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THE EFFECT OF INTERACTION BETWEEN HUMAN UROKINASE AND ITS COMPETITIVE INHIBITOR, Nα-BENZOYL-L-ARGININE AMIDE, ON REDUCTION OF A SPECIFIC SS BOND RELATED TO ENZYMATIC ACTIVITY*

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SUMMARY: The 55- (H-UK) and 36-kDa forms (L-UK) of human urinary urokinase lost most of esterase activity toward acetyl-glycyl-L-lysine methyl ester upon reductive cleavage of 3 SS bonds with dithiothreitol in the presence of the competitive inhibitor, Nα-benzoyl-L-arginine amide (BAA), bound to polyacrylyl azide with $C_{16}N_3$-arm (PAA) at 0.3 M guanidine, a threshold point of the native state where a protein-denaturating transition began. One of the 3 SS bonds was protected from reduction, with an unaltered activity, under the similar conditions except for replacement of BAA-PAA conjugate by glycine-PAA conjugate. This "specific" SS bond was reduced and, after the other SH groups produced were blocked with iodoacetamide (IAM), selectively reoxidized, which resulted in complete reactivation. The intact B-chain isolated from H-UK was completely inactivated when its specific SS bond was reduced and selectively alkylated with IAM after the other SH groups were reversibly blocked with 5, 5'-dithiobis(2-nitrobenzoic acid), which was finally removed. The results indicate that a single specific SS bond is essential for retaining a conformation necessary to activity exhibition.

Human urokinase (UK[¶]) [EC3.4.21.31], a plasminogen activator produced in the kidney and found in urine, is divided into H-UK (51-55 kDa) and L-UK (31-36 kDa). H-UK is composed of the A- and B-chains (1), while L-UK has the mini-chain (2, 3) besides the B-chain with the active site serine (1, 4). We have purified both the UK forms, which are homogeneous in terms of molecular weight and immunogenicity (5) and different in their electrophoretical properties (6), stabilities against heat and pH (7) and ionization modes of alkalized tyrosyl residues (8).

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¶ ABBREVIATIONS: H-(L-)UK, 55-(36-)kDa form of urokinase; BAA, Nα-benzoyl-L-arginine amide; PAA, polyacrylyl azide derivative; GuHCl, guanidine hydrochloride; DTT, dithiothreitol; IAM, iodoacetamide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); AGLME, acetyl-glycine-L-lysine methyl ester

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In the previous work (9), we determined the secondary structures of H- and L-UK by CD spectral analysis and showed that both the UK forms retain native conformations below 0.3 M GuHCl, above which a protein-denaturating transition began with randomization. In this paper we report that, at the threshold point of the native state, one specific SS bond related to activity exhibition is exposed to the solvent in the presence of the competitive inhibitor BAA (10) bound to PAA, although buried within the molecule in the absence of BAA-PAA conjugate or denaturants.

MATERIALS AND METHODS

Materials---H- (55 kDa) and L-UK (36 kDa) were obtained from human urine as previously described (5). Their homogeneities were certified by SDS-PAGE, Ouchterlony's double immunodiffusion, isotachoelectrophoresis and isoelectric focusing (5, 6). The specific activities of H- and L-UK were 1.20×10^5 and 1.52×10^5 IU/mg protein, respectively. GuHCl (Nakarai, Kyoto) was recrystallized from an ethanol-benzene mixture and then from water. AGLME was purchased from Peptide Institute, Osaka. DTT, DTNB and IAM of the guaranteed grade were obtained from Wako, Osaka.

BAA- and Glycine-PAA Conjugates---The conjugates were synthesized in a series of the following steps essentially as described by Maciag et al. (11). Enzacryl AH (Aldrich) was activated with $NaNO_3$ and coupled to 1, 6-hexanediamine. The primary amine of the bifunctional agent bound reacted with succinic anhydride to form a carboxyl group, which reacted further with 1, 6-hexanediamine in the presense of [3-(3-dimethylaminopropyl)]ethyl carbodiimide (Aldrich). Nα-benzoyl-L-arginine (Sigma) or glycine (6 mmol) was coupled to the substituted Enzacryl AH (5 g/30 ml) with the carbodiimide. The conjugates were rinsed with 200 mM Tris-HCl buffer (pH 8.1) containing 300 mM NaCl and 2 mM EDTA (Buffer A). According to quantitative analysis of the unbound carboxylic acid by the hydroxamate-Fe(III) chelation method (22), amounts of BAA and glycine amide were 0.81 and 0.95 mmol/g PAA, respectively, where a $C_{16}N_3$ arm intervened between Enzacryl AH and the amino acid amide.

Reduction of SS Bonds and Their Alkylation---To a 100 µl UK solution (130 µM), 154.5 wet mg (150 ul) of BAA- or glycine-PAA conjugate and, after 10 min, 750 µl of 5.3 M GuHCl were added at 37°C. After incubation for 2 hr, the protein was reduced by addition with 25 µl of 300 mM DTT for 6 hr at 0°C with insufflated nitrogen. Addition of 25 µl of 1 M IAM was followed by stirring for 1 hr at 0°C in the dark. The reference experiments were carried out by replacing each agent dissolved in Buffer A with Buffer A alone. The mixture containing PAA gel was made to 4.0 M in GuHCl and filtrated. The residue was suspended in 1 ml of 4.0 M GuHCl and filtrated again. This procedure was repeated three times at 4°C. From the filtrate collected and condensed on an Amicon Diaflo PM-10, excess agents were removed by passage through a Sephadex G-25 column (1.2 x 30 cm).

Contents of Protein SH Groups---The SH contents were colorimetrically determined with DTNB as described by Ellman (12). To a 0.5 ml solution of UK or its derivative more concentrated than 13 µM, was added 2.5 ml of freshly prepared 5 mM DTNB in Buffer A containing 7.2 M or no GuHCl. After 2 hr its absorption spectrum was measured against a DTNB blank with a Hitachi 124 double-beam spectrophotometer. The molar extinction coefficients of the colored anion generated from DTNB in 6 M and no GuHCl were assumed to be 1.39×10^4 and 1.36×10^4 at 412 nm, respectively (13).

SDS-Polyacrylamide Gel Electrophoresis and Densitometry---UK denatured with 1% SDS in the absence of reductants at 100°C for 4 min (5.0 ug in 10 ul) was

subjected to electrophoresis according to the method of Weber and Osborn (14) as previously described (5). After stained with Amide Black 10B and destained, the gel was scanned with a Schoeffel Co. densitometer.

Esterase Activity toward AGLME---The activity was measured by the method of Walton (15) as previously described (8). The amounts of carboxyl groups produced by hydrolysis of AGLME were determined with a Radiometer, Inc. pH-stat apparatus.

Amounts of Proteins---The concentrations of H- and L-UK were colorimetrically determined by the method of Lowry et al. (16) with bovine serum albumin for calibration.

Reoxidation of open SS bonds---Reduced UK (17.6 μM) was gently stirred at 18°C for 24 hr in Buffer A without EDTA in the presence of 50 μM $CuSO_4$.

RESULTS

H- and L-UK contained no SH groups as determined with DTNB at 6 M GuHCl and, when reduced with DTT at 4 M GuHCl, reacted with 22 and 12 DTNB molecules, indicating that 11 and 6 SS bonds were contained, respectively (Table 1). One SS bond in each UK form was so labile as to be cleft with DTT in the absence of denaturants. The numbers of SH groups were greater in H- and L-UK reduced with DTT at 0.3 M GuHCl in the presence of BAA-PAA conjugate (6 residues/mol) than of glycine-PAA conjugate (4 residues/mol). The difference of 2 SH groups in each UK form is ascribed to one SS bond that is made to be reactive through interaction of UK with BAA.

Treatment with DTT in the absence of GuHCl made most of H-UK molecules split into 2 chains and decreased M_r of L-UK by 3 kDa as judged by SDS-PAGE (Fig. 1). This reaction was accompanied by cleavage of one SS bond (Table 1), by which alone therefore the 2 chains were connected.

H- and L-UK whose single interchain SS bond was reduced with DTT in the absence of GuHCl retained their initial esterase activity (Table 2). Both UK forms were completely inactivated when reduced with DTT at 4.0 M GuHCl. H- and L-UK retained most of activity when reduced with DTT at 0.3 M GuHCl in the presence of glycine-PAA conjugate. The similar reduction except for

Table 1. Effects of BAA-PAA conjugate on reduction of SS bonds in UK.

	-DTT	DTT	glycine-PAA, DTT		BAA-PAA, DTT	
GuHCl (M)	4.0		0.0	0.3	0.0	0.3
H-UK	0.12	21.67	1.95	4.02	1.83	6.34
L-UK	0.02	11.88	1.81	4.12	1.84	6.16

The number of SH groups in UK (13 μM) treated with 7.5 mM DTT at 0, 0.3 or 4 M GuHCl in the presence or absence of glycine- or BAA-PAA conjugate (154.5 wet mg/ml) was determined at 6 M GuHCl by the DTNB method (12) after removal of excess agents by passage through a Sephadex G-25 column.

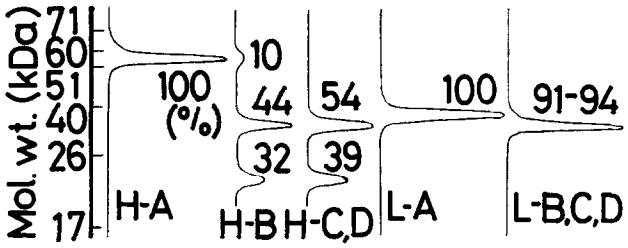

Fig. 1. SDS-PAGE and densitometry of reduced UK. H- and L-UK (A) were reduced at 0 (B), 0.3 (C), 4.0 (D) M GuHCl as described in the legend to Table 1. Five μg of the protein was subjected to SDS-PAGE in the absence of reductants. The density of each band is shown as a percentage to that of untreated UK. Addition of BAA-PAA conjugate did not affect the results.

replacement of the conjugate by BAA-PAA conjugate deprived both UK forms of most of activity, suggesting that UK interacted with BAA even at 0.3 M GuHCl. The effect of BAA-PAA conjugate on the inactivation corresponds to additional cleavage of a "specific" SS bond (Table 1) related to activity exhibition.

H-UK (I in Fig. 2) reduced with DTT in the absence of denaturants gave 3 peaks (II) in the chromatograms of Sephadex G-75. The protein in the first peak was unreduced H-UK. The secondly eluted protein showed a specific activity similar to that of L-UK but was identified as the B-chain because of a M_r value slightly lower than that of L-UK and contents of 1 SH group originating from an interchain SS bond cleft (Table 3). The third protein was the inactive A-chain. The single SH group in the B-chain was alkylated with IAM at 4 M GuHCl (III), which did not alter the activity.

This B-chain derivative was reduced with DTT at 0.3 M GuHCl in the absence of BAA-PAA conjugate (IV in Table 4), which resulted in cleavage of 1 "non-specific" SS bond with retention of most of activity. The 2 resultant SH

Table 2. Enzymatic activity of UK reduced with DTT in the presence of BAA-PAA conjugate at various concentrations of GuHCl.

	Glycine-PAA conjugate			BAA-PAA conjugate		
GuHCl (M)	0.0	0.3	4.0	0.0	0.3	4.0
H-UK	12.2 (99.7)	11.5 (94.2)	0.0 (0.0)	12.3(100.6)	3.4 (27.8)	0.0 (0.0)
L-UK	16.2(102.9)	13.7 (87.5)	0.0 (0.0)	15.6 (99.4)	3.1 (19.7)	0.0 (0.0)

Specific activities (10^4 IU/mg protein) (relative activities (%)) of UK treated as in the legend to Table 1 are tabulated. Esterase activities toward AGLME were assayed by the method of Walton (15) and the protein amounts were determined by the method of Lowry et al. (16).

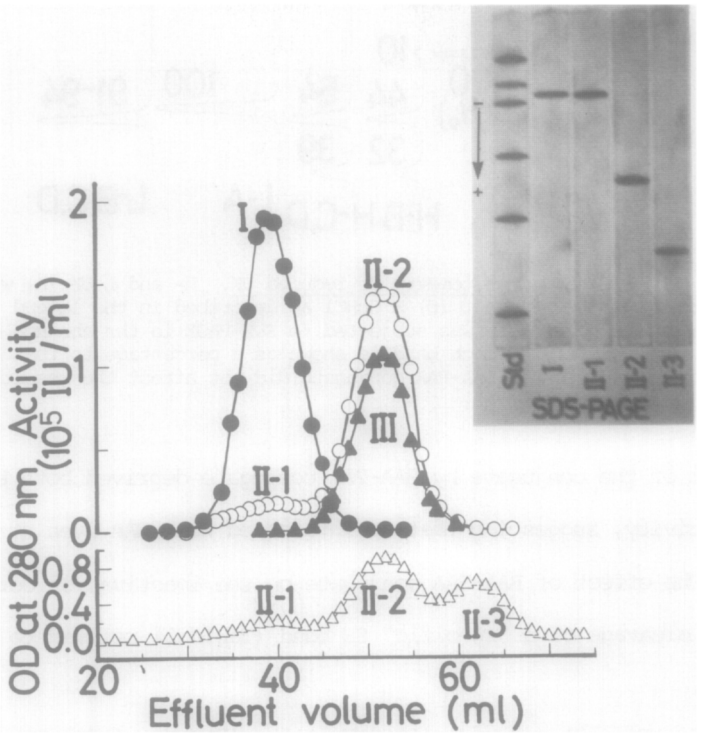

Fig. 2. Column chromatography of reduced UK on Sephadex G-75. H-UK (12.3 mg protein; 15.42x10^5 IU) was loaded on a Sephadex G-75 column (1.6x46 cm) euilibrated with Buffer A and eluted. The effluent (I) was subjected to measurements of OD at 280 nm and esterase activity toward AGLME. The protein (effluent volume: 32-48 ml) was reduced with 7.5 mM DTT and desalted on Sephadex G-25. The protein condensated to 2.7 ml on an Amicon Diaflo membrane PM-10 was subjected to gel filtration similarly (II). The protein in the second peak was alkylated with 25 mM IAM at 4 M GuHCl and treated similarly (III).

groups were alkylated with IMA at 4 M GuHCl (V), which decreased the activity slightly. The alkylated B-chain was further reduced with DTT at 0.3 M GuHCl in the presence of BAA-PAA conjugate (VI) to lose most of activity with cleavage of a "specific" SS bond. Modification of the 2 resultant SH groups with DTNB at 4 M GuHCl (VII) induced complete inactivation, indicating that the 2 SH groups were not reoxidized to a SS bond through SH-SS interchange catalyzed by DTNB (17) as observed for the other proteins (18, 19). Removal of the 2 modifier groups with DTT reproduced 2 unblocked SH groups (VIII), which were thereafter reoxidized in the presence of Cu(II) ions to form a SS bond *in situ* with a concomitant reactivation.

Table 3. Isolation of the active B-chain from reduced H-UK.

UK derivatives	Total activity (10^5 IU)	Specific activity (10^4 IU/mg protein)	SS content (residues/mol)	SH content	M_r (kDa)
I	14.03	12.6	10.83	0.19	55
II-1	1.64	11.2	10.74	0.08	55
II-2	10.69	20.5	4.86	0.83	33
II-3	0.00	0.0	4.53	0.87	23
III	8.06	18.3	5.34	0.00	33

The fractions in the chromatograms in Fig. 2 were subjected to measurements of esterase activity toward AGLME, SH contents before and after reduction with DTT at 6 M GuHCl, protein amounts and M_r values by SDS-PAGE (14).

The intact B-chain was reduced with DTT at 0.3 M GuHCl in the presence of BAA-PAA conjugate (X in Table 5), which resulted in cleavage of 2 SS bonds and inactivation. Upon reaction of this reduced B-chain with DTNB in the absence of BAA-PAA conjugate at 0.3 M GuHCl, were blocked all 5 SH groups (XI) involving a SH group originating from a interchain SS bond and, without the denaturant, 3 out of 5 SH groups were blocked (XII). The 2 unblocked SH groups were alkylated with IAM (XIII). Further, the 3 modifier groups from DTNB were eliminated from the 3 modified SH groups with DTT (XIV). Two out of the 3 unblocked SH groups were reoxidized in the presence of Cu(II) ions (XV). This protein was inactive though intact except for alkylation of a "specific" SS bond cleft. On the similar reoxidation the B-chain intact except for 2 cleft SS bonds (X) was reactivated (XVI). The results indicate that the "specific" SS bond is essential for activity exhibition.

Table 4. Reversible modification of a specific SS bond with DTNB.

UK derivatives	Treatments	Activity: 10^4 IU/mg protein, (%)	SS content (residues/mol)	SH content (residues/mol)
IV	0.3 M GuHCl, DTT	15.9 (86.9)	4.29	1.70
V	4.0 M GuHCl, 25 mM IAM	14.1 (77.3)	4.34	0.00
VI	BAA-PAA, 0.3 M GuHCl, DTT	2.1 (11.5)	3.28	1.94
VII	4.0 M GuHCl, 10 mM DTNB	0.0 (0.0)	-	0.00
VIII	DTT	2.0 (10.9)	3.10	1.89
IX	50 μM $CuSO_4$	12.5 (68.3)	4.06	0.41

UK derivatives were prepared from III in Table 3 in the successive treatments indicated and characterized in terms of activities, SS and SH contents and protein amounts as in Table 3. The derivative VII was obtained by reaction of VI (19.1 μM) with 10 mM DTNB at 4 M GuHCl in Buffer A and subsquent desalting on a Sephadex G-25. Treatment of VII with 7.5 mM DTT in Buffer A liberated the modifier groups to form VIII after the similar desalting.

Table 5. Selective reduction and alkylation of a specific SS bond.

UK derivatives	Treatments	Activity: 10^4 IU/mg protein (%)	SS content (residues/mol)	SH content (residues/mol)
X	BAA-PAA, 0.3 M GuHCl, DTT	4.6 (22.4)	3.35	4.91
XI	0.3 M GuHCl, 10 mM DTNB	0.0 (0.0)	-	0.04
XII	10 mM DTNB	4.3 (20.9)	-	1.73
XIII	4.0 M GuHCl, 25 mM IAM	0.0 (0.0)	-	0.00
XIV	DTT	0.0 (0.0)	3.22	3.12
XV	50 μM $CuSO_4$	0.0 (0.0)	3.70	1.14
XVI	50 μM $CuSO_4$	17.1 (83.4)	4.59	1.26

UK derivative X was prepared by reduction of II-2 in Table 3 with 7.5 mM DTT at 0.3 M GuHCl in the presence of BAA-PAA. The derivatives XI, XII-XV and XVI were obtained from X in the (successive) treatment(s) indicated, respectively. The activities and SS and SH contents were determined as in Table 3.

DISCUSSION

The results of reversible and selective modification of a specific SS bond in H- and L-UK (Tables 4 & 5) show that it is essential for exhibiting enzymatic activity. The single SS bond is buried within the molecule in the native state irrespective of addition of the competitive inhibitor BAA (10) bound to PAA, but, at its threshold point (0.3 M GuHCl) in protein-denaturation process (9), exposed to the solvent only in the presence of BAA-PAA (Table 1). The specific SS bond therefore is sensitized to DTT by synergistic effects of conformational changes due to both "induced fit" (20) of UK for BAA and partial denaturation with 0.3 M GuHCl. At 0.3 M GuHCl, both the initial activity and ordered structures such as the helice (12-14%) and β-pleated sheets (17-21%) are metastably retained but altered upon further addition of GuHCl (9). The metastable conformation seems to be facilely altered by a stimulus such as binding to inhibitors.

UK is classified into the serine proteases (1, 4), where SS (42-58) bond adjacent to the catalytic His 57 is absolutely conserved and probably maintains the appropriate disposition of this histidyl residue (21). No SS bonds in the serine proteases have been proved to be obligatory for activity exhibition. The specific SS bond in UK may adjoin His 57 topologically.

The specific SS bond is exposed at 0.3 M GuHCl in the presence of BAA-PAA whether the nonspecific SS bond whose cleavage alone induces no inactivation is reduced and carboxamidomethylated (VI in Table 4) or not (X in Table 5), indicating spatially distant location of the 2 SS bonds. The spe-

cific SS bond, however, after cleft, is exposed at 0.3 M GuHCl even in the absence of the inhibitor (XI), and buried only in the absence of GuHCl (XII). This indicates that the 2 SH groups originating from the specific SS bond become less masked dynamically by stimulation with denaturing agents, but, without denaturants, are statically buried possibly owing to the hydrophobicity (23) as before the cleavage in spite of lengthened SS-distance of 2.08 Å to more than 4.8 Å (24).

A SS bond in the native lysozyme, although located at the surface of the molecule (25), sluggishly reacts with reductants (26) possibly owing to non-covalent binding to the side-chains in the vicinity (27). Two SH groups at the surface of the native hemoglobin are also unreactive presumably through interaction with a few neighboring hydrophobic side-chains (28). These suggest that the low availability for the single specific SS bond and its open form in UK may not be caused by the spatial location but by their microenvironment.

The single interchain SS bond in UK is fully exposed so as to be reduced with DTT even in the absence of denaturants (II in Table 3). This is true of interchain SS bonds in γ-globulin (29) and insulin (30). The 2 SS bonds reduced at 0.3 M GuHCl in the absence or presence of BAA-PAA seem to be half-buried in the native UK. All the other 8 and 3 SS bonds in H- and L-UK, respectively, are non-reactive. The high ratio of SS contents in the A-chain to the M_r value (5 residues/23 kDa) probably makes its domains kringle, which may be resistant against reductants owing to low availability for all the 5 SS bonds.

The conformational changes in UK induced by selective reduction of the specific SS bond and interaction with BAA at 0.3 M GuHCl are under way to be analyzed by circular dichroic technics.

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