, then once from water.

Circular Dichroism (CD)—CD measurements were achieved with a JASCO automatic recording spectropolarimeter, model J-40. Instrument performance was calibrated with D-pantolactone¹⁰⁾ and D-camphor-10-sulfonic acid¹¹⁾ as standards at 220 and 290 nm, respectively. The CD data were expressed in terms of mean residue ellipticity, $[\theta]$, in $\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. The mean residue weight was calculated from the amino acid composition.²⁾ CD measurements were conducted in 20-, 10-, 5-, 2- and 1-mm cells at a protein concentration of 30 mg/dl; near and below this concentration, $[\theta]$ in the far ultraviolet (UV) was confirmed to be independent of UK concentration, indicating the absence, in this such a concentration range, of protein aggregation as affecting optical-rotatory activity. UK solution was adjusted to 25°C using a thermostatically controlled cell holder under constant nitrogen flush.

Secondary Structure of Protein—The secondary structure of UK was determined on the basis of CD spectra in the far UV (200–240 nm) essentially by the best-curve-fitting method of Chang *et al.*¹²⁾

Plasminogen Activator Activity—Using the International Standard preparation for calibration, the activity was determined by the fibrin tube method of Ploug and Kjeldgaard.¹³⁾

Protein Amount—The protein amount was determined colorimetrically by the method of Lowry *et al.*¹⁴⁾ with bovine serum albumin as a standard.

Effects of pH on CD Spectra—H- and L-UK were dissolved in 20 mM McIlvaine buffer for wide pH range, $\mu=0.15$ KCl, pH 1.0–13.0 (Buffer I), at a protein concentration of 30 mg/dl and readjusted to pH 1.0–13.0. After being left at 25°C for 24 h, UK solutions were subjected to CD measurements. The pH values were remeasured immediately after the CD measurements.

Protein Denaturation with Gdn-HCl—The stock solution of Gdn-HCl dissolved in 50 mM Tris-HCl buffer–300 mM NaCl, pH 8.6 (Buffer II) was added gradually to Buffer II containing UK. This solution was readjusted to pH 8.6 with a trace of 2 N HCl or KOH solution and incubated at 37°C for 24 h. Under these conditions but without addition of Gdn-HCl, both UK forms were confirmed to retain their plasminogen activator activities for at least 48 h.⁸⁾ After the incubation, UK solutions were left for 2 h at 25°C and subjected to CD measurements. To correct the baseline, successive CD measurements were conducted with the reference which contained no UK but the same concentration of Gdn-HCl as in the UK samples. Plasminogen activator activity was determined at 3 and 6 min after the counterparts of the same duplicate samples as used for CD measurements had been diluted 50-, 100-, 200- and 600-fold with Buffer II. The UK samples were diluted further 17-fold and assayed essentially according to the method of Ploug and Kjeldgaard.¹³⁾ The plasminogen activator activities at various Gdn-HCl concentrations were obtained by extrapolating the activity values at 3 and 6 min to zero time. This two-step dilution was confirmed to exclude the direct effect of Gdn-HCl on the fibrinolysis rate even in the case of 6 M Gdn-HCl solution, which was diluted finally to 7.2 mM at the maximum in the assay system. The direct addition of Gdn-HCl at concentrations below 7.2 mM to the assay system had no effect on the activity.

Results

Secondary Structures of H- and L-UK determined by CD Spectral Analysis

The CD spectra of H- and L-UK in the far UV region (Fig. 1) were measured under the optimal conditions for maintaining their plasminogen activator activities, at pH 8.6 and an ionic strength of 0.15.⁸⁾ The fractions of helix, β -form, β -turn and unordered structure were determined (Table I) from the mean residue ellipticities at 200–240 nm by the best-curve-fitting method.

The standard deviations of the fractions of helix, β -form, β -turn and unordered structure, which were determined on the basis of CD spectra measured several times, were 0.003, 0.004, 0.001 and 0.002 for H-UK and 0.003, 0.004, 0.002 and 0.000 for L-UK, respectively, showing high reproducibility of the CD measurements used for determination of the secondary structure of UK.

The calculated CD spectra (Fig. 1), which were obtained from the secondary structure determined by the CD analysis, were in rough accordance with the observed CD spectra for

the most part, but deviated somewhat near the negative extremum in the far UV. Below the wavelength of the extremum, aromatic groups begin to show remarkably high extinction coefficients¹⁵⁾ and the CD of the helix shows marked chain-length dependence.¹⁶⁾ The coincidence of this curve-fitting of H- and L-UK was not compared unfavorably with that of eight kinds of proteins reported by Chang *et al.*¹²⁾

The helical contents in both UK forms were comparatively low as water-soluble enzymes (Table I). The content of prolyl residues was pointed out to be inversely related to the α -helical content.¹⁷⁾ A protein with a prolyl residue content of less than 3% can possess more than 50% α -helix, while a protein with more than 8% proline is almost necessarily random-coiled. Both H- and L-UK contain relatively many prolyl residues (5.8 and 5.7%, respectively)²⁾ as

TABLE I. Secondary Structures of H- and L-UK determined by CD Analysis

	Fractions of the whole protein molecule			
	Helix	β -Form	β -Turn	Unordered structure
H-UK	0.14	0.23	0.26	0.37
L-UK	0.12	0.17	0.32	0.40

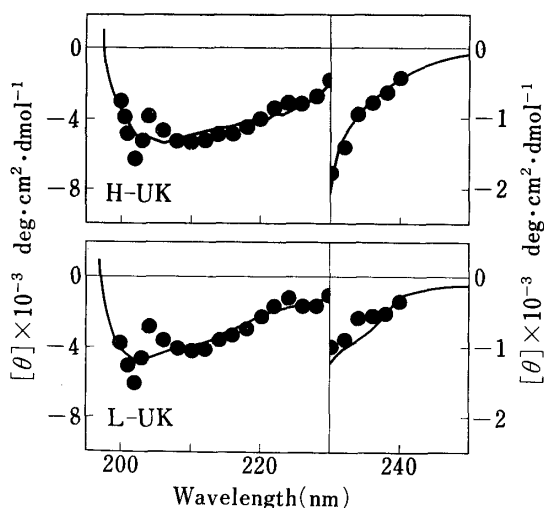


Fig. 1. CD Spectra in the Far UV of highly Purified H- and L-UK

A similar CD spectrum was observed for UK solutions of 5–30 mg protein/dl at pH 8.2–9.0. A calculated mean residue ellipticity was obtained as the sum of ellipticities attributed to the helix, β -form, β -turn and unordered structure, on the basis of the secondary structure of UK determined by the best-curve-fitting method for CD spectra.

—, the observed CD spectrum; ●, the calculated mean residue ellipticity.

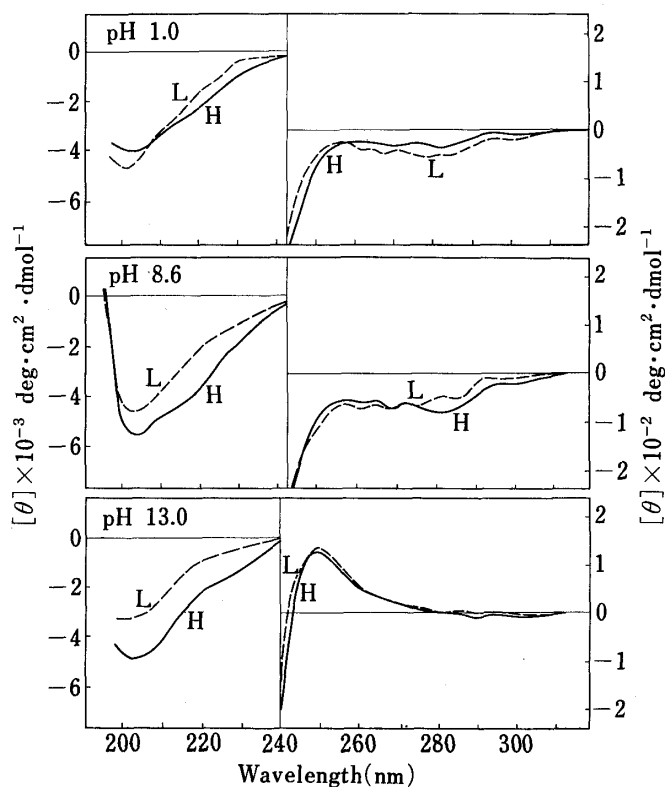


Fig. 2. CD Spectra in the Far and Near UV at Various pH's

CD spectra were measured for UK of 30 mg protein/dl in 20 mM McIlvaine buffer, $\mu=0.15$; KCl (Buffer I), after the solution had been left for 24 h at 25°C. The pH values were remeasured immediately after the CD measurements. There was no significant difference in CD spectra of UK in Buffer I (pH 1.0, 8.6 and 13.0) and other buffers with corresponding pH (20 mM; $\mu=0.15$; KCl): KCl-HCl buffer at pH 1.0; Tris-HCl buffer at pH 8.6; Na_2HPO_4 -NaOH buffer at pH 13.0. H, H-UK; L, L-UK.

compared with many other water-soluble enzymes, *e.g.* egg-white lysozyme, 1.6%; ribonuclease A, 3.2%; π -chymotrypsin A, 3.0%. Accordingly, the low helicity of both UK forms is not unreasonable from the standpoint of proline content.

Effects of pH Change on CD Spectra in the Near and Far UV Regions

The changes of CD spectra were investigated for both UK forms in response to pH change at a fixed ionic strength ($\mu=0.15$; KCl), roughly in the near UV (Fig. 2) and in detail in the far UV (Fig. 3).

In the near UV, several humps were observed and all CD bands showed negative Cotton effects for both UK forms at pH 1.0 and 8.6. Alkalinization of UK rendered these troughs of CD bands shallow on the whole and caused the emergence of a positive band at 250 nm, which was assumed to be attributable to the dissociation of phenolic groups of tyrosyl residues to phenoxylate ions as reported upon alkalinization of some other proteins and synthetic poly-

peptides.¹⁸⁻²¹ The enhancement of the CD band at 250 nm induced by alkalinization was pronouncedly large in its ellipticity as compared with other CD bands of UK in the near UV.

The minima of the negative ellipticities at 198–207 nm are shown as a function of pH in Fig. 3. The ellipticities of both UK forms at 198–207 nm had almost fixed values between pH 8.0 and 9.5 with constant minimal wavelengths. The plateau of the pH-stability curve of plasminogen activator activity of both UK forms was shown in a previous paper⁸) to be from pH 8.2 to 9.0. Therefore, the pH range between 8.2 and 9.0 was considered to be favorable both for the maintenance of enzymic activity and the stability of ellipticity to pH variation for H- and L-UK. In order to investigate the conformational alteration of UK produced by a denaturant, accordingly, it is recommended that CD spectra in the far UV should be measured at $\text{pH } 8.6 \pm 0.4$.

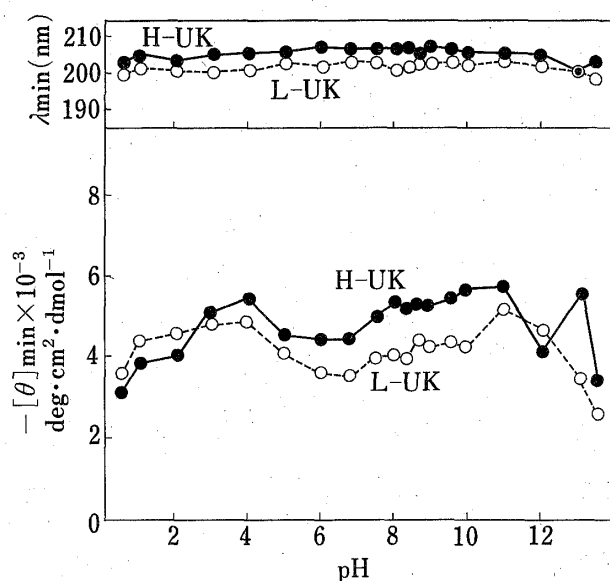


Fig. 3. Dependence on pH of Minimal Ellipticity and Its Wavelength in the Far UV for H- and L-UK

CD spectra at 200–250 nm were obtained for UK dissolved in Buffer I. The minimal ellipticity and its wavelength in a single trough of the CD spectra measured in the same manner as in Fig. 2 are expressed as a function of pH.

Alteration of CD Spectra after Denaturation with Gdn-HCl

CD spectra were measured after UK had been denatured with Gdn-HCl, which rarely gives rise to side-reactions such as cyanate formation from urea.²² The trough at 202–204 nm tended to become shallower as the concentration of Gdn-HCl added to either UK form was increased. However, the trough of H-UK deepened at Gdn-HCl concentrations as low as 0.50–1.00 M (Fig. 4).

The ellipticity in the far UV was minimal at 204 and 202 nm for H- and L-UK, respectively, with no added Gdn-HCl. The ellipticities at these wavelengths are shown as a function of Gdn-HCl concentration for H- and L-UK in Fig. 5. This variation of the minimal ellipticity with Gdn-HCl concentration showed that the denaturation of UK with Gdn-HCl was not a typical two-state process involving only the native and denatured states, as observed for some proteins.^{23,24} The Gdn-HCl denaturation processes of H- and L-UK were implied to consist of “four states and three transitions” and “three states and two transitions,” respec-

tively, taking into consideration that the measurement errors for the mean residue ellipticity near the trough at 202–204 nm were less than 2%.

Below 0.50 M Gdn-HCl, both UK forms retained almost fixed ellipticities. These starting states are designated as native (N). The first transition of H-UK occurred at 0.50–0.75 M. This transition is assumed not to be a process of simple randomization of the ordered structure, because the trough of the CD band at 204 nm deepened gradually at a constant wavelength of the minimal ellipticity until Gdn-HCl concentration exceeded 1.00 M. The first transition of L-UK is assumed to occur at 0.50–2.00 M Gdn-HCl, because the ellipticity was lowered gradually. This suggests that L-UK was gradually randomized in the first transition in contrast with H-UK.

The second and third transitions of H-UK corresponded to Gdn-HCl concentrations of 1.25–2.00 M and 3.00–4.00 M, respectively. These transitions are inferred to be accompanied with partial disruption of the folded polypeptide chains, since the CD spectra more closely

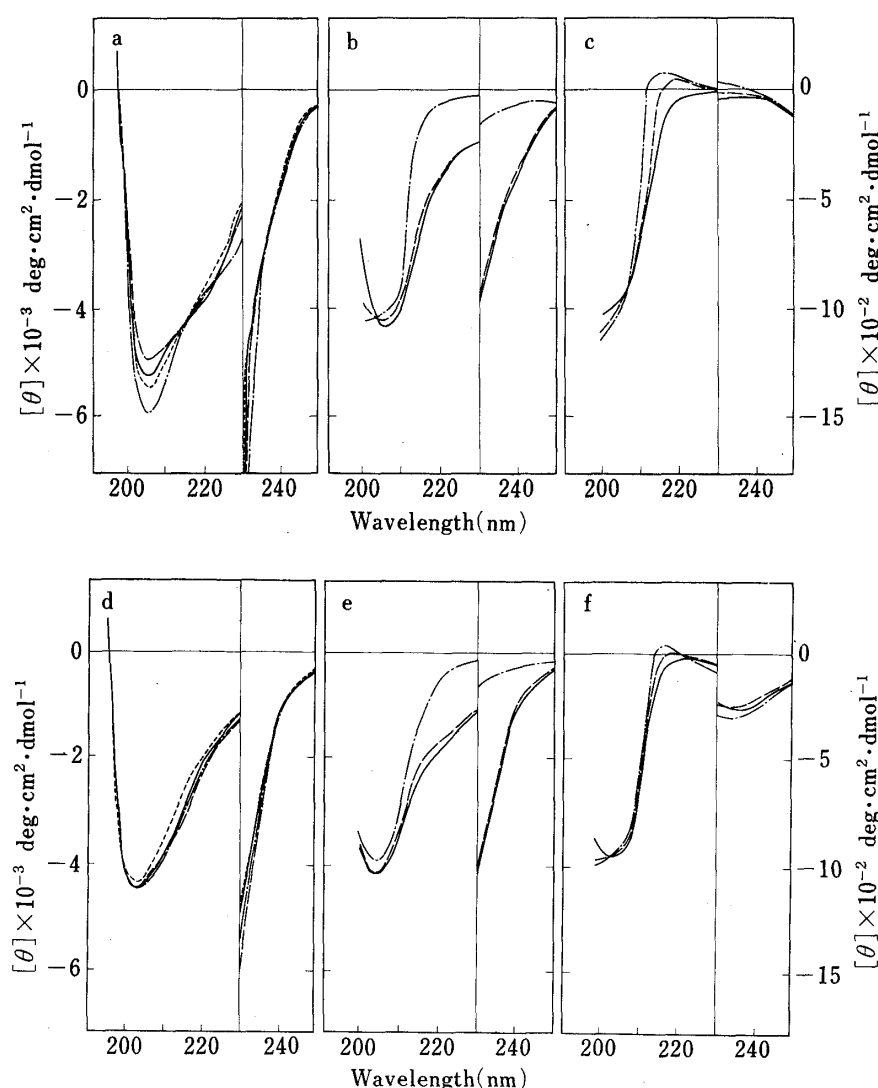


Fig. 4. CD Spectra of H- and L-UK in the Far UV at Various Gdn-HCl Concentrations

CD spectra were measured for UK's which had been treated for 24 h at 37°C with Gdn-HCl dissolved in 50 mM Tris-HCl buffer-300 mM NaCl, pH 8.60, then left for 2 h at 25°C. The Gdn-HCl concentrations are indicated in terms of M:

H-UK	{	(a)	{	(d)	—, 0.00; ———, 0.50; — · — · —, 1.00; ·····, 1.50.
		(b)		(e)	—, 2.00; ———, 2.50; — · — · —, 3.00.
		(c)		(f)	—, 4.00; ———, 5.00; — · — · —, 6.00.

resembled those of random coil as the Gdn-HCl was increased. The second state of H-UK at 0.75–1.25 M Gdn-HCl showed an ellipticity larger than that of N. This was observed for H-UK but not for L-UK. At 2.00–3.00 M Gdn-HCl, H- and L-UK were in the intermediate state (I) corresponding to the third and second states, respectively.

Above 4.00 M H- and L-UK reached the final state designated as the denatured state (D). The threshold concentration of Gdn-HCl, above which the CD spectra scarcely altered with further increase of the denaturant concentration was 3.50–4.00 M for both UK forms. Accordingly, from the viewpoint of the variation of the minimal ellipticity in the far UV with Gdn-HCl concentration, the denaturation processes of H- and L-UK were similar to each other, except for the intervention of the second state at 0.75–1.25 M Gdn-HCl between N and I for H-UK alone.

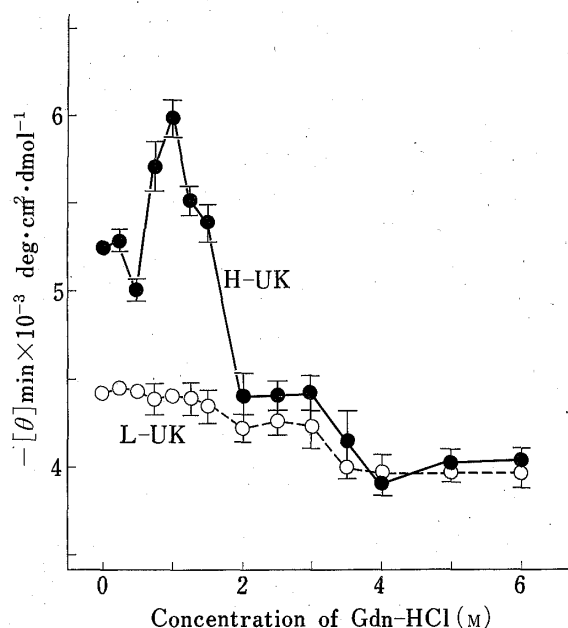


Fig. 5. Minimal Ellipticity in the Far UV as a Function of Gdn-HCl Concentration

The mean residue ellipticity at the wavelength of minimal ellipticity in the CD spectrum (illustrated by Fig. 4) is plotted against Gdn-HCl concentration. Each point and bar represent the average and standard deviation, respectively, obtained from three independent experiments. A point without a bar has a standard deviation of less than 50 $\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$.

forms rapidly restored their activities, indicating that irreversible inactivation requires a higher Gdn-HCl concentration and/or a longer exposure duration to the denaturant.

H-UK retained its activity completely after treatment with Gdn-HCl below 0.75 M and was activated by 18% at maximum with 0.75–1.25 M Gdn-HCl (Fig. 7). In appearance, this slight activation of H-UK corresponds to the increase in negativity of the ellipticity at 204 nm and is regarded as reflecting an activated state (A) such as is known to exist for some proteins.²⁵⁾ In contrast with H-UK, L-UK failed to maintain the initial activity completely even at a concentration of Gdn-HCl as low as 0.50 M. Therefore, in terms of resistance to such low concentrations of the denaturant, H-UK was superior to L-UK from the standpoints of both activity retention and the stability of the negative ellipticity at 202–204 nm (Fig. 5). Above 1.50 M Gdn-HCl, the alteration of enzymic activity exhibited by H-UK as a function of Gdn-HCl concentration was similar to that by L-UK.

The activity enhancement of H-UK is considered not to be an artifact for the following reasons.

Alteration of Plasminogen Activator Activity after Gdn-HCl Denaturation

Plasminogen activator activity was determined after treatment of UK with Gdn-HCl followed by dilution of the denaturant. The activity could not be directly measured without diluting Gdn-HCl. This is because the effects of Gdn-HCl on the denaturations of fibrin clot and plasminogen and on the hydrolytic action of plasmin toward fibrin clot were detected in the assay system¹³⁾ where fibrinolysis was conducted at the same Gdn-HCl concentration as contained in the UK sample used for CD measurement.

The plasminogen activator activity which was lost by Gdn-HCl denaturation was not recovered within 6 min after UK solution containing Gdn-HCl was diluted (Fig. 6). Furthermore, there was no significant difference in activity recovery between 3 and 6 min after diluting Gdn-HCl. These observations indicate that activity values within 6 min were similar to those before the dilution of Gdn-HCl. Even at a Gdn-HCl concentration as high as 4.00 M, both UK

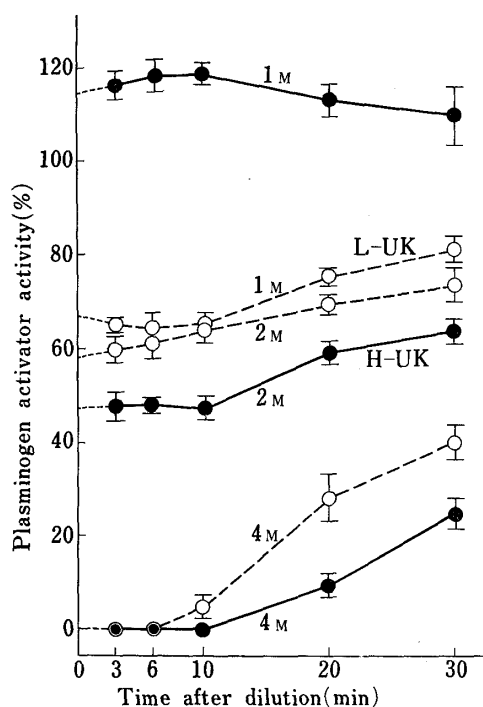


Fig. 6. Time Course of Plasminogen Activator Activity after diluting H- and L-UK treated at Various Gdn-HCl Concentrations

UK was treated with Gdn-HCl in the same manner as in Fig. 4. The Gdn-HCl-treated UK was diluted 50-, 100-, 200- and 600-fold. The UK samples were further 17-fold diluted. At the indicated time after the first dilution, the UK solutions were subjected to assay of plasminogen activator activity. Each point and bar represent the average and standard deviation obtained from three independent experiments. A point without a bar has a standard deviation of less than a relative activity of 1.5%. A dotted line represents the extrapolation to zero time of the activity values at 3 and 6 min.

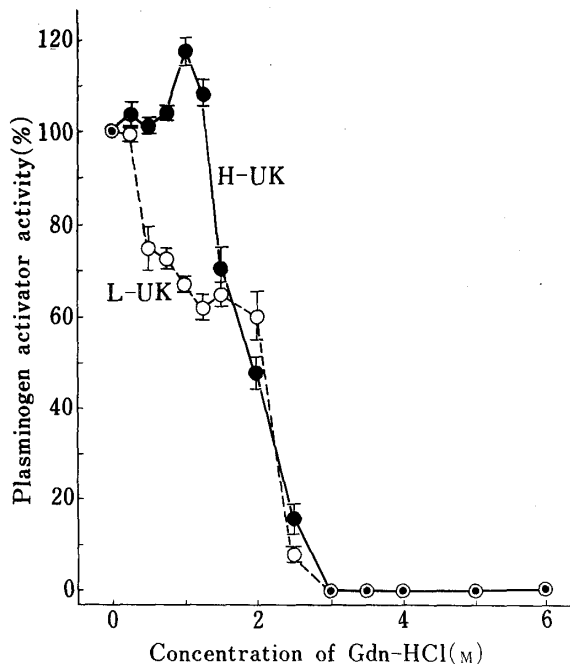


Fig. 7. Plasminogen Activator Activity of Gdn-HCl-Treated H- and L-UK determined by the Successive Dilution Method

UK treated with the indicated concentration of Gdn-HCl was diluted and assayed for plasminogen activator activity in the same manner as in Fig. 6. The indicated activity values were obtained by extrapolating the values at 3 and 6 min to zero time. Each point and bar represent the average and standard deviation obtained from three independent experiments. A point without a bar has a standard deviation of less than a relative activity of 1.0%.

(1) The fibrinolytic activity was scarcely potentiated when Gdn-HCl was directly added to the assay system at the same denaturant concentration as for the activation of H-UK. This ruled out the possibility that the activity potentiation of H-UK was related to the activation by Gdn-HCl of plasmin which was formed from plasminogen by UK in the assay system.¹³⁾

TABLE II. Alterations of Fractions of Helix and β -Form from Their Initial Values determined by CD Analysis in the Native, Activated, Intermediate and Denatured States of Gdn-HCl Denaturation for H- and L-UK

	Changes in fractions													
	Helix				β -Form									
	N	A	I	D	N	A	I	D	N	A	I	D		
H-UK	0.00	+0.01	-0.01	+0.02	-0.02	-0.06	-0.02	-0.00	-0.06	-0.01	-0.10	-0.09	-0.18	-0.11
L-UK	0.00	—	0.00	-0.02	-0.01	-0.02	-0.00	—	-0.06	-0.05	-0.16	-0.10		

(2) An ionic strength effect of Gdn-HCl cannot explain either the activation of H-UK or the CD enhancement of H-UK treated with 0.75–1.25 M Gdn-HCl, since the addition of 1.0 M KCl to H-UK without any added Gdn-HCl had no effect on the plasminogen activator activity or the CD spectra in the far UV.

Discussion

The variation with Gdn-HCl concentration of the ellipticity at the minimal wavelength (Fig. 5) suggests that the Gdn-HCl denaturation processes of H- and L-UK were not two-state transitions but consisted of "four states and three transitions" and "three states and two transitions," respectively. The secondary structures of the four states in the Gdn-HCl denaturation process of H-UK were studied by the best-curve-fitting method¹²⁾ based on the CD spectra at 200–240 nm. The helix contents were estimated to be 0.14–0.15 in N, 0.13–0.16 in A, 0.12 in I and 0.08 in D, and the β -form contents were 0.21–0.23 in N, 0.17–0.22 in A, 0.13–0.14 in I and 0.05–0.12 in D. In the same manner as for H-UK, the helical fractions were evaluated for L-UK as 0.12 in N and I and 0.10–0.11 in D, and the β -form fractions as 0.15–0.17 in N, 0.11–0.12 in I and 0.01–0.07 in D.

These estimations for the helical and β -form fractions are probably relatively reliable. This is because the helical and β -form contents determined by the CD analysis correlated well with the results of X-ray crystallography for the majority of 18 proteins studied by Chang *et al.*¹²⁾ In contrast estimates of β -turn content were poorly correlated with the results of X-ray diffraction analysis. Moreover, the calculated CD spectra (Fig. 1), which were obtained from the secondary structures determined by the CD analysis, were in reasonably good agreement with the observed CD spectra even for Gdn-HCl-denatured H- and L-UK as compared with the coincidence between the calculated and observed spectra for eight proteins investigated by Chang *et al.*¹²⁾ The standard deviations, furthermore, were as small as 1.7–3.2% and 2.4–3.7% of the contents of the two ordered structures determined on the basis of CD spectra measured several times for H- and L-UK, respectively.

Helix and β -form fractions in N, A, I and D determined by the CD analysis were compared to their initial fractions of UK without any added Gdn-HCl (Table II). At 2.00–3.00 M Gdn-HCl, corresponding to I, the fractions of both helix and β -form were essentially fixed within a narrow range of 0.00–0.01 for H- and L-UK. This corresponded to the almost unchanged minimal ellipticities with increase of Gdn-HCl concentration for I of both UK forms (Fig. 5). Thus the Gdn-HCl denaturation processes of H- and L-UK were not two-state transitions, but, besides N and D, include I where the secondary structure was inferred to be stabilized in spite of the increase of Gdn-HCl concentration.

At 0.75–1.25 M Gdn-HCl, corresponding to A of H-UK, the helix content was observed to increase (Table II) and reached a maximum at 1.00 M Gdn-HCl (changes of the helical fraction from its initial fraction in UK with no added Gdn-HCl: 0.75 M Gdn-HCl, +0.01; 1.00 M, +0.02; 1.25 M, –0.02). No other states of the UK forms showed such an increase in helicity, which corresponded to the increase in the minimal ellipticity at 204 nm (Fig. 5) for H-UK treated with 0.75–1.25 M Gdn-HCl. On the other hand, L-UK showed reduced minimal ellipticity at 202 nm at the same Gdn-HCl concentration.

The enhancement in plasminogen activator activity in state A as a characteristic of H-UK (Fig. 7) should be elucidated by further investigations such as CD measurements in the near UV for Gdn-HCl-denatured UK and in the far UV for renatured UK. The present data imply that the activity potentiation of H-UK might be closely related to the slight increase of the helix content in the N→A transition (changes of the helical fraction relative to that in the whole protein molecule: 0.00–0.25 M Gdn-HCl, 0.14; 0.50–0.75 M, 0.15; 1.00 M, 0.16) for the following reasons.

(1) The increase of the helix was not sudden, but steady. In view of the precision in determining the reference conformation, the CD analysis is considered to be more reliable for helical content than for β -form content.¹²⁾

(2) No other transitions of the UK forms resulted in activation or showed such a steady increase in helicity. Of all five transitions of the two UK forms, the N→A transition of H-UK was the only transition that brought about both activity enhancement and steadily increased helicity.

(3) The activity was enhanced with the increase of the helix content in the N→A transition. The activation of H-UK was maximal at 1.00 M Gdn-HCl in state A. At the same Gdn-HCl concentration, the increases of the helicity and of the ellipticity at 204 nm were also maximal. In the A→I transition, furthermore, the degrees of activation fell with the decreases of the helix and of the minimal ellipticity. These findings show that the change of activity with Gdn-HCl concentration closely paralleled the changes of helicity and minimal ellipticity with Gdn-HCl concentration in the N→A and A→I transitions.

(4) L-UK also lost a part of the β -form content in the first transition, as did H-UK, but lost considerable (27–38%) activity at 0.75–1.25 M Gdn-HCl in contrast with H-UK. It is difficult, therefore, to conclude that the activation of H-UK is due more to the β -form reduction than to the helical increment in the N→A transition.

These considerations suggest that the additional formation of a small amount of helix might alter the conformation of the active site and/or its vicinity so as to increase the catalytic activity and/or affinity toward plasminogen in the case of H-UK. In the case of L-UK, its lack of the light polypeptide chain which H-UK possesses^{3,4)} may prevent such a change.

Thus, the Gdn-HCl denaturation processes were shown to include I for both UK forms. In the case of H-UK, A intervened between N and I. These findings show that the denaturation processes consisted of “four states-three transitions” for H-UK and “three states-two transitions” for L-UK.

Acknowledgement The authors wish to express their thanks to Dr. Isamu Utsumi, general manager of the Pharmaceuticals Research Center, Kanebo, Ltd., for his encouragement and to Miss Yuko Nakata for her technical assistance.

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