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Binding of Sodium Dodecyl Sulfate to Aprotinin and Its Effect on the Aprotinin Molecule

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When the basic protease inhibitor aprotinin was treated with sodium dodecyl sulfate (SDS), it showed an increased apparent molecular weight upon SDS gel electrophoresis. The amount of SDS bound to aprotinin was much lower than that bound to aprotinin treated with SDS and mercaptoethanol (ME). This result suggests that the increased apparent molecular weight upon SDS gel electrophoresis is not due to dimerization of aprotinin in the presence of SDS, but rather derives from a decrease of SDS binding to aprotinin.

Aprotinin treated with SDS retained both the inhibitory activity against trypsin and immunoreactivity against antiaprotinin immunoglobulin G (IgG), whereas aprotinin treated with SDS and ME showed no inhibitory activity against trypsin but did retain immunoreactivity against antiaprotinin IgG. These observations indicate that aprotinin resists denaturation by SDS, but is considerably affected by treatment with SDS and ME.

Keywords——aprotinin; sodium dodecyl sulfate; dimerization; inhibitory activity; immunoreactivity

A basic protease inhibitor from bovine organs, aprotinin, is used to treat acute pancreatitis and acute circulatory failure, as it has inhibitory effects on trypsin, chymotrypsin, plasmin and kallikrein.[1] In solution, aprotinin exists in two states, a monomer (M_r, 6500) and a dimer (M_r, 13000).[2] Any change in the two states of aprotinin alters the aprotinin activity.[3] As shown in Fig. 1 (I), aprotinin migrated at nearly the same position as cytochrome c (M_r, 12500) after treatment with 1% sodium dodecyl sulfate (SDS), whereas it migrated at a position corresponding to a molecular weight of about 6500 after treatment with 1% SDS and 1% mercaptoethanol (ME) (Fig. 1 (II)). In general, decrease in SDS binding correlates fairly well with decrease in electrophoretic mobility on SDS gels, resulting in an increased apparent molecular weight upon SDS gel electrophoresis.[4] In the present study, binding of SDS to aprotinin and the effect of SDS on the aprotinin molecule were investigated in order to clarify whether the increased apparent molecular weight upon SDS polyacrylamide gel electrophoresis (SDS–PAGE) is related to dimerization of aprotinin in the presence of SDS or whether it is due to a decrease in SDS binding to aprotinin.

Experimental

Materials——Aprotinin, separated from a commercial sample of Trasylol (Bayer, A.G., W. Germany) on a Sephadex G-50 column and freeze-dried as described previously,[5] was dissolved in 0.9% NaCl solution and used in this study. SDS, acrylamide, *N,N'*-methylenebisacrylamide and 2-ME were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Insulin chain B, cytochrome c and trypsin inhibitor from soybean were obtained from Boehringer Mannheim GmbH (W. Germany), cellulose tubing (VT 351) from Visking Company (U.S.A.), nitrocellulose sheet (pore size 0.45 μm) from Toyo Roshi (Japan), and trypsin (3 times recrystallized) from Miles Laboratories (U.S.A.). Triton X-100, benzoyl-L-arginyl-β-naphthylamide (BANA), agarose and Tween 20 were obtained from Nakarai Chemicals, Ltd. (Japan), Fast blue B from E. Merck (W. Germany), ovalbumin from

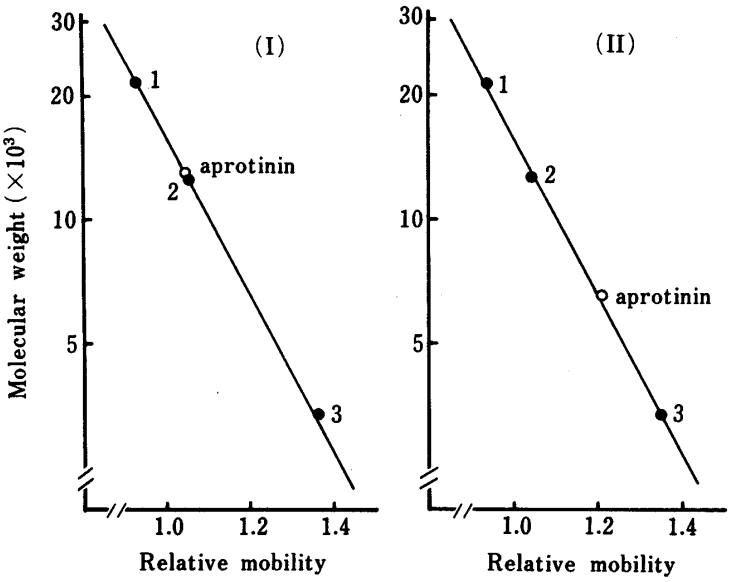

Fig. 1. Molecular Weight Determination of Aprotinin by Disc Gel Electrophoresis in the Presence of SDS

Calibration proteins were as follows: 1, trypsin inhibitor from soybean ($M_r = 21500$); 2, cytochrome c ($M_r = 12500$); 3, insulin chain B ($M_r = 3400$). Aprotinin and calibration proteins were treated at 37 °C for 3 h with 1% SDS (I) or with 1% SDS and 1% ME (II) and then allowed to stand at room temperature for 3 h before electrophoresis. Relative mobility was calculated from the following equation. Relative mobility = gel length before staining × distance of protein migration/gel length after staining × distance of dye (bromophenol blue) migration.

Seikagaku Kogyo Co., Ltd. (Japan), and bovine serum albumin (BSA) (fraction V) from Armour Pharmaceutical Company (U.S.A.). Antiaprotinin immunoglobulin G (IgG) was prepared as described previously.[5] Mouse antirabbit IgG-horseradish peroxidase was kindly given by Dr. S. Kawaguchi (Department of Microbiology and Immunology, Shimane Medical University). All other chemicals were of the purest grade available commercially.

SDS–PAGE——Aprotinin (140 μg/ml) was incubated with 1% SDS or with 1%SDS and 1% ME at 37 °C for 3 h, and then allowed to stand at room temperature for 3 h before electrophoresis. For disc gel electrophoresis, 7.5% gel and electrode buffer, using the standard SDS–phosphate system, were prepared according to Weber and Osborn.[4] Samples were electrophoresed for 140 min at 8 mA/tube (7 cm long, 6 mm diameter). Then the gels were stained and destained as described by Weber and Osborn.[4] For slab gel electrophoresis, 3% (stacking) and 7.5% (separation) gels and electrode buffer were prepared according to Laemmli.[6] In this case, samples were electrophoresed for 2 h at 20 mA per slab (12 × 13.5 cm, 2 mm thick). The gel was stained in a solution containing 0.25% Coomassie brilliant blue R, 50% methanol and 7% acetic acid, and destained in 30% methanol and 10% acetic acid.

SDS Binding to Aprotinin——After treatment of 1% ME and/or 1% SDS as described above for SDS–PAGE, 1 ml of aprotinin solution (140 μg/ml) was sealed into cellulose tubing (TV 351, molecular weight cut-off 3500), and dialyzed against 0.9% NaCl solution (200 ml per 1 ml of aprotinin solution; changed every 8 h, 3 changes) to remove excess ME and/or SDS. The amounts of SDS inside and outside the dialysis sack were measured according to Pitt-Rivers and Impiombato.[7] For the calculation of the amount of SDS bound to aprotinin, it was assumed that the concentration of free SDS inside the dialysis sack was equal to that outside at equilibrium. Thus, the amount of SDS bound to aprotinin was calculated by subtracting the amount of free SDS inside the dialysis sack from that of total SDS (bound and free SDS) inside the dialysis sack.

Electrophoretic Transfer——Electrophoretic transfer of aprotinin from a polyacrylamide slab gel to a nitrocellulose sheet was performed according to Towbin *et al.* for SDS gel.[8] After completion of the run, the sheet was cut longitudinally between the appropriate lanes. The blot was stained in a solution containing 0.1% amido black, 45% methanol and 10% acetic acid, and destained in 30% methanol and 10% acetic acid.

Immunological Detection of Aprotinin on Nitrocellulose——The electrophoretic blots (not stained with amido black) were soaked in a solution containing 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2, 0.05% Tween 20 (PBS/Tween 20) and 0.5% ovalbumin at 4 °C overnight. The blots were washed for a total of 30 min with five successive 100 ml portions of PBS/Tween 20. The protein-saturated blots were incubated for 2 h at room temperature with antiaprotinin IgG (500 μg/ml) diluted with a solution containing PBS/Tween 20 and 0.5%BSA, and then washed as above. The blots were next incubated for 2 h at room temperature with mouse antirabbit IgG-horseradish peroxidase (0.8 μg/ml) diluted with PBS/Tween 20/BSA, then again washed as above and dipped into 50 mM Tris–

HCl buffer (pH 7.5) for a brief rinse. Antigenic bands were stained in 50 mM Tris–HCl buffer (pH 7.5) containing 3,3′-diaminobenzidine (0.3 mg/ml) and 0.016% hydrogen peroxide.

Detection of Aprotinin Activity Using the Simultaneous Azo-Coupling Method——After SDS–PAGE, the slab gel was washed with Triton X-100, and then rinsed thoroughly with distilled water, according to Granelli-Piperno and Reich.[9] The washed gel was immersed in trypsin solution (300 μg/ml in 0.1 M Tris–HCl buffer, pH 6.5, containing 0.1% $CaCl_2$) for 30 min at 37 °C, and then transferred to another tray for incubation at 37 °C for 30 min in a humid environment. The gel was laid carefully onto a BANA–agarose–Fast blue B plate prepared by the method of Lojda *et al.*,[10] with some modifications; that is, the solution containing BANA, Fast blue B and agarose was spread evenly on the surface of a glass plate (5 ml per 5 × 10 cm glass plate), and allowed to form a firm gel. The unit was incubated at 37 °C for 1 h in a humid environment. Then the gel was dipped for 5 min into 2% copper sulfate and washed for 5 min with distilled water at room temperature. The aprotinin activity against trypsin was seen as a yellow area on the blue-violet background.

Results and Discussion

The following results were obtained. 1. Both in disc gel electrophoresis and in slab gel electrophoresis, aprotinin migrated to nearly the same position as cytochrome c after treatment with SDS, whereas it migrated to a position corresponding to a molecular weight of about 6500 after treatment with SDS and ME. 2. In the treatments with SDS and with SDS and ME, the amounts of SDS bound to aprotinin were 0.18 μg SDS/μg of aprotinin and 1.22 μg SDS/μg of aprotinin, respectively (mean of three determinations). 3. Aprotinin treated with SDS retained both the inhibitory activity against trypsin and immunoreactivity against antiaprotinin IgG, whereas aprotinin treated with SDS and ME showed no inhibitory activity against trypsin but did retain immunoreactivity against antiaprotinin IgG (Fig.2). 4. After being washed with Triton X-100 and with distilled water, the electrophoresed gel was

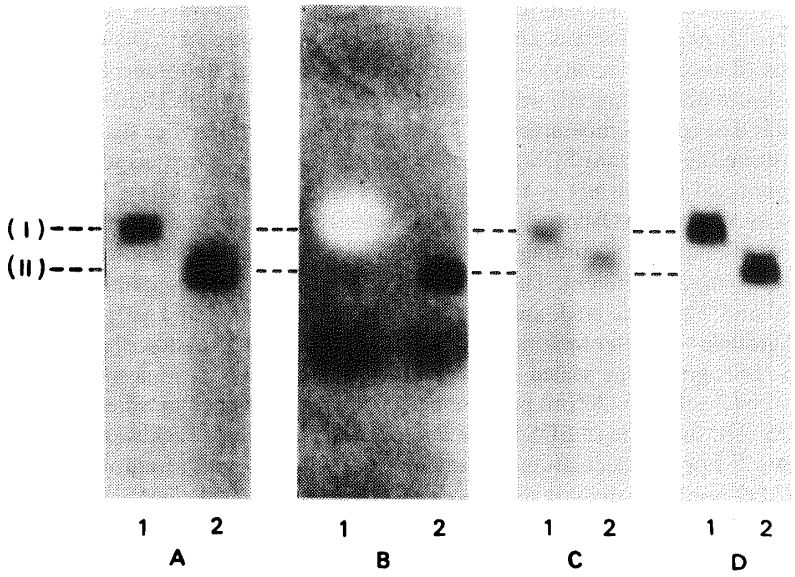

Fig. 2. A: Coomassie Brilliant Blue R Staining after Slab SDS–PAGE of Aprotinin
B: Inhibitory Activity of Aprotinin on the Gel Against Trypsin Demonstrated by Using the Simultaneous Azo-Coupling Method
C: Amido Black 10 B Staining after Blotting onto a Nitrocellulose Sheet
D: Immunoreactivity Against Antiaprotinin IgG after Blotting onto a Nitrocellulose Sheet

Lanes 1 and 2 show the results of treatments of aprotinin before slab gel electrophoresis; aprotinin was treated at 37 °C for 3 h with 1% SDS (lane 1) or with 1% SDS and 1% ME (lane 2) and then allowed to stand at room temperature for 3 h. (I) and (II) show the migration positions of aprotinin. Direction of migration is from top to bottom. Inhibitory activity of aprotinin on the gel against trypsin is shown in B, lane 1.

immersed in 10 mM Tris–HCl buffer, pH 8.5, and was gassed with 100% O_2 for 1 h or with air for 20 h. The trypsin-inhibitory activity of aprotinin that had been treated with SDS and ME, detected by the simultaneous azo-coupling method, was not recovered.

For the accurate measurement of the amount of SDS bound to aprotinin by a colorimetric method, it was necessary to remove the excess free SDS. For this reason, the conditions of dialysis described in Experimental (SDS binding to aprotinin) were used. The value of 1.22 μg SDS/μg of aprotinin obtained after the treatment with SDS and ME was somewhat lower than the usual value of 1.4.[4] However, there was a clear difference in amount of SDS bound to aprotinin between the treatments with SDS and with SDS and ME. Result 2 suggests that the increased apparent molecular weight upon SDS gel electrophoresis is not due to dimerization of aprotinin in the presence of SDS, but rather is due to a decrease in SDS binding to aprotinin. Aprotinin has three disulfide bonds,[11] and failure to reduce the disulfide bonds restricts the conformational freedom of the polypeptide and decreases the extent of SDS binding.[7] Results 3 and 4 show that an irreversible change was elicited in the aprotinin molecule after treatment with SDS and ME, whereas in the treatment with SDS, reactive sites in the aprotinin molecule, which have inhibitory activity against trypsin and immunoreactivity against antiaprotinin IgG, were well retained. Result 3 also suggests that the antigenic sites of aprotinin, in which two or three such sites exist,[12] are present at sites other than the reactive sites against trypsin.

Aprotinin shows remarkable stability; it retains its inhibitory activity toward trypsin or kallikrein despite exposure to high temperature, acid, alkali and proteolytic degradation.[2b] The present results show that aprotinin also resists denaturation by SDS, but a remarkable change is elicited in the aprotinin molecule by treatment with SDS and ME.

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