Silvius, J. R., Mak, N., & McElhaney, R. N. (1980) *Biochim. Biophys. Acta 597*, 199.
Siminovitch, D. J., Wong, P. T. T., & Mantsch, H. H. (1987) *Biochim. Biophys. Acta 900*, 167.
Six, L., Ruess, K.-P., & Lieflander, M. (1983) *Tetrahedron Lett. 24*, 1229.
Slater, J. L., & Huang, C. (1987) *Biophys. J. 52*, 667.
Slomiany, B. L., Murty, V. L. N., Liau, Y. H., & Slomiany, A. (1987) *Prog. Lipid Res. 26*, 29.
Snyder, R. G. (1967) *J. Chem. Phys. 47*, 1316.
Sorokoumova, G. M., Gusev, D. G., Vasilenko, I. A., Kaplun, A. P., & Shvets, V. I. (1987) *Bioorg. Khim. 13*, 1579; (1988) *Sov. J. Bioorg. Chem. (Engl. Transl) 13*, 860–868.
Steim, R. S., & Sutherland, G. B. B. M. (1954) *J. Chem. Phys. 22*, 1993.
Tomoaia-Cotisel, M., Chifu, E., Sen, A., & Quinn, P. J. (1982) in *Biochemistry and Metabolism of Plant Lipids* (Wintermans, J. F. G. M., & Kuiper, P. J. C., Eds.) p 393, Elsevier Biomedical Press, New York.
Tomoaia-Cotisel, M., Sen, A., & Quinn, P. J. (1983) *J. Colloid Interface Sci. 94*, 391.
Tomoaia-Cotisel, M., Zsakó, J., Chifu, E., & Quinn, P. J. (1987) *Biochem. J. 248*, 877.
Tristram-Nagle, S., Weiner, M. C., Yang, C.-P., & Nagle, J. F. (1987) *Biochemistry 26*, 4288.
Wickberg, B. (1958) *Acta Chem. Scand. 12*, 1187.
Wiegandt, H. (1985) in *Glucolipids*, Elsevier, New York.
Wieslander, A., Ulmius, J., Lindblom, G., & Fontell, K. (1978) *Biochim. Biophys. Acta 512*, 241.
Wieslander, A., Christiansson, A., Rilfors, L., Khan, A., Johansson, L. B.-A., & Lindblom, G. (1981a) *FEBS Lett. 124*, 273.
Wieslander, A., Rilfors, L., Johansson, L. B.-A., & Lindblom, G. (1918b) *Biochemistry 20*, 730.
Wong, P. T. T., & Mantsch H. H. (1985) *J. Chem. Phys. 83*, 3268.
Wong, P. T. T., & Mantsch, H. H. (1988) *Chem. Phys. Lipids 46*, 213.
Wong, P. T. T., & Huang, C. (1989) *Biochemistry 28*, 1259.
Wong, P. T. T., Moffatt, D. J., & Baudais, F. L. (1985) *Appl. Spectrosc. 39*, 733.
Wong, P. T. T., Siminovitch, D. J., & Mantsch, H. H. (1988) *Biochim. Biophys. Acta 947*, 139.
Wynn, C. H., Marsden, A., & Robson, B. (1986) *J. Theor. Biol. 119*, 81.

Affinity Chromatography Purification of Angiotensin II Receptor Using Photoactivable Biotinylated Probes†

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Received January 30, 1990; Revised Manuscript Received May 11, 1990

ABSTRACT: We have developed biotinylated photoactivable probes that are suitable for covalent labeling of angiotensin II (AII) receptors and the subsequent purification of covalent complexes through immobilized avidin or streptavidin. One of these probes, biotin-$NH(CH_2)_2SS(CH_2)_2CO$-[Ala^1,$Phe(4N_3)^8$]AII, which contains a cleavable disulfide bridge in its spacer arm and which displays, in its radioiodinated form, very high affinity for AII receptors ($K_d \sim 1$ nM), proved to be suitable for indirect affinity chromatography of rat liver receptor with facilitated recovery from avidin gels by use of reducing agents. This consistuted the central step of an efficient partial purification scheme involving hydroxylapatite chromatography, streptavidin chromatography, and thiopropyl-Sepharose chromatography. SDS–PAGE analysis and autoradiography established the identity of the purified entity (molecular weight 65K) as the AII receptor. Possible ways of completing purification to homogeneity and extrapolation of the protocols to a preparative scale are discussed, as well as the potential contribution of our new probes to the study of the structural properties of angiotensin receptors.

The inability of solubilized angiotensin II receptors to bind angiotensin II (AII:[1] Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) has considerably hampered the development of a simple purification by classical affinity techniques and hence progress in the elucidation of their structural and molecular properties. In spite of the data from Soffer's (Sen et al., 1984; Bandhyopadhyay et al., 1988; Kiron & Soffer, 1989) and Blalock's (Elton et al., 1988) groups, purification protocols providing sequencable amounts of receptors are not yet available. The ability to efficiently covalently label the receptor with AII derivatives bearing an azidophenylalanine at their C-termini (Escher et al., 1978; Guillemette et al., 1985, 1986) and the previous demonstration that biotinylation through N-terminal modifications of the AII molecule preserved affinity for the

†This work was supported by Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, and Fondation pour la Recherche Médicale.

[1] Abbreviations: AII, angiotensin II; [Sar^1]AII, [1-sarcosine]angiotensin II; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethanesulfonyl fluoride; TFMS, trifluoromethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; Ahx, aminohexanoic acid; Bio-AII-N_3, biotin-Ahx-[Ala^1,$Phe(4N_3)^8$]AII; Bio-S-S-AII(N_3), biotin-$NH(CH_2)_2SS(CH_2)_2CO$-[Ala^1,$Phe(4N_3)^8$]AII.

0006-2960/90/0429-8943$02.50/0

receptors (Bonnafous et al., 1988a,b) prompted us to develop an indirect affinity chromatography strategy for rat liver angiotensin II receptor purification involving (1) covalent labeling of purified rat liver plasma membranes with biotinylated, photoactivable, and radioiodinated AII derivatives and (2) solubilization and adsorption of biotin-tagged hormone–receptor complexes on immobilized avidin or streptavidin. A similar approach was proposed by Finn et al. (1985) for ACTH receptors and by Brennan and Levine (1987) for PTH receptors.

The present work describes the properties of several original biotinylated azido probes, synthesized in our laboratory, and the various problems raised by their application to the purpose indicated above. A major problem was the low yield of receptor recovery from the avidin gels, under an excess of biotin, resulting from the very high affinity of biotin for avidin: difficulties were overcome by the design of biotinylated derivatives bearing a cleavable disulfide bridge between their biotin and hormone moieties. One of these probes, biotin-$NH(CH_2)_2SS(CH_2)_2CO$-[Ala^1,$Phe(4N_3)^8$]AII [Bio-S-S-AII(N_3)] was successfully applied to the development of an efficient partial purification scheme in experiments starting at the nanomole level of membrane receptor. The potential applications of this protocol to the determination of the structural and physicochemical properties of angiotensin receptors are discussed. The constraints of purification to homogeneity and its extrapolation to a preparative scale are also evaluated with respect to the problem of primary structure determination, which is presently investigated by more recent or unpredicted approaches (Jackson et al., 1988).

Materials and Methods

Materials. Biotin, avidin, and streptavidin were from Sigma, Triton X-100 was from Pierce, hydroxylapatite (Bio-Gel-HTP) was from Bio-Rad, and activated Sepharose 4B and thiopropyl-Sepharose 6MB were from Pharmacia.

Synthesis of Biotinylated Photoactivable Probes. Bio-Ahx-[Ala^1,$Phe(4N_3)^8$]AII was synthesized as previously described (Seyer et al., 1989). Bio-$NH(CH_2)_2SS(CH_2)_2CO$-[Ala^1,$Phe(4N_3)^8$]AII was obtained by reacting Bio-NH-$(CH_2)_2SS(CH_2)_2COOSu$ (Pierce) with [Ala^1,$Phe(4N_3)^8$]AII (synthesized by solid-phase peptide synthesis) as described by Seyer et al. (unpublished results). The probes were purified by reverse-phase HPLC and characterized by NMR spectroscopy (360 MHz). Solutions of the azido probes were calibrated by UV spectroscopy (ϵ_{250nm} = 12 500).

Iodination and Radioiodination. Unlabeled monoiodo derivatives of the probes were obtained by the action of ICl (Mc Farlane, 1958) at a 1/1 peptide/ICl stoichiometry, allowing optimal formation of monoiodinated compound; the latter was separated from starting peptide and diiodinated compound by HPLC as previously described (Bonnafous et al., 1988). The monoiodinated derivative was identical with the compound obtained by direct introduction of iodotyrosine in the probe synthesis protocol, followed by NMR characterization of the final product. Radioiodination was carried out in 0.5 M phosphate, pH 7.0, with ^{125}INa (Amersham) and iodogen-coated polypropylene tubes (Fraker & Speck, 1978) (iodide, peptide, and iodogen were in the following proportions: 1/20/70). The large excess of peptide over radioactive iodide allows optimal iodine incorporation as monoradioiodinated compound, little diiodinated compound being found under these conditions. The reaction lasted 2.5 min at room temperature, in the dark. The radioiodinated probes were isolated by HPLC, using acetonitrile gradients in 0.1% trifluoroacetic acid. Probe samples of appropriate specific radioactivities were obtained by mixing labeled and unlabeled compounds.

Membrane Preparation. Purified liver membranes from Wistar male rats were prepared according to Neville (1968) up to step 11. Routinely, 120–180 mg of membrane proteins was obtained from 16 livers. Membranes were stored in liquid nitrogen. Pools from 6–12 separate preparations were used for purification experiments.

Binding Studies. Equilibrium binding experiments of the iodinated probes to purified rat liver plasma membranes were carried out as previously described (Bonnafous et al., 1988): membrane samples (50 μg) were incubated for 30 min at 30 °C with ligand solutions with or without 0.12 mM *N*-ethylmaleimide (NEM) (90-μL final volume) in the binding buffer (50 mM phosphate, pH 7.4, 5 mM $MgCl_2$, 1 mg/mL BSA, 1 mg/mL bacitracine).

Photoaffinity Labeling and Solubilization. After saturation of the binding sites with the radioiodinated probes (8–10 nM, in binding buffer), in the presence of NEM when required (0.5 mM for 2 mg/mL membrane protein concentrations), in the absence or presence of 10^{-6} M unlabeled [Sar^1]AII, the membranes (1–2 mg/mL) were washed with cold phosphate buffer, pH 7.4, containing 5 mM $MgCl_2$ and 0.5 mM NEM. The membranes were then irradiated for 5 min at 254 nm (five TUV 6 Philips lamps, temperature 0 °C, 1–2 mg/mL membranes displayed as 1–2 mm thick layers). After two additional washings with 10 mM phosphate, pH 6.0, 5 mM EDTA, 1 mM PMSF (pH 7.5 or 6.0 in more recent experiments), and ±0.5 mM NEM, the pelleted membranes were solubilized with 10 mM phosphate, pH 6.0, 5 mM EDTA, 1 mM PMSF, ±0.5 mM NEM, and 0.5–1.5% Triton X-100 (1–2.5 mg/mL membrane protein) added at room temperature and then cooled to 0 °C; after 1.5 h, the detergent-treated samples were centrifuged for 1 h at 200000*g*; the ratioactivities found in the supernatants represented an accurate evaluation of the solubilized receptor since there was no contamination by ligand dissociated from nonsolubilized complexes (identical values were obtained in labeling assays carried out with [Sar^1]angiotensin derivatives, which give stable complexes). The solubilized membranes were treated for 1 h at 30 °C to dissociate noncovalently bound probe; the latter was eliminated by gel filtration through Trisacryl GF 05 (IBF-LKB) or Sephadex G-50 (Pharmacia) or Bio-Gel P-30 (Bio-Rad) before SDS–PAGE analysis according to Laemmli (1970). Alternatively, the covalent complexes were selectively adsorbed to hydroxylapatite in a batchwise procedure, 50 μL of sedimented gel/mg of starting membrane protein being used; elution was achieved with 0.3 M phosphate buffer, pH 6.0, 0.5% Triton X-100, 1% SDS, 5 mM EDTA, 1 mM PMSF, and 0.5 mM NEM when necessary.

Adsorption of Biotinylated Covalent Complexes to Immobilized Avidin or Streptavidin. Succinylavidin–Sepharose (1 mg of avidin/mL of gel) was prepared according to Finn et al. (1984). The succinylation step was omitted in the preparation of streptavidin–Sepharose (1 mg/mL of gel). Covalent complex adsorption was carried out as described under Receptor Purification, except that a batchwise procedure was adopted for analytical purposes (maximal adsorption time 4–5 h). SDS (1%) was added to samples recovered from gel filtration.

Receptor Purification. A total of 2 g of rat liver plasma membranes was incubated for 30 min at 30 °C with 8 nM ^{125}I-Bio-S-S-AII(N_3) and 0.5 mM NEM (+10^{-6} M [Sar^1]AII for the "nonspecific" assay) in 1 L of binding medium (50 mM phosphate, pH 7.4, 5 mM $MgCl_2$, 1 mg/mL BSA, 1 mg/mL bacitracin). The membranes were washed, photolyzed, and

solubilized (1.5% Triton X-100 for 2.5 mg/mL membrane protein) as described above. After dissociation of noncovalently bound ligand and EDTA complexation with the appropriate amount of $MgCl_2$ (10 mM final concentration), the solubilized sample was incubated with gentle shaking in 100 mL of hydroxylapatite–Bio-Gel P-30 for 1 h at room temperature (hydroxylapatite gel has been previously equilibrated in 10 mM phosphate, pH 6.0, 1 mM PMSF, 0.5 mM NEM, and 0.5% Triton X-100). The gel was then packed into a column and rinsed thoroughly. Receptor was eluted with 0.3 M phosphate, pH 6.0, 0.5% Triton X-100, 1% SDS, 5 mM EDTA, 1 mM PMSF, and 0.5 mM NEM (volume about 50 mL), added with NaCl (0.1 M final concentration), and incubated for 1 h at 30 °C.

The samples were then chromatographed (flow rate 20 mL/h; 12–15 °C to avoid SDS precipitation) on a 5-mL streptavidin column equilibrated in the following buffer: 10 mM phosphate, pH 6.0, 0.5% Triton X-100, 5 mM EDTA, 1 mM PMSF, and 0.5 mM NEM. The column was washed with 15 mL of each of the following media: buffer + 1% SDS; buffer + 1 M NaCl; buffer, pH 8.0; 10 mM phosphate, pH 8.0, + 0.1% SDS.

Elution of the complexes was achieved by gentle agitation (15 min at room temperature) of the streptavidin gel with 5 volumes of 10 mM phosphate, pH 8.0, 50 mM DTT, and 1% SDS. The streptavidin eluate was freed of most of its DTT content by three successive ultrafiltration steps through YM30 Diaflo membranes (Amicon), separated by dilution with 10 mM phosphate, pH 6.0, and 0.1% Triton X-100 (slightly acidic medium avoids reoxidation of SH functions generated on the spacer arm). The resultant concentrated sample (~2 mL) was mixed with 0.5 mL of thiopropyl-Sepharose 6MB (sedimented gel previously equilibrated in 10 mM phosphate, pH 6.0, and 0.1% Triton X-100): The pH of the resulting suspension was adjusted to 7.5–7.8 by the addition of 1 M phosphate, pH 8.2. After 2 h of gentle agitation, the gel was poured into a small column and rinsed with 10 mM phosphate, pH 8.0, and 0.1% SDS, before elution with the same buffer supplemented with 50 mM DTT. The thiopropyl-Sepharose eluate was concentrated in Amicon Centricon P30 microconcentrators (three cycles separated by 5-fold dilution with 0.1% SDS and 10 mM phosphate, pH 8.0). The lyophilized samples were finally analyzed by SDS–PAGE, in reducing conditions, and autoradiography.

Deglycosylation. Solubilized biotinylated probe–receptor covalent complexes were chemically deglycosylated by TFMS as described by Edge et al. (1981). Maximal deglycosylation (occurring both at N- and O-linked oligosaccharides) was obtained after 5 min of treatment. TFMS treatment induced important losses of iodine from the ligand tyrosine estimated at 80–90%.

Protein Measurements. Plasma membrane proteins were estimated according to Lowry et al. (1951). Proteins in solubilized membranes, hydroxylapatite, streptavidin, and thiopropyl-Sepharose eluates were measured by the fluorescamine method (Castell et al., 1979) applied to 6 N HCl hydrolyzed samples.

Results

Probe Structure: Iodination and Radioiodination. Two synthetic biotinylated probes have been used. Their structures are shown in Figure 1: Bio-Ahx-[Ala^1,$Phe(4N_3)^8$]AII [Bio-AII(N_3)] (I); Bio-NH$(CH_2)_2$SS$(CH_2)_2$CO-[Ala^1,$Phe(4N_3)^8$]AII [Bio-S-S-AII(N_3)] (II).

Aminohexanoic acid (Ahx) has been introduced as spacer arm in probe I. The Asp residue of AII has been replaced by

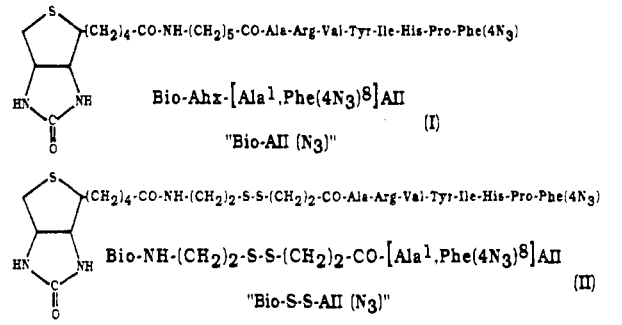

FIGURE 1: Structure of biotinylated photoactivable angiotensins.

Ala to make these probes resistant to *Staphylococcus aureus* V8 protease, which we intend to use in fragmentation experiments. This replacement did not effect the affinity for the receptor (data not shown for the Asp analogue of compound II).

Monoiodinated derivatives of these probes were classically obtained by action of iodine monochloride (McFarlane, 1958), followed by HPLC separation as previously described (Bonnafous et al., 1988a).

Radioiodination was carried out from [^{125}I]iodide with iodogene as the oxidizing agent (Fraker & Speck, 1978). HPLC separation of the radioiodinated compounds is represented in Figure 2a, which refers to probe II (similar profiles were obtained for radioiodination of both probes): compound B was the desired compound: it comigrated with the unlabeled monoiodo derivative (Figure 2b); as previously demonstrated for another biotinylated angiotensin (Bonnafous et al., 1988a), the more polar compound (A) was oxidized on biotin (biotin sulfoxide); the latter was discarded because it was uncompletely separated from the excess noniodinated, nonoxidized starting peptide. We could find conditions allowing minimal biotin oxidation (24%) with suitable iodine incorporation. A minor amount of diiodinated compound C was formed.

Binding Properties of the Radioiodinated Probes. Equilibrium binding of the two radioiodinated probes was studied on purified rat liver membranes (Figure 3). The K_d value derived from Scatchard analysis was 0.9 nM for Bio-AII(N_3) (Figure 3a), the binding capacity being similar to that obtained for ^{125}I[Sar^1]AII within the same experiments.

Accurate evaluation of the binding properties of Bio-S-S-AII(N_3) required the addition of *N*-ethylmaleimide (NEM) to the assays, since some premature cleavage of the disulfide bridge occurred, presumably due to membrane thiol action; this was evident since the yield of covalent probe–receptor complex adsorption to avidin gels (see next paragraph) was reduced in the absence of NEM. Although high NEM concentrations inhibited binding of AII to its receptor (by reaction with an essential SH group), it was possible to find concentrations of NEM that exert no drastic effect on the binding parameters when added to membranes at the same time as the ligand (Figure 3b). The K_d values (1.3 nM without NEM and 1.1 nM in the presence of NEM) are close to that of Bio-AII(N_3), which is consistent with the similar structural properties of the two probes.

The presence of NEM in the binding assays significantly reduced (by 40%) nonspecific binding, probably by reducing thiol–disulfide exchange reactions and thus possible covalent linking of the probe to membrane SH functions.

Nonspecific binding was limited to acceptable values (25–35% for 8–10 nM concentrations of the probes). Indeed, as shown under Receptor Purification, it decreased throughout the purification steps to reach undetected levels in SDS–PAGE analysis.

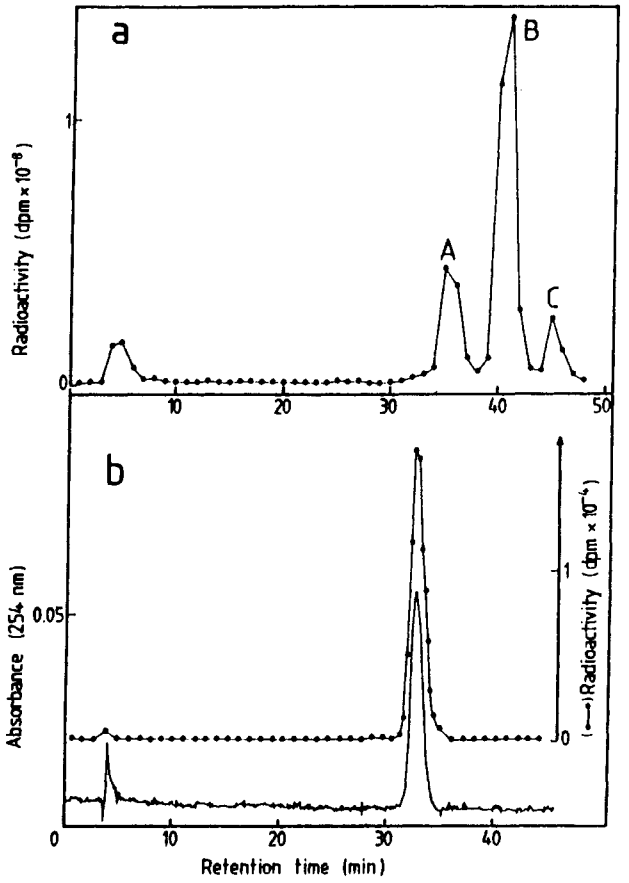

FIGURE 2: Radioiodination of biotinylated azidoangiotensins. (a) Radioiodination of Bio-S-S-AII(N_3). 10 nmol of biotinylated probe II was treated for 2.5 min with 1 mCi of ^{125}INa (sp radioactivity 1800–2200 Ci/mmol) in an iodogen-coated polypropylene tube as described under Materials and Methods. The radioiodinated products were separated by HPLC on a Waters Bondapack TM-C_{18} column (0.4 × 25 cm) with acetonitrile gradients in 0.1% trifluoroacetic acid (TFA) (gradient profile: 10–25% acetonitrile in 10 min; 25–50% acetonitrile in 45 min). (b) Identification of ^{125}I-Bio-S-S-AII(N_3). A mixture of 7 nmol of unlabeled monoiodo-Bio-S-S-AII(N_3) and trace amounts of radioiodinated compound B was analyzed by HPLC, with simultaneous absorbance and radioactivity detections. 1-mL fractions were collected, except in the elution area of the compounds where 0.3-mL fractions were analyzed. N.B. Different columns were used for experiments a and b, which explains variations in retention times.

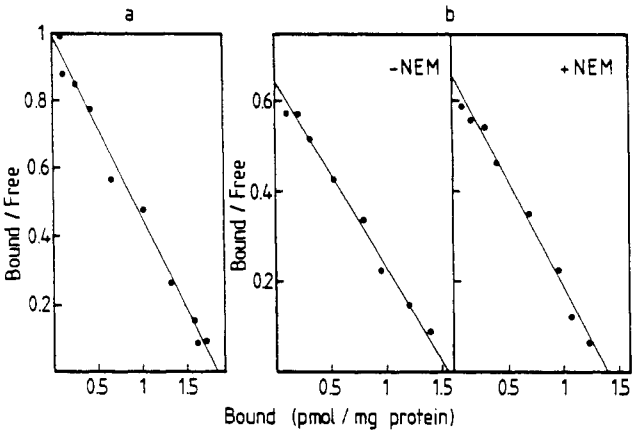

FIGURE 3: Equilibrium binding of radioiodinated biotinylated azidoangiotensins on rat liver plasma membranes. Membranes (50 μg/90 μL assay) were incubated for 30 min at 30 °C with various concentrations of radioiodinated Bio-AII(N_3) and Bio-S-S-AII(N_3) (sp radioactivities 99 and 348 Ci/mmol, respectively). Nonspecific binding was evaluated by adding 1 μM [Sar^1]AII to the assays; binding was unchanged when an excess of monoiodo unlabeled probes was used instead of [Sar^1]AII. Each experimental value is the mean of triplicate assays. (a) Scatchard plots relative to the specific binding of ^{125}I-Bio-AII(N_3). The binding parameters for this typical experiment are K_d = 0.92 ± 0.05 nM and B_{max} = 1830 ± 62 fmol/mg. The figure is representative of four separate experiments (K_d values ranging from 0.8 to 1.8 nM). (b) Scatchard plots relative to the specific binding of ^{125}I-Bio-S-S-AII(N_3) in the absence (left panel) or the presence (right panel) of 0.12 mM NEM. The binding parameters for this typical experiment are as follows: (−NEM) K_d = 1.32 ± 0.06 nM and B_{max} = 1576 ± 47 fmol/mg; (+NEM) K_d = 1.14 ± 0.06 nM and B_{max} = 1370 ± 41 fmol/mg. The figure is representative of three separate experiments (K_d values ranging from 1.3 to 1.9 nM).

These binding studies revealed labeling of a single class of high-affinity sites, consistent with the recent reevaluation of the correlation between binding sites and biological properties of angiotensin II in hepatocytes (Bouscarel et al., 1988). This, together with the previous finding that a parent non-azido biotinylated probe displayed agonist properties (Bonnafous et al., 1988a), ascertains that our two probes actually label angiotensin II receptors.

Covalent Labeling of the Receptor with Biotinylated Probes. The purification strategy requires covalent labeling of the receptor before solubilization. After equilibrium binding of the probes, the washed liver membranes were submitted to UV irradiation (254 nm, optimal time 5 min). The membranes were solubilized with Triton X-100. Dissociated noncovalently bound ligand could be eliminated by gel filtration; alternatively, in recently deviced purification protocols (see Receptor Purification), unbound ligand was eliminated by selective adsorption of covalent complexes to hydroxylapatite gels. The yields of probe–receptor complex solubilization by Triton X-100, which could be accounted for by radioactivities found in centrifugation supernatants of detergent-treated membranes (see Materials and Methods), were in the range 55–60%. Although SDS solubilization was more efficient (90%), it proved insuitable for further hydroxylapatite adsorption of the complexes.

The yield of photoaffinity covalent labeling repeatedly reached 20–25% and, as expected, was not dependent upon the biotinylated ligand used. Attempts to increase this yield by repetitive azido-probe loading and irradiation cycles were not successful.

SDS–PAGE analysis, in reducing conditions, of the covalent complexes revealed specific labeling of a 65K apparent molecular weight species (Figure 4A). The unexplained minor specific labeling observed at higher molecular weights (Figure 4A, lane a) was observed only occasionally, irrespective of the ligand used. This pattern of rat liver receptor photoaffinity labeling was similar to that previously found with [Sar^1,Phe(4N_3)^8]AII as ligand and was not significantly different when electrophoresis was carried out in nonreducing conditions (Guillemette et al., 1985, 1986).

The glycoprotein nature of the receptor was verified by chemical deglycosylation experiments (Figure 4A, lane c). An apparent molecular weight of 40K for the trifluoromethanesulfonic acid deglycosylated receptor is consistent with the molecular weight of the protein core usually found for G protein coupled receptors.

Indirect Affinity Chromatography of Biotinylated Covalent Hormone–Receptor Complexes on Avidin or Streptavidin Gels. In addition to their high affinity for the receptor, a second steric requirement of our biotinylated probes lies in the possible recognition of the solubilized probe–receptor complexes by immobilized avidin or streptavidin. Several possibilities for the development of indirect affinity chromatography have been investigated according to the scheme detailed in Figure 5.

Positive results were obtained with Bio-AII(N_3). Efficient

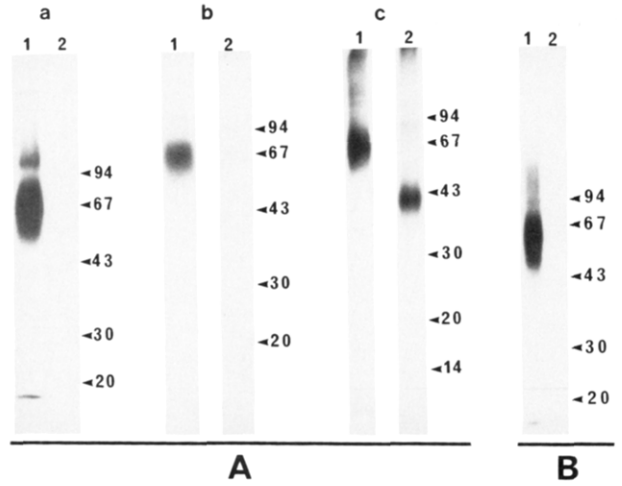

FIGURE 4: Electrophoresis pattern of photolabeled AII receptors. (A) Photoaffinity labeling of rat liver AII receptor with biotinylated azidoangiotensins. Rat liver membranes (2 mg/mL) were incubated in binding buffer with 8 nM radioiodinated Bio-AII(N_3) or Bio-S-S-AII(N_3). Washed membranes were photolabeled as described under Materials and Methods. Triton X-100 solubilized membranes were either filtrated on Trisacryl GF 05 [experiment with Bio-AII(N_3)] or adsorbed to hydroxylapatite [experiment with Bio-S-S-AII(N_3)] before SDS–PAGE analysis in reducing conditions and autoradiography. Molecular weights of protein standards are indicated in K. (a) Labeling with ^{125}I-Bio-AII(N_3) alone (lane 1) or in the presence of 1 μM [Sar^1]AII (lane 2) on 12.5% acrylamide gel. (b) Labeling with ^{125}I-Bio-S-S-AII(N_3) alone (lane 1) or in the presence of 1 μM [Sar^1]AII (lane 2) on 12.5% acrylamide gel. (c) Electrophoretic pattern of the chemically deglycosylated receptor. Liver receptor labeled with Bio-AII(N_3) was deglycosylated with TFMS according to Edge et al. (1981) before SDS–PAGE analysis (15% acrylamide gels). Lane 1: Control receptor. Lane 2: Deglycosylated receptor. (B) Electrophoresis pattern of purified AII receptor. Lane 1: About 2 pmol of purified receptor (thiopropyl-Sepharose eluate, see Materials and Methods) initially labeled with ^{125}I-Bio-S-S-AII(N_3) was analyzed by SDS–PAGE analysis (12.5% acrylamide gels) and autoradiography. Lane 2: Control nonspecific assay. An excess of unlabeled [Sar^1]AII was present at the initial labeling step. Molecular weights of protein standards are indicated in K.

Table I: Adsorption and Elution of Solubilized Biotinylated Probe–Receptor Covalent Complexes on Avidin– or Streptavidin–Sepharose[a]

	adsorption (%)		elution	
	–NEM	+NEM	conditions	%
Bio-AII(N_3)	75		–biotin (5 mg/mL)	10
			–formic acid in 0.15% Triton X-100	70
Bio-S-S-AII(N_3)	33	82	–50 mM DTT	43
			–50 mM DTT, +1% SDS	82

[a] Membranes (2 mg/mL) were labeled with 8 nM radioiodinated Bio-AII(N_3) or Bio-S-S-AII(N_3) in the presence or absence of 0.5 mM NEM. Photolabeling solubilization and hydroxylapatite chromatography and avidin adsorption were carried out as described under Materials and Methods. 70% formic acid in 0.15% Triton X-100 solutions was used for drastic elution.

binding (70–80%) of the complexes to avidin–Sepharose was obtained (Table I). This adsorption was biotin specific since it was abolished in the presence of an excess of biotin. Pretreatment of the samples by 1% SDS increased the rate of the binding and somewhat improved (by about 10%) the maximal extent of adsorption. It is noteworthy that addition of SDS to Triton X-100 solubilized samples has little dissociating effect on the immobilized avidin tetrameric structure (see comments in purification experiments). However, receptor recovery with an excess of biotin was rather poor (10–12%). Drastic elution by 70% formic acid, although almost quantitative, induced

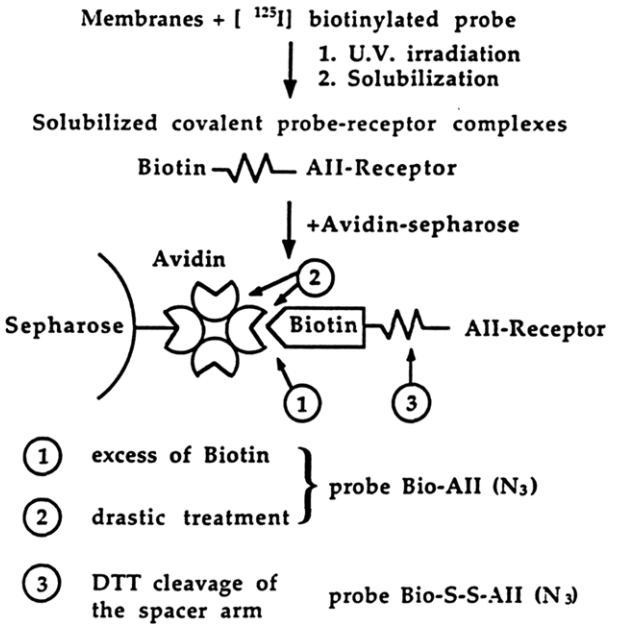

FIGURE 5: Indirect affinity chromatography of AII receptor using biotinylated azido probes.

recovery of large amounts of avidin and moreover was responsible for undesired receptor aggregation. The use of probes containing iminobiotin instead of biotin (Orr et al., 1986) was not a convenient solution since their nonspecific interactions with liver membranes were very high.

This problem was foreseen and had led us to synthesize probes possessing a cleavable spacer arm. However, difficulties were encountered in achieving efficient and reproducible adsorption yields of complexes obtained with Bio-S-S-AII(N_3) in the experimental conditions used for Bio-AII(N_3) (Table I). Parallel experiments carried out in the presence of *N*-ethylmaleimide indicated that these lower values resulted from premature cleavage of the probe disulfide bridge: When added to membranes at the same time as the ligand, and kept present in all buffers used up to the avidin chromatography step, NEM fully restored the efficiency of probe–receptor complex binding to avidin gels (Table I) without important reduction (limited to 10–15% in the experimental conditions used) of the initial extent of receptor saturation. Receptor recovery from the affinity gels could be achieved with dithiothreitol (DTT). The elution yield increased from 43% to 82% when SDS was present, most probably as a result of improved accessibility of the spacer arm to DTT action; indeed, we controlled that SDS by itself induced negligible elution of adsorbed complexes (or biotinylated ligand alone) by mere dissociation of avidin subunits. However, avidin or streptavidin release, although not being responsible for receptor recovery, cannot be ignored in terms of protein purification since the avidin which is efficient for probe–receptor complex binding was estimated to represent no more than 1/1000 of total immobilized avidin; the contaminating streptavidin can be specifically eliminated (see next paragraph).

Scheme for Angiotensin II Receptor Purification. A purification protocol was established by experiments starting from nanomole amounts of membrane receptors. ^{125}I-Bio-S-S-AII(N_3) was used as the biotinylated photoactivable probe. Results of a typical experiment are presented in Table II.

(*A*) *Hydroxylapatite Chromatography.* Triton X-100 solubilization of the covalent complexes was consistent with their selective adsorption on hydroxylapatite–Bio-Gel P-30: this step allowed elimination of the free dissociated ligand, partial purification (from 2- and 4-fold, varying between ex-

Table II: Purification of Covalent Biotinylated Probe–Receptor Complexes[a]

step	protein (mg)	receptor (pmol)	nonspecific radioactivity (% of total radioactivity)	yield of receptor recovery (%)		receptor purification (x-fold)	
				step	overall	step	overall
photolabeled membranes	1800	300		100	100		
solubilization	630	165	22	55	55	1.6	1.6
hydroxylapatite chromatography	74	84	18	51	28	4.3	6.9
streptavidin chromatography		63	10	75	21	780	5400
YM30 Diaflo concentration		50.4		80	17		
thiopropyl-Sepharose chromatography		35.3	7	70	12		
Centricon P30 concentration	0.035	31	7	87	10		

[a] Two grams of rat liver plasma membranes was labeled with 8 nM ^{125}I-Bio-S-S-AII(N_3) (sp radioactivity 11 Ci/mmol). The values for nonspecific radioactivities were obtained in a parallel assay carried out on the same membrane amount in the presence of 1 μM [Sar^1]AII. The specific receptor activity of the washed membranes was 0.7 pmol/mg of protein, the nonspecific binding being 31% of the total binding.

periments), and concentration of the receptor samples, which appeared to be an important experimental convenience for the streptavidin chromatography step.

(B) Streptavidin–Sepharose/Thiopropyl-Sepharose Chromatography. Adsorption to streptavidin–Sepharose columns, in the presence of 1% SDS, was very efficient (75–85%); as previously mentioned for analytical assays, the first washing medium, which contained 1% SDS, released undetectable amounts of complexes. DTT elution never exceeded 40% in the absence of SDS but was increased up to 80% in the presence of 1% SDS. The DTT-eluted receptor was freed from excess DTT and then adsorbed at high yield (80–90%) on Thiopropyl-Sepharose 6MB, through the thiol function which has been generated on the spacer arm or possibly through SH functions generated by DTT reduction of intramolecular disulfide bridges of the receptor. This step, which constitutes in itself a way of concentrating receptor samples, eliminated any released streptavidin, which possesses no cysteine residue (Argarana et al., 1986), and possibly other contaminating proteins.

The efficiency of the thiopropyl-Sepharose step for streptavidin elimination was verified by pilot control experiments carried out with immobilized ^{125}I-streptavidin; the amount of streptavidin released upon DTT + SDS treatment was about 35 μg/mL of gel; the remaining amount in the thiopropyl-Sepharose eluates was lowered to 1.5 μg and was exclusively found as the monomeric subunit (15K, as revealed by electrophoresis and autoradiographic analysis). Avidin, which possesses cysteine residues (De Lange & Huang, 1971), should not be used instead of streptavidin: in similar control experiments using immobilized ^{125}I-avidin the amount of initially released avidin was 80 μg/mL of gel and, as predicted, was not significantly eliminated by thiopropyl-Sepharose chromatography.

The thiopropyl-Sepharose-adsorbed receptor was recovered once more by DTT elution in the presence of SDS; it was further concentrated in Centricon P30 microconcentrators and analyzed by SDS–PAGE.

The autoradiographic pattern of the purified receptor was similar to that of the crude solubilized sample (Figure 4B). A "nonspecific assay" was carried out in the same experiment, on the same amount of starting membranes labeled with the biotinylated probe in the presence of an excess of [Sar^1]AII; no labeling was observed at the end of the whole purification procedure. Thus, the purified entity is actually an angiotensin II receptor. Confirmation was afforded by chemical deglycosylation of the purified receptor, which then shifted its apparent molecular weight to 40K (not shown).

Protein estimation in the thiopropyl-Sepharose eluates and receptor evaluation from the radioactivity of the probe indicated that the purity of the receptor was about 7%. The overall purification factor was about 6000, the combined affinity steps by themselves allowing an 800-fold purification. Silver-stained receptor could not be detected, probably because of the amount analyzed and the heterogeneity of the electrophoretic pattern. A sample (3 pmol) of purified receptor preparation, previously treated by the *S. aureus* V8 protease, which has no effect on the receptor, was submitted to SDS–PAGE separation; the receptor was electroblotted onto a poly(vinyl difluoride) membrane (Immobilon P, Millipore); amino acid analysis after 6 N HCl digestion together with radioactivity determination allowed evaluation of the receptor purity in the electroblotted material (it was increased to 25%). These data will have to be taken into account in order to define a likely strategy for completion of the purification of the receptor or receptor fragments to homogeneity (see Discussion).

The reproducibility of the described protocol could be established in five experiments carried out on 1–2 g of purified membranes. The adsorption and elution yields at the two affinity steps and the final purification factor of receptor purification were found to be constant (relative variations <10%).

Discussion

Until now, molecular and structural data relating to AII receptors have been restricted to the evaluation of apparent molecular weights after covalent labeling with radioactive probes (Escher et al., 1978; Guillemette et al., 1985, 1986; Guillemette & Escher, 1983; Capponi & Catt, 1980; Paglin & Jamieson, 1982; Carson et al., 1987; Kwok & Moore, 1985). Receptor purification, an obligatory first step for the most classical method of primary structure determination, has been obstructed by the difficulties in binding AII to solubilized preparations. Purification procedures previously described by Sen et al. (1984) have been abandoned. More recent work (Bandhyapadhyay et al., 1988; Kiron & Soffer, 1989) by the same group resulted in purification of a 70K AII binding protein from rabbit liver by protocols devoid of any affinity step; because of the unexpected optimal AII binding conditions of this protein and its main localization in cytosol rather than in plasma membranes, its relationship to the plasma membrane AII receptor remains undefined.

Using a more unusual approach, Elton et al. (1988) purified a protein recognized by antibodies to an angiotensin "complementary peptide". However, more experimental data dealing with the purification itself and critical reevalution of the theory involved (Goldstein & Brutlag, 1989) appear to be required for a fuller assessment of the function of this protein.

We demonstrate the ability to covalently label rat liver AII receptors with several original biotinylated photoactivable

probes which display very high affinity for the receptors; the yield (20–25%) was sufficient to envisage purification of the resulting covalent complexes through avidin or streptavidin chromatography. One of the synthetic probes Bio-S-S-AII-(N_3), which possesses a cleavable disulfide bridge inside its spacer arm, was successfully applied in a substantial partial purification of the receptor, thereby demonstrating the efficacy of the indirect affinity chromatography step; the identity of the purified glycoprotein to AII receptors was clearly established. These results represent, in themselves, a feasible methodological alternative for purification of unstable receptors. Although it was suggested by Finn et al. (1985), this strategy has not given rise to any concrete application until now. In a more recent work from the same group (Hofmann et al., 1988), no figure dealing with efficiency of ACTH receptor purification was available; in this situation, the problem of receptor recovery from avidin gels was solved by replacing biotin with dethiobiotin and eluting the receptor with guanidinium chloride. We have proposed the introduction of a cleavable disulfide bridge between AII and biotin and the use of streptavidin instead of avidin; any streptavidin released upon DTT + SDS elution can be eliminated by specific adsorption of the receptor on thiopropyl-Sepharose through the cleaved spacer arm of the probe. One can predict that these combined affinity steps might be applied to other receptors or ligand binding proteins.

The major disadvantage of our stragegy for AII receptor purification is the requirement for covalent labeling, which is accompanied by an important loss of receptor. Despite the fact that the yields of the other steps are quite satisfactory, scale up of the amounts of starting material is required. The scale up and simplification of liver membrane preparations consistent with acceptable initial nonspecific binding and the possibility to use other biological sources are under investigation, in order to complete purification to homogeneity. This scale up should in itself improve receptor purity by minimizing the relative blank values in protein estimation in the latest stages of the purification scheme. Generalization of the described protocols can easily be predicted; we have already verified that it can be applied to receptors from other tissues (unpublished results). Receptor deglycosylation or fragmentation followed by electroelution or electroblotting of fragments from electrophoresis gels might constitute a means of eliminating the last contaminating proteins. Indeed, we demonstrated electroblotting, at high yield, of the intact receptor or fragments onto supports suitable for protein sequencing.

Recent data suggest that the mas oncogene product, which shares structural properties with G protein coupled receptors (Young et al., 1986, 1988), is related to a "cerebral" type of angiotensin receptor (Jackson et al., 1988); in spite of unsolved questions (Brown, 1989), they constitute a potential starting point for cloning the genes encoding AII receptors and possibly demonstrating the existence of receptor subtypes for which pharmacological evidence is just emerging (Whitebread et al., 1989; Chiu et al., 1989) by the use of selective peptidic or nonpeptidic antagonists (Whitebread et al., 1989; Wong et al., 1989). Anyhow, whichever cloning strategy is successful, purification and physicochemical characterization of these receptors (or mas oncogene products) will be required. Moreover, the described scheme for AII receptor purification might in principle be applied to the characterization and isolation of receptor fragments which are involved in the hormone binding domains, in various tissues or expression systems. The presently described purification procedure, once scaled up, should be efficient enough to provide samples for animal immunization and anti-receptor antibody production. When applied at analytical or semipreparative levels, it should also facilitate the insight into some classical aspects of the cell biology of angiotensin receptors.

Acknowledgments

We thank M. Collet, F. Martin, and J. Bernard for technical assistance and M. Paolucci for preparing the manuscript.

Registry No. AII, 11128-99-7; Bio-AII-N_3, 115082-72-9; Bio-S-S-AII(N_3), 128359-05-7; [^{125}I]-Bio-S-S-AII(N_3), 128359-06-8.

References

Argarana, C. E., Kuntz, I. D., Birken, S., Axel, R., & Cantor, C. R. (1986) *Nucleic Acids Res. 14*, 1871–1882.

Bandhyopadhyay, S. K., Rosenberg, E., Kiron, M. A. R., & Soffer, R. L. (1988) *Arch. Biochem. Biophys. 263*, 272–280.

Bonnafous, J. C., Tencê, M., Seyer, R., Marie, J., Aumelas, A., & Jard, S. (1988a) *Biochem. J. 251*, 873–880.

Bonnafous, J. C., Seyer, R., Tencê, M., Marie, J., Kabbaj, M., & Aumelas, A. (1988b) *J. Recept. Res. 8*, 295–309.

Bouscarel, B., Blackmore, P. F., & Exton, J. M. (1988) *J. Biol. Chem. 263*, 14913–14919.

Brenman, D. P., & Levine, M. A. (1987) *J. Biol. Chem. 262*, 14795–14800.

Brown, K. (1989) *Trends Pharmacol. Sci. 10*, 87–89.

Capponi, A. M., & Catt, K. J. (1980) *J. Biol. Chem. 255*, 12081–12086.

Carson, M. C., Harper, C. M. L., Baukal, A. J., Aguilera, G., & Catt, K. J. (1987) *Mol. Endocrinol. 1*, 147–153.

Castell, J. V., Cervera, M., & Marco, R. (1979) *Anal. Biochem. 99*, 379–391.

Chiu, A. T., Herblin, W. F., McCall, D., Ardecky, R. J., Carini, D. J., Duncia, J. V., Pease, L. J., Wong, P. C., Wexler, R. R., Johnson, A. L., & Timmermans, B. M. W. M. (1989) *Biochem. Biophys. Res. Commun. 165*, 196–203.

De Lange, R. J., & Huang, T. S. (1971) *J. Biol. Chem. 246*, 698–709.

Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, L. E., Jr., & Weber, P. (1981) *Anal. Biochem. 118*, 131–137.

Elton, T. S., Dion, L. D., Bost, K. C., Oparil, S., & Blalock, J. E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2518–2522.

Escher, E., Nguyen, T. M. D., Robert, H., St-Pierre, S., & Regoli, D. (1978) *J. Med. Chem. 21*, 860–864.

Finn, F. M., Titus, G., Horstman, D., & Hofmann, K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7328–7332.

Finn, F. M., Stehle, C. J., & Hofmann, K. (1985) *Biochemistry 24*, 1960–1965.

Fraker, P. J., & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun. 80*, 849–857.

Goldstein, A., & Brutlag, D. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 86*, 42–45.

Guillemette, G., & Escher, E. (1983) *Biochem. J. 22*, 5591–5596.

Guillemette, G., Guillon, G., Marie, J., Pantaloni, C., Balestre, M. N., Escher, E., & Jard, S. (1985) *J. Recept. Res. 4*, 267–281.

Guillemette, G., Guillon, G., Marie, J., Balestre, M. N., Escher, E., & Jard, S. (1986) *Mol. Pharmacol. 30*, 544–551.

Hoffmann, K., Stehle, C. J., & Finn, F. M. (1988) *Endocrinology 123*, 1355–1363.

Jackson, T. R., Blair, L. A. C., Marshall, J., Goedert, M., & Hanley, M. R. (1988) *Nature 335*, 437–440.

Kiron, M. A. R., & Soffer, R. L. (1989) *J. Biol. Chem. 264*, 4138–4142.

Kwok, Y. G., & Moore, G. J. (1985) *Eur. J. Pharmacol. 115*, 53–58.

Laemmli, U. K. (1970) *Nature 227*, 680-685.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Mc Farlane, A. S. (1958) *Nature 182*, 53.
Neville, D. M., Jr. (1968) *Biochim. Biophys. Acta 154*, 540-552.
Orr, G. A., Heney, G. C., & Zeheb, R. (1986) *Methods Enzymol. 122*, 83-94.
Paglin, S., & Jamieson, J. D. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3739-3743.
Sen, I., Bull, H. G., & Soffer, R. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1679-1683.
Seyer, R., Aumelas, A., Tencé, M., Marie, J., Kabbaj, M., Bonnafous, J. C., Jard, S., & Castro, B. (1989) *Int. J. Pept. Protein Res. 34*, 235-245.
Whitebread, S., Mele, M., Kamber, B., & de Gasparo, M. (1989) *Biochem. Biophys. Res. Commun. 163*, 284-291.
Wong, P. C., Price, W. A., Chiu, A. T., Thoolen, M. J. M. C., Duncia, J. V., Johnson, A. L., & Timmermans, P. B. W. M. (1989) *Hypertension 13*, 489-497.
Young, D., Waitches, G., Birchmeier, C., Fasano, O., & Wigler, M. (1986) *Cell 45*, 711-719.
Young, D., O'Neil, K., Jessel, T., & Wigler, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5339-5342.

Heparin Binding Domain of Antithrombin III: Characterization Using a Synthetic Peptide Directed Polyclonal Antibody†

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Received April 12, 1990; Revised Manuscript Received May 22, 1990

ABSTRACT: Antithrombin III (ATIII) is a plasma-borne serine protease inhibitor that apparently forms covalent complexes with thrombin. The interaction between ATIII and thrombin is enhanced several thousandfold by the glycosaminoglycan, heparin. We have previously proposed that the heparin binding site of ATIII resides within a region extending from amino acid residues 114-156 [Smith, J. W., & Knauer, D. J. (1987) *J. Biol. Chem. 262*, 11964-11972]. Computer-assisted analysis of this region revealed the presence of a 22 amino acid domain (residues 124-145), part of which shows a strong potential for the formation of an amphipathic helix: hydrophobic on one face and highly positively charged on the other. In the presence studies, polyclonal antisera were generated against a synthetic peptide corresponding to residues 124-145 in native human ATIII. Affinity-purified IgG from these antisera, as well as monovalent Fab's derived from them, specifically blocked the binding of heparin to ATIII. Additionally, occupancy of the heparin binding site by these same monovalent and bivalent IgG's at least partially substituted for heparin, accelerating linkage formation between ATIII and thrombin. These results provide the first immunological evidence that region 124-145 is directly involved in the binding of heparin to ATIII and that an antibody-induced conformational change within this region can mediate ATIII activation.

Antithrombin III (ATIII)[1] is a key regulatory molecule in the control of intravascular clotting (Rosenberg & Damus, 1973; Rosenberg, 1977). ATIII inactivates a number of serine proteases that participate in the cascade, but principally controls clot formation by acting as a suicide inhibitor of thrombin. Apparently, a covalent linkage is formed between ATIII and thrombin at the active-site serine of the protease, and this linkage formation is accelerated several thousandfold by heparin (Jordan et al., 1980). It should be noted, however, that this covalent linkage is only inferred from chemical stability, and has never been directly demonstrated. Evidence suggests that the mechanism of this enhancement is attributable both to a conformational change induced in ATIII by heparin, which renders it more susceptible to proteolytic cleavage by thrombin, and to multiple binding sites on the linear heparin polymer that bring ATIII and thrombin into close proximity (Stone et al., 1982; Einarsson & Andersson, 1977; Nesheim et al., 1986).

The structure of the heparin binding site of ATIII has been of considerable interest, since the elucidation of it structure is the first step in understanding the mechanism of heparin activation. Nuclear magnetic resonance studies (Gettins & Wooten, 1987), as well as analysis of CNBr fragments of ATIII (Rosenfeld & Danishefsky, 1986), suggest that the heparin binding site of ATIII resides within the first one-third to half of the amino-terminal end of the molecule. In support of this, chemical modification experiments have implicated that lysine residues 107, 125, 133, and 136 are involved in the binding of heparin (Liu & Chang, 1987; Peterson et al., 1987; Chang, 1989).

† This work was supported by Grant 2R01-GM34001 from the National Institutes of Health and Grant 86-S107 from the American Heart Association.
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[1] Abbreviations: ATIII, antithrombin III; Th, thrombin; ^{125}I-F-heparin, ^{125}I-labeled fluoresceinamine heparin; RP-HPLC, reverse-phase high-performance liquid chromatography; IgG, immunoglobulin G; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline; SPDP, succinimidyl (2-pyridyldithio)propionate; DTT, dithiothreitol; ACN, acetonitrile; BSA, bovine serum albumin; NP40, Nonidet P-40; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-2960/90/0429-8950$02.50/0