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A Novel Crosslinking Reagent and Its Application for the Detection and Isolation of Heparin-Binding Protein(s) on the Platelet Surface

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A new hetero-bifunctional photo crosslinking reagent, 2-(4-azidoanilyl)-4-(4-azabicyclo-[2,2,2]hexylammonio)-6-morpholino-1,3,5-triazine chloride, was designed to detect and isolate heparin-binding protein(s) that may act as heparin-receptor(s) on the platelet surface. In a preliminary study using ethanol as a model substrate, the reagent was shown to react with the alcoholic hydroxy group under mild conditions and its crosslinking photoreactivity was high. The reagent effectively formed similar covalent bonds with heparin, while preserving its anticoagulant anti-Xa activity. [^{3}H]Heparin labeled with this reagent crosslinked to antithrombin III very specifically but not to ovalbumin, as analyzed by the Bio-imaging Analyzer System (BAS™, Fuji Photo Film, Tokyo). Affinity crosslinking of [^{3}H]heparin was then used to detect heparin-binding proteins on the surface of intact platelets. Several discrete protein bands were detected by the BAS-imaging of SDS-PAGE.

Key words: affinity, crosslinking, heparin, platelet, protein.

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Affinity crosslinking can be a simple and direct way to identify the specific molecules involved in a binding process of biological significance. Many crosslinking reagents have been used in which the functional reactive groups of the crosslinker are tailored to the species of molecules thought to be involved in the binding reaction. Typically, to identify an unknown receptor, the ligand is first labeled with the crosslinker. Then, the complex is crosslinked to vicinal receptors, by virtue of the ligand's affinity for the target protein. For the study of glycosaminoglycan-protein interactions, it is most important that the labeling step does not interfere with high affinity or the biological function of the glycosaminoglycan.

The anticoagulant heparin is a highly sulfated polysaccharide that is now known to have many biological activities beyond its conventional interaction with antithrombin III (*1*). For example, pharmaceutical heparin binds to platelets and may directly alter platelet function (*2, 3*), inducing many side effects. Recently, low molecular weight heparins (LMWHs) prepared by the depolymerization of native heparins have been used clinically instead of the native large molecular weight heparin, mainly in Europe and Japan (*4*). We previously found that the method of depolymerization significantly affected the heparin's affinity for platelets (*5*) and that a specific disaccharide sequence in heparin may be a key unit for the binding of heparin to platelets (*6*). The interaction between heparin and platelets may be mediated by the binding interaction of heparin with specific proteins on the cell surface of platelet: heparin receptors.

To identify the specific heparin-binding proteins on the platelet surface, we have designed a new hetero-bifunctional crosslinker. One arm of the crosslinker reacts nondestructively with heparin under mild conditions, and the other arm contains a photo-activatable reactive group which covalently binds well to proteins. In this paper, we describe the preparation of the reagent, its basic properties and an application for the detection and isolation of heparin-binding proteins using intact human platelets.

MATERIALS AND METHODS

Instruments—For the NMR measurements, JEOL EX-270 (270 MHz, Tokyo) or GSX-400 (400 MHz) was used. Mass spectrometry (MS) was done using FAB-MS (SX-102, JEOL) and ESI-MS (API-III, SCIEX-Parkin Elmer, Thornhill, Ontario, Canada, and Mariner™, Perceptive Biosystems, Framingham, MA, USA). For imaging of radioactivity in gels and blots, BAS-3000 and BAS-2000 systems (Fuji Photo Film, Tokyo) were used.

Preparation of the Crosslinker (AA-D, 1)—The preparation of a novel crosslinker, 2-(4-azidoanilyl)-4-(4-azabicyclo[2,2,2]hexylammonio)-6-morpholino-1,3,5-triazine chloride (**1**, abbreviated as AA-D), is shown in Fig. 1. 4-Azidoaniline hydrochloride **2** (170.6 mg, 1 mmol, Sigma

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Abbreviations: ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline; NMR, nuclear magnetic resonance spectroscopy; TLC, thin layer chromatography.

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Aldrich Chem., St. Louis, MS, USA) was dissolved in 5 ml of acetone, and triethylamine (697 μl, 5 mmol, Wako Chem., Osaka) was added at 0°C with stirring. After 30 min, the precipitated powder was removed by filtration, and the filtrate was evaporated to remove excess triethylamine. The residual syrup was dissolved in 5 ml of acetone, and cyanuric chloride (277 mg, 1.5 mmol, Nacalai Tesque, Kyoto) dissolved in 5 ml of acetone was added portionwisely at 0°C. The reaction mixture was stirred overnight at room temperature. To the solution, morpholine (227 μl, 2.6 mmol, Nacalai Tesque) in 5 ml of acetone was added at 0°C, then the reaction mixture was stirred for 3 h at room temperature. After removing the solvent with a stream of nitrogen, the residue was suspended in diethyl ether. The insoluble material was removed by filtration. The solvent of the filtrate was removed by evaporation. The residue was subjected to silica-gel column chromatography (20 g, No. 9385, Merck, Darmstat, Germany) eluted with chloroform to obtain 103 mg of compound **4** and 318 mg of 2-(4-azidoanilyl)-4,6-dichloro-1,3,5-triazine (**3**). The latter compound was dissolved in dichloromethane and treated with morpholine again to form further **4** under similar conditions described above. The total yield of **4** from **2** was 81%. ^{1}H-NMR data of **4** (400 MHz, in $CDCl_3$): δ 7.00 (2H, d, ^3J=8.8 Hz) and 7.50 (2H, d, ^3J=8.8 Hz), δ 7.26 (1H, s), δ 3.73 and 3.83 (8H, m). FAB-MS (positive mode): 333.1 $(M+H)^+$, 304.1 $(M+H-N_2)^+$.

Compound **4** (182 mg, 0.49 mmol) was dissolved in 20 ml of dichloromethane, and 1,4-diazabicyclo[2,2,2]octane (DABCO, 75 mg, 0.67 mmol, Nacalai Tesque) dissolved in 10 ml of the same solvent was added with portionwise at room temperature. The reaction mixture was stirred for 8 h, then the solvent was removed by evaporation. Addition of chloroform and acetone (8:1, v/v) to the residual syrup yielded the product **1** as a white powder, which was isolated by filtration and washed with the same mixed solvent. Analysis of **1** by ^{1}H-NMR and FAB-MS indicated the absence of contaminating compounds. ^{1}H-NMR (400 MHz, in DMSO-d_6): δ 7.13 (2H, d, ^3J=8.6 Hz), δ 7.71 (3H, broad multiplet), δ 3.18 (6H, t, ^3J=7.3 Hz) and δ 3.76 (14H, m). FAB-MS (positive): 409.0 $(M-Cl)^+$, 381 $(M-Cl-N_2)^+$. Yield: 181 mg (80%).

From the filtrate, 2-(4-azidoanilyl)-4-(4-chloroethylpiperazinyl)-6-morpholino-1,3,5-triazine (**5**) was obtained in 18% yield by silica-gel column chromatography (20 g, No. 9385, Merck). ^{1}H NMR of **5** (270 MHz, in $CDCl_3$): δ 7.54 (2H, d, ^3J=8.6 Hz), δ 6.95 (2H, d, ^3J=8.6 Hz), δ 6.66 (1H, s), δ 3.76 (12H, m), δ 3.62 (2H, t, ^3J=7.3 Hz), δ 2.79 (2H, t, ^3J=7.3 Hz), δ 2.54 (4H, dd, ^3J=5.6 and 5.1 Hz). FAB-MS (positive): 445.2 $(M+H)^+$, 417.2 $(M+H-N_2)^+$.

Reaction of AA-D (1) with Ethanol: Preparation of 2-(4-Azidoanilyl)-4-Ethoxy-6-Morpholino-1,3,5-Triazine (6)—AA-D (27 mg, 61 mmol) was dissolved in 5 ml of ethanol, then 1 ml of 0.1 M aqueous sodium hydroxide was added with stirring. Completion of the reaction within 10 min was confirmed by TLC (Silica-gel 60 F_{254}, No. 5715, Merck). After removing the solvent by evaporation, the product was purified by silica-gel column chromatography (20 g, No. 9385, Merck) to give 2-(4-azidoanilyl)-4-ethoxy-6-morpholino-1,3,5-triazine (**6**). The yield was 20.8 mg (quantitative). ^{1}H NMR (270 MHz, in $CDCl_3$): δ 7.54 (2H, d, ^3J=8.9 Hz), δ 7.00 (3H, m), δ 4.36 (2H, q, ^3J=7.3 Hz), δ 3.84 (4H, m), δ 3.74 (4H, m), δ 1.39 (3H, d, ^3J=7.3 Hz). ^{13}C-NMR (100 MHz, in $CDCl_3$) δ 170.9, 166.4, 165.4, 136.0, 134.9, 121.8, 119.6, 66.9, 63.2, 44.2, and 14.7. FAB-MS (positive): 343.0 $(M+H)^+$, 315.0 $(M+H-N_2)^+$.

Labeling of Heparins with AA-D—Porcine intestinal pharmaceutical heparin (1.5 mg, Celsus, Cincinnati, OH, USA, average MW: 17,500) was dissolved in 150 μl of 0.2 M sodium bicarbonate buffer (pH 8.4) and added to 300 μl of solution of AA-D reagent (2 mg/ml in the same buffer). After incubation of the mixture at room temperature overnight, 150 μl of a solution of D-glucose (100 mg/ml in 0.2 M sodium bicarbonate buffer) was added to quench the excess reagent. Then 600 μl of the solution was applied to a column of Sephadex G-25 (1 cm×25 cm, Pharmacia Biotech, Uppsala, Sweden) and eluted with distilled water. The elution profile was monitored by absorbance at 280 nm. Void fractions were collected and lyophilized to obtain AA-D-labeled heparin.

Labeling of [^{3}H]heparin (1.5 mg, New England Nuclear, 0.55 mCi/mg, average MW: 15,500) was performed with 0.6 mg of AA-D and the fractions obtained by G-25 column chromatography were also monitored by scintillation countering.

Photoreaction of AA-D-Labeled [^{3}H]Heparin with Purified Heparin-Binding and Non-Heparin-Binding Proteins—Purified antithrombin III or ovalbumin (Pharmacia Biotech) was dissolved in 5 μl of HBSS buffer (pH 7.4) (5 mg/ml). To the solution, [^{3}H]heparin labeled with AA-D (abbreviated as AA-D-[^{3}H]Hep) dissolved in HBSS buffer was added (15 μl; 1 mg/ml of HBSS buffer; total radioactivity: 6.5 μCi). The solution was incubated for 10 min at room temperature under room light, then exposed to UV light (254 nm) from a 4-cm distance by use of a Spectroline model #Q-22SNF (Spectronics, Westbury, NY, USA) at room temperature for various times. After irradiation, the protein/heparin mixture was separated by SDS-PAGE (10% gel, reduced) and electrotransferred to two PVDF membranes by standard Western blotting techniques. One membrane was exposed to [^{3}H]-Imaging Plate and analyzed by the BAS™ system (BAS-2000 or BAS-3000). The other membrane was gold-stained to visualize the protein bands (Owl Separation Systems, Woburn, MA, USA). The radioactivity of the separated bands were quantified by the BAS™ system's software.

For a competitive binding experiment, unlabeled heparin (Celsus) was added in a range of concentrations to the reaction mixture containing AA-D-[^{3}H]Hep and ATIII.

Photo Crosslinking of AA-D-Labeled [^{3}H]Heparin (AA-D-[^{3}H]Hep) to Platelet Membrane Surface Proteins—Fresh human platelets from healthy normal donors were separated and washed as previously described (5), then suspended at 1.5×10^6 cells/μl in HBSS buffer. The washed platelet suspension (300 μl, 1.5×10^6 cells/μl) was mixed with AA-D-[^{3}H]Hep (40.8 μCi, 0.96 mg/ml, 96 μg in 100 μl of HBSS). After a 5 min preincubation, photo-irradiation was performed as described above for 15 min with gentle stirring. After separation from the suspension by centrifugation, the platelets were washed 3 times with PBS, then dissolved in PBS containing 1% Triton X-100 (Sigma Aldrich). After centrifugation to remove Triton-insoluble material, the obtained supernatant was analyzed by SDS-PAGE followed by BAS™-imaging of the blots.

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RESULTS AND DISCUSSION

Preparation of AA-D Reagent and Its Reactions with Alcohols and Heparin—The preparation of AA-D reagent is summarized in Fig. 1. It can be done under room light. AA-D was obtained as a precipitate. A simple filtration followed by washing yielded pure AA-D, as indicated by FAB-MS and ^{1}H NMR of the powder. The by-product, 2-(4-azidoanilyl)-4-(4-chloroethylpiperazinyl)-6-morpholino-1,3,5-triazine **5**, was obtained in 18% yield by silica-gel column chromatography of the filtrate.

AA-D was then allowed to react with ethanol to demonstrate its ability to form a covalent bond *via* free hydroxy groups in a simplified system. The product of the reaction under slightly alkaline conditions was visualized as a single spot on TLC (Fig. 2). From a large-scale preparation, 2-(4-azidoanilyl)-4-ethoxy-6-morpholino-1,3,5-triazine **6** was obtained in a quantitative yield. Under similar conditions, AA-D reagent also reacted with 2-propanol to give 2-(4-azidoanilyl)-4-(2-propoxy)-6-morpholino-1,3,5-triazine in a high yield (data not shown). These results demonstrated that the AA-D reagent reacts effectively with both primary and secondary hydroxy groups in high yields.

To test the photoreactivity of the azide end of AA-D, ethanol labeled with AA-D was exposed in ethanol to UV light (254 nm) for 5 min from a 4-cm distance by use of a Spectroline model #Q-22SNF (Spectronics). The single spot on TLC changed to several spots with lower R_f (Fig. 2), indicating that the photoreaction of **6** with the solvent

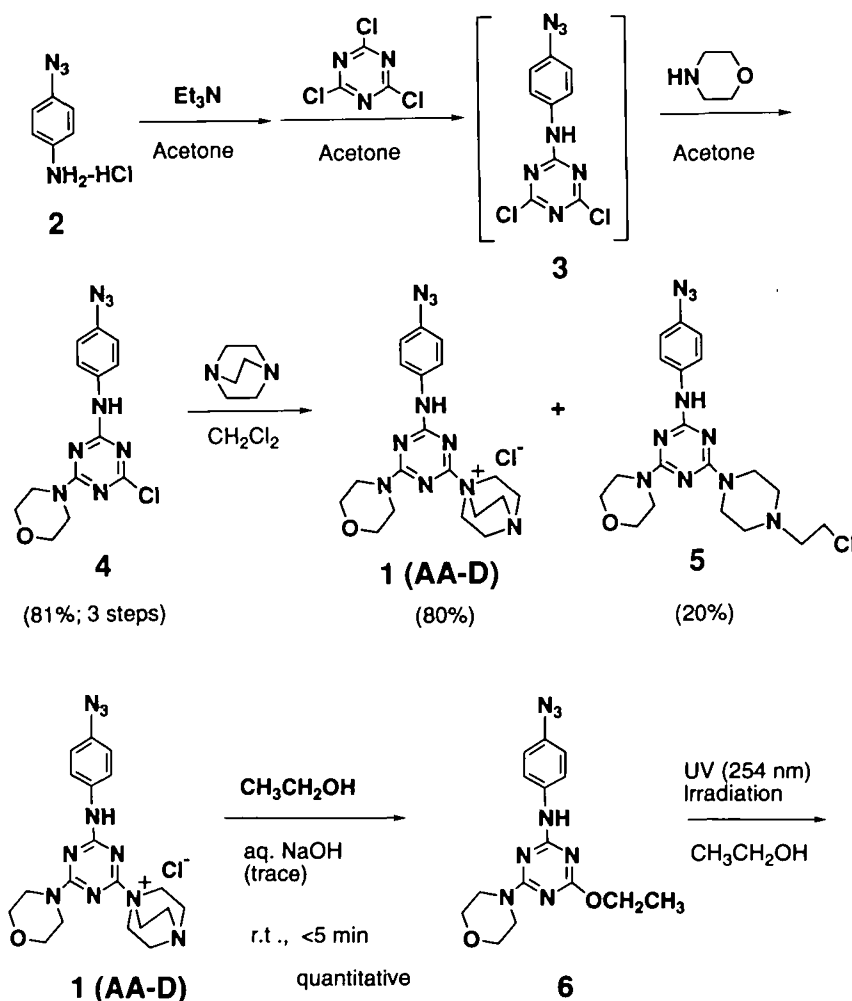

Fig. 1. **Preparation of the hetero-bifunctional crosslinker, 2-(4-azidoanilyl)-4-(4-azabicyclo-[2,2,2]hexylammonio)-6-morpholino-1,3,5-triazine chloride 1 (abbreviated as AA-D).**

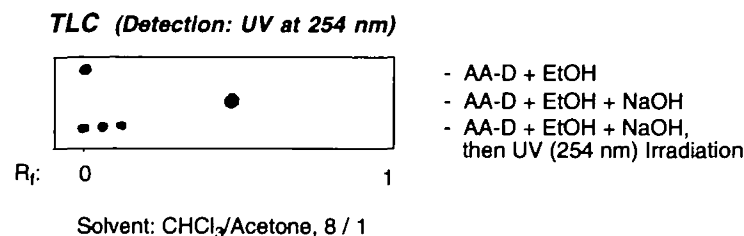

Fig. 2. **Labeling of ethanol with AA-D, and subsequent photo-crosslinking to ethanol.** TLC analysis of products is shown.

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ethanol occurred, creating covalently bound ethanol/AA-D/ethanol complexes. An ion peak ($m/z=383$) corresponding to the complex, $(M-Cl-DABCO+CH_3CH_2O-N_2+CH_3CH_2OH+Na)^+$, was observed in the ESI-MS analysis. These results indicate the high crosslinking activity of the azide group in the AA-D reagent by the UV irradiation.

Heparin or [^{3}H]heparin was then labeled with AA-D reagent in the same way with fluorescein-containing reagent (7). Figure 3 shows a chromatographic profile obtained on the labeling of [^{3}H]heparin. Labeled [^{3}H]heparin (abbreviated as AA-D-[^{3}H]Hep) was obtained in the void fractions, as indicated by co-migration of the radioactivity of heparin and the UV absorption of the AA-D residue. Labeled cold heparin (AA-D-Hep) was obtained similarly.

In a typical experiment, the reaction of 0.65 mg/ml of AA-D with 22 mg/ml of heparin yielded an intensity of AA-D labeling of 142% (1.42 molecule of AA-D was labeled on one heparin molecule on average). After bonding with AA-D, the anticoagulant activity of heparin as measured by anti-Xa assay (5) was unchanged at 305 units/mg. Labeling of heparin with AA-D did not alter its anticoagulant potency.

Specificity in Crosslinking of AA-D-[^{3}H]Hep with Antithrombin III and/or Ovalbumin—To test the specificity of the heparin affinity crosslinking, we attempted to crosslink the AA-D-[^{3}H]Hep to a known heparin-binding protein (antithrombin III), as well as a protein (ovalbumin) that does not bind heparin. Numerous controls were used, including AA-D-[^{3}H]Hep mixed with either of the proteins or both using a range of UV exposures from none to 16 min. The reaction products were analyzed by SDS-PAGE, followed by blotting and BAS™-imaging. Figure 4 shows that the radio-labeled heparin was successfully crosslinked to antithrombin III, but not to ovalbumin. The radioactivity of protein bands corresponding to antithrombin III became more intense with increasing irradiation of UV light, whereas almost no bands were observed at the position of ovalbumin. The molecular mass of the 1 to 1 conjugate of ATIII and heparin should be about 75 kDa. Since heparin is a highly sulfated polysaccharide, the conjugate should be highly negatively charged. In the SDS-PAGE, negatively charged compounds migrate faster than expected from the molecular mass alone. Therefore, the heparin-bearing ATIII migrates to almost the same position to ATIII itself in 15% SDS-PAGE gel.

As BAS™-imaging gives highly quantitative results over

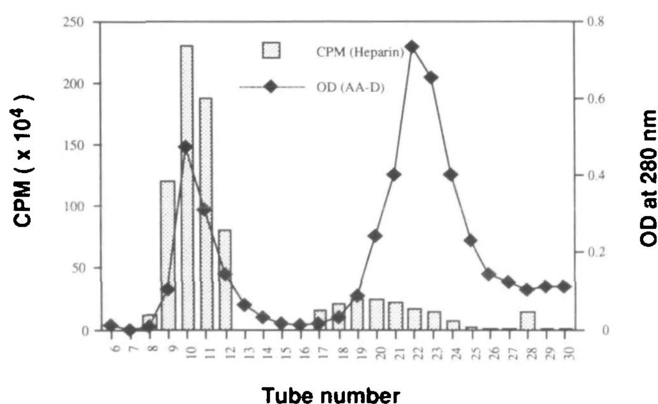

Fig. 3. **Labeling of [^{3}H]heparin with AA-D.** See text for detailed methods. [^{3}H]Heparin was dissolved in 0.2 M sodium bicarbonate buffer and AA-D reagent was added. The labeled [^{3}H]heparin (AA-D-[^{3}H]Hep) was obtained in the first peak.

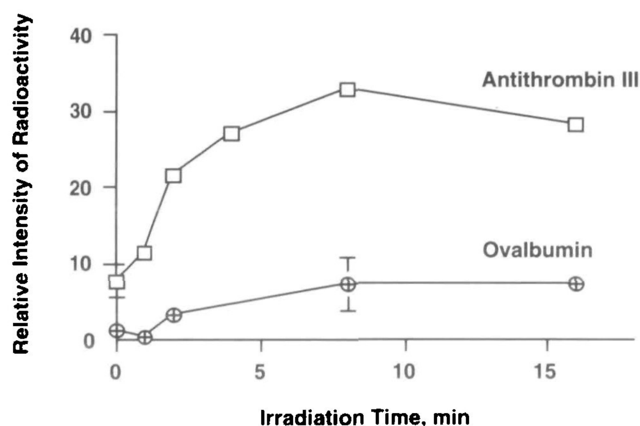

Fig. 5. **Quantification of binding specificity and efficiency.** Intensity of bands corresponding to Fig. 4 was analyzed by BAS™ and plotted against duration of UV light irradiation.

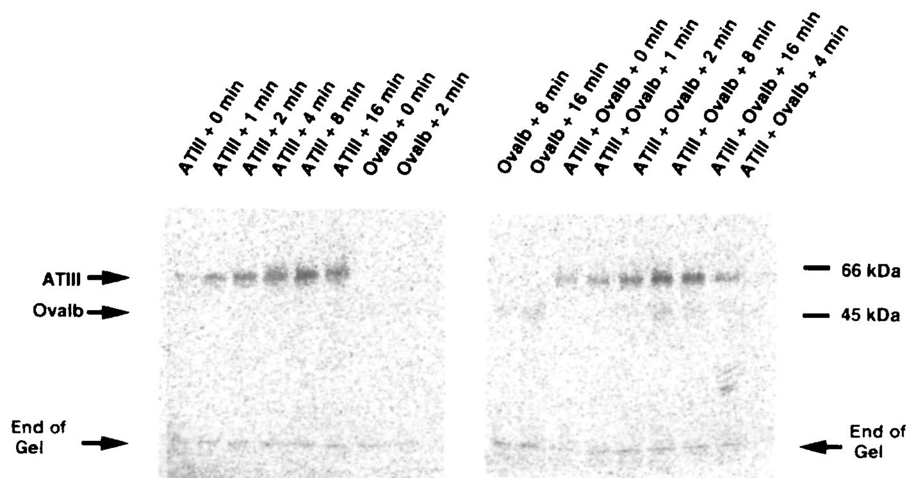

Fig. 4. **Specificity in photo-affinity crosslinking of AA-D-[^{3}H]Hep to model proteins.** See text for detailed methods.

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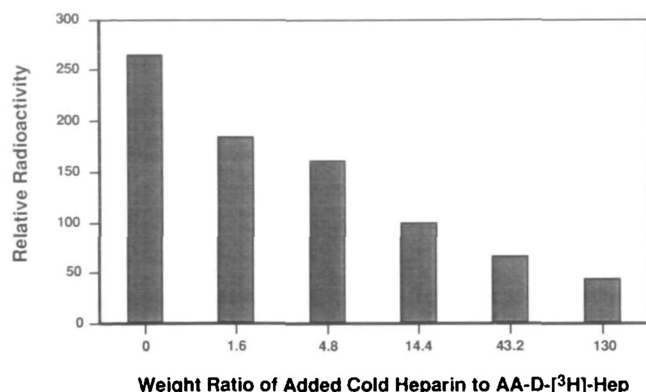

Fig. 6. **Inhibition of photo-affinity crosslinking by unlabeled heparin.**

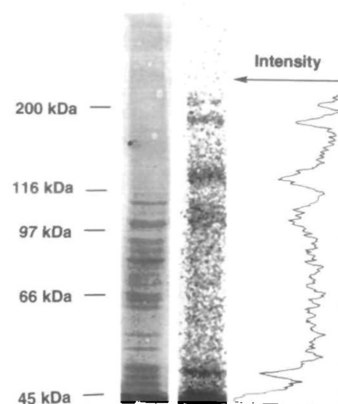

Fig. 7. **Heparin photo-affinity crosslinking to platelet surface proteins.** The lefthand lane shows gold-stained protein bands from the Western blot of 6% reduced gel. The middle hand lane show the radioactive bands from the same gel. The righthand lane shows the tracer of the radioactive bands in the middle lane. See text for detailed methods. Molecular mass standards are indicated.

a wide dynamic range, thus the relative intensity of radioactive heparin bound to proteins was able to be estimated (Fig. 5). The radioactivities in the ovalbumin band were at the background level, whereas those in the antithrombin III steadily rose with increased time of irradiation (with a peak at 8 min exposure).

To further prove the specificity of the crosslinking, the same experiment was performed with antithrombin III in the presence of increasing concentrations of unlabeled heparin. As shown in Fig. 6, the corresponding intensity of the band of antithrombin III decreased steadily as the concentration of unlabeled heparin increased. These results strongly suggest that the photo-crosslinking reaction based on the binding of AA-D-[^{3}H]Hep is highly selective for heparin-binding proteins.

Detection of Heparin-Binding Protein(s) on the Surface of Platelets—Washed platelets were incubated with AA-D-[^{3}H]Hep in HBSS for 5 min, then expose to the UV light for 15 min with gentle stirring. The platelets were washed 3 times with PBS containing glucose, precipitated by centrifugation, and solubilized in PBS containing 1% Triton X-100. As estimated by the scintillation counting of the solubilized platelet pellet, about 7% of AA-D-[^{3}H]Hep was incorporated into the platelets. The dissolved platelet proteins were subjected to SDS-PAGE, electrotransferred, stained, and analyzed by BAS^{TM}-imaging as described above. Figure 7 shows the images side by side. Several bands (165-200, 155-180, 126-140, 107-113, and about 50 kDa) were visualized. Using independent, complementary techniques to identify heparin-binding proteins in the platelet membrane fraction, we have detected similar heparin-binding protein bands in this range (*8*). We are currently attempting to isolate and identify these heparin-binding platelet surface proteins.

In conclusion, we have reported the synthesis and application of a novel heterobifunctional photo-affinity crosslinking reagent that is especially suited to the study of protein-glycosaminoglycan interactions. The crosslinker was shown to label heparin efficiently and non-destructively without lose of heparin's affinity for specific proteins. This reagent may have a wide range of applications for studying the interactions of heparins and other glycosaminoglycans with cells and proteins.

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