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Deglycosylation and fragmentation of purified rat liver angiotensin II receptor: application to the mapping of hormone-binding domains

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We report new structural data about the rat liver angiotensin II receptor, which belongs to the AT_1 subclass. This receptor has been purified at analytical or semi-preparative levels by a previously described strategy involving its photolabelling with a biotinylated azido probe and selective adsorption of the covalent probe–receptor complexes to immobilized streptavidin [Marie, Seyer, Lombard, Desarnaud, Aumelas, Jard and Bonnafous (1990) Biochemistry **29**, 8943–8950]. Chemical or enzymic deglycosylation of the purified receptor has shown a shift in its molecular mass from 65 kDa to 40 kDa. Fragmentation of the purified receptor was carried out with V8 protease from *Staphylococcus aureus*, CNBr and trypsin. It was possible to find trypsin-treatment conditions which allowed production of a 6 kDa probe–fragment complex with a satisfactory yield. Attempts to localize this small fragment (5 kDa after subtraction of the probe contribution) in the recently published rat AT_1 receptor sequence are reported. As expected, this fragment is not glycosylated; moreover, its further fragmentation by CNBr induces a very slight decrease in its size. These data support the hypothesis that a receptor sequence comprising the third transmembrane domain and adjacent portions of extra- and intracellular loops is involved in photolabelling by the C-terminal azidophenylalanine of the angiotensin-derived probe. These preliminary results are discussed in terms of future prospects for the characterization of hormone-binding domains of angiotensin II receptors.

INTRODUCTION

Recently, two different groups reported the expression cloning of the angiotensin II (AII) receptor (AT_1 subtype) from bovine adrenals (Sasaki et al., 1991) and rat vascular smooth-muscle cells (Murphy et al., 1991). Before these reports, none of the various cloning strategies had proved to be successful. Purification of AII receptors, which constitutes the first step of the most classical way of primary-structure determination, has been greatly delayed by the loss of their binding properties upon detergent solubilization. We recently described an original protocol for the purification, at a semi-preparative scale, of the rat liver AII receptor (AT_1 subtype) which involves photoaffinity labelling with biotinylated azido derivatives, followed by indirect affinity chromotography on streptavidin columns and thiopropyl-Sepharose chromatography (Marie et al., 1990). The experiments described in the present paper constitute progress in the physicochemical characterization of AII receptors: we have undertaken to elucidate the problems raised by deglycosylation, fragmentation of purified rat liver receptor and electroblotting of intact receptor or receptor fragments on to supports suitable for protein sequencing. These developments constitute a prerequisite for mapping studies, especially future isolation and sequencing of receptor domains involved in hormone binding and the understanding, at the molecular level, of hormone–receptor interaction. Before this aim can be reached, interpretation of trypsin- and CNBr-fragmentation data, obtained at an analytical level, can provide evidence for the possible localization of receptor domains which have been covalently bound to the photoactivable angiotensin derivative. The recent pharmacological discrimination of AII receptor subtypes (Whitebread et al., 1989; Chiu et al., 1989; Duncia et al., 1990; Dudley et al., 1990) reinforces the interest of these investigations.

MATERIALS AND METHODS

Materials

The biotinylated (Bio) photoactivable probe used for AII receptor pholabelling and purification, Bio-NH-$(CH_2)_2$-S-S-$(CH_2)_2$-CO-[Ala^1,$Phe(4N_3)^8$]-AII [Bio-S-S-AII(N_3)] was synthesized as previously described (Seyer et al., 1989; Seyer and Aumelas, 1990). Solutions of the azido probes were calibrated by u.v. spectroscopy ($\epsilon_{250} = 12\,500\ M^{-1} \cdot cm^{-1}$). The radioiodinated and unlabelled monoiodinated probes were obtained as previously described (Bonnafous et al., 1988; Marie et al., 1990). Probe samples of appropriate specific radioactivities were obtained by mixing labelled and unlabelled compounds.

Biotin and streptavidin were from Sigma, and activated Sepharose 4B and thiopropyl-Sepharose 6B were from Pharmacia–LKB; streptavidin–Sepharose (1 mg/ml of gel) was prepared as described by Finn et al. (1984), except that the succinylation step was omitted.

Immobilon P membranes were purchased from Millipore, trypsin of sequencing grade (EC 3.4.21.4) and *Staphylococcus aureus* V8 protease (endoproteinase Glu-C; EC 3.4.21.19) were from Boehringer, CNBr was from Sigma, Centricon P30 microconcentrators were from Amicon, hydroxyapatite (Bio-Gel P30) was from Biorad, Triton X-100 was from Pierce, and N-glycanase [peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase, or PGNase F (EC 3.5.1.52) from Genzyme.

Electrophoresis reagents were obtained from Serva and Bio-Rad, and low-molecular-mass standard kit (14400–97400 Da)

Abbreviations used: AII, angiotensin II; Bio, biotinyl; TFMS, trifluoromethanesulphonic acid; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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and a horse heart myoglobin fragments kit (2512–16949 Da) were from Pharmacia–LKB.

Membrane preparations

Purified liver membranes from Wistar male rats were prepared as described by Neville (1968) up to step 11. About 10 mg of purified membranes was obtained from one liver. Membranes were stored in liquid nitrogen.

Receptor purification

AII receptor was purified from rat liver plasma membranes by using the biotinylated photoactivable probe [^{125}I]-Bio-S-S-AII(N_3) as described previously (Marie et al., 1990), except that indirect affinity chromatography of biotinylated covalent hormone–receptor complexes on streptavidin–Sepharose and thiopropyl-Sepharose gels was carried out in batchwise procedures for analytical purposes.

Briefly, radioiodinated probe solutions (5–8 nM) and *N*-ethylmaleimide (0.3 mM final concn.), were simultaneously added, under agitation, to pre-warmed membranes (200–400 mg, 2 mg/ml membrane protein concentration in binding medium: 50 mM phosphate, pH 7.4, 5 mM $MgCl_2$, 1 mg/ml BSA, 1 mg/ml bacitracin).

After incubation for 30 min at 30 °C, membranes were washed and photolysed at 254 nm for 5 min. After two additional washings, membranes were solubilized for 90 min at 0 °C in the presence of 1.5 % Triton X-100 and centrifuged at 200000 *g* for 1 h.

Covalent complexes were separated from the free dissociated ligand through their selective adsorption to hydroxyapatite Bio-Gel P-30 (50 μl of sedimented gel/mg of starting membrane protein).

Hydroxyapatite eluates were incubated under gentle agitation (3–4 h) with streptavidin–Sepharose gel (250 μl of gel/100 mg of starting membrane protein). The gel was washed and elution was performed by cleavage of the probe-disulphide bridge under gentle agitation (15 min at room temperature) in 5 vol. of 10 mM phosphate (pH 8.0)/50 mM dithiothreitol (DTT)/1 % SDS.

The streptavidin eluate was freed of most of its DTT content by ultrafiltration through Amicon Centricon P30 micro-concentrations (three cycles involving dilution with 5 vol. of 10 mM phosphate (pH 6.0)/0.1 % Triton X-100.

The resulting concentrated sample (300–400 μl) was mixed with thiopropyl-Sepharose 6B equilibrated in 10 mM phosphate, pH 6.0, and 0.1 % Triton X-100 (25 μl of sedimented gel/100 mg of starting membrane protein). Efficient adsorption was achieved after gentle agitation for 2 h at room temperature. After washing with 10 mM phosphate (pH 8.0)/0.1 % SDS, elution was performed by gentle agitation for 15 min at room temperature with 50 mM DTT in washing buffer.

The thiopropyl-Sepharose eluate was dialysed and concentrated through Amicon Centricon P30 microconcentrator [three cycles involving 5-fold dilutions with 100 mM Tris/HCl (pH 8.5)/0.1 % SDS or 10 mM phosphate (pH 8.0)/0.1 % SDS.

In some experiments, alkylation was carried out by treating thiopropyl-Sepharose eluates in 100 mM Tris/HCl (pH 8.5)/0.1 % SDS/50 mM iodoacetate for 2 h in the dark. The excess of iodoacetate was then eliminated by three ultrafiltration cycles.

Fragmentation and deglycosylation experiments were carried out on thiopropyl-Sepharose eluates. A receptor purity of 10 % was taken as a basis for enzyme/protein calculations.

Chemical and enzymic deglycosylation of the AII receptor and receptor fragments

Crude or purified probe–receptor covalent complexes (or derived fragments) were chemically deglycosylated with trifluoromethanesulphonic acid (TFMS) as described by Edge et al. (1981).

N-glycanase digestion of samples of thiopropyl-Sepharose eluates (final volume 30 μl, containing about 3–4 fmol of denatured and reduced receptor) was carried out in 10 mM phosphate (pH 8.0)/0.1 % SDS/2 % Triton X-100/45 mM DTT/10 mM phenanthroline, at 35 °C in the presence of 30–54 units of N-glycanase/ml.

Fragmentation of purified AII receptor or receptor fragments

Staph. aureus V8 protease treatment of purified probe–receptor complexes was performed in 10 mM phosphate (pH 8.0)/0.1 % SDS at 37 °C for 1 h by V8 protease at a protease/protein ratio of 15:1. Reaction was stopped by addition of phenylmethanesulphonyl fluoride (PMSF) at a final concentration of 1 mM.

Trypsin digestion of purified probe–receptor complexes was performed over various time periods with different concentrations of trypsin. The reaction was carried out at 30 °C in 50 μl of 100 mM Tris/HCl buffer, pH 8.5, containing 30–50 fmol of the purified receptor, 0.1 % SDS and 10 mM $CaCl_2$. Reaction was stopped with a 4-fold molar excess of soybean trypsin inhibitor. In control experiments, trypsin was omitted.

CNBr cleavage was carried out on samples containing 230–300 fmol of purified receptor in 100 mM Tris/HCl (pH 8.5)/0.1 % SDS. Samples were freeze-dried and then dissolved in 200 μl of aq. 70 % (v/v) formic acid. CNBr dissolved in 100 μl of 70 % formic acid was then added to a final concentration of 100 mg/ml. As a control assay, a sample of the receptor was treated with 70 % formic acid without CNBr. The mixtures were incubated in the dark for 24 h at room temperature under nitrogen. CNBr and formic acid were removed by dilution of the freeze-dried samples with 1 vol. of water and freeze-drying. This step was repeated three times. Similar experimental conditions have been applied to the 6 kDa probe–fragment complex previously isolated by Tricine/SDS/PAGE, except that the CNBr concentration was increased to 200 mg/ml.

SDS/PAGE

SDS/PAGE was performed under reducing and denaturing conditions. Samples were treated for 1 h at 30 °C in a medium containing 80 mM Tris/HCl (pH 6.8), 2 % SDS, 10 % glycerol, 0.1 M DTT and Coomassie Brilliant Blue or Bromophenol Blue.

Samples were first analysed by classical SDS/PAGE on slab gels (200 mm × 200 mm × 1.5 mm) in accordance with the method of Laemmli (1970), 10–15 %-acrylamide running gels overlaid by a 5 %-acrylamide stacking gel (electrophoresis conditions: 14–16 h, 50 V). Analysis of low-molecular-mass peptides was carried out by Tricine/SDS/PAGE on slab gels (100 mm × 140 mm × 1.5 mm) as described by Schägger and von Jagow (1987): a 16.5 %-polyacrylamide running gel was overlaid by a 10 %-polyacrylamide spacer gel and by a 4 %-polyacrylamide stacking gel (electrophoresis conditions: 30 V for 1 h, then 85 V for 18 h).

Gels were stained with Coomassie Brilliant Blue. Dried gels were autoradiographed at −80 °C with Kodak XAR-5 films and intensifying screens.

Radioactivity profiles were measured on fresh or dried gels by cutting the lanes into 2 mm bands and counting them in a γ-

radiation counter. Overall radioactivity recoveries were 80–85% for fresh gels and 60–65% for dried gels.

When desired, autoradiograms were submitted to densitometry analysis.

Electroelution and electroblotting

Electroelution of receptor or receptor fragments from polyacrylamide gels was performed at 200 V in a Biotrap apparatus (Schleicher and Schuell). The gel was cut into strips; the strips containing the intact receptor or fragments to be analysed were detected by the ^{125}I-labelled angiotensin which is covalently bound to them, then placed in the elution chamber delimited by two permeable membranes; the eluted proteins were collected in the elution trap, delimited on its anodic side by a dialysis membrane (5 kDa cut-off). The extent of electroelution could be controlled at any time by measuring the remaining radioactivity in the gel pieces.

In electroblotting experiments, a piece of polyvinylidene difluoride membrane (Immobilon P; Millipore) (Matsudaira, 1987) was applied against the inside face of the dialysis membrane which delimits the anodic side of the elution trap. This Immobilon membrane was previously soaked in methanol for 10 s, then immersed in electroblotting buffer (25 mM Tris, 150 mM glycine, 0.1% SDS, 20% methanol).

After electroblotting, the Immobilon piece was counted in a γ-radiation counter.

RESULTS

Receptor purification

The protocol for the rat liver AII receptor purification has been previously described (Marie et al., 1990); since these experiments were carried out on 1–2 nmol of starting membrane receptors (1–2 g of purified rat liver plasma membranes), it was necessary to verify that this protocol could be applied to routine analytical investigations. This was easily achieved for receptor amounts as low as 5–10 pmol of starting membrane receptor. The experiments described in this paper were performed with 200–400 mg of membrane protein. Compared with larger-scale purification, the two affinity steps (streptavidin–Sepharose and thiopropyl-Sepharose) were carried out in batchwise procedures. The yields of the various steps are collected in Table 1; they are as good as, or even better than, those obtained in larger-scale experiments.

The purification protocol, summarized in Figure 1, is based on the covalent labelling of the membrane receptor with the biotinylated probe Bio-S-S-AII(N_3). The central step of the purification is constituted by the selective adsorption of the covalent complexes to streptavidin–Sepharose gels; receptor elution was achieved by DTT cleavage of the disulphide bridge of the probe. Its accessibility to DTT was improved by adding SDS to the elution medium; however, as a side effect, SDS induced release of streptavidin subunits from the affinity gels. The eluted receptor was then adsorbed on a thiopropyl-Sepharose matrix through the thiol function generated by DTT cleavage of the spacer arm (or possibly through thiol functions resulting from reduction of receptor intramolecular disulphide bridges) (Marie et al., 1990); the contaminating streptavidin was not adsorbed on thiopropyl-Sepharose, since it possesses no cysteine residue (Argarana et al., 1986). In experiments carried out with 1–2 nmol of starting membrane receptor (1–2 g of purified rat liver plasma membranes), the purity of the receptor was estimated to be 6–10% in the thiopropyl-Sepharose eluates and 25% in samples further treated by *Staph. aureus* V8 protease (see 'Fragmentation of the AII receptor' below) then submitted to electrophoretic separation and electrotransfer. At these two steps, in a typical experiment, the specific activities of the receptor

Table 1 Scheme for rat liver AII receptor purification

The yields of the various steps have been calculated from measurements of the probe radioactivity. The values indicated in the left-hand column were obtained in a typical experiment carried out on 400 mg of starting membrane protein. When calculated in 12 different experiments, the ranges of variation of the yields were 24–30% for affinity labelling, 61–70% for solubilization, 65–81% and 74–80% for adsorption and elution from streptavidin gels, 75–88% and 84–90% for adsorption and elution from thiopropyl-Sepharose gels. *Data from Marie et al. (1990).

Purification steps		Receptor recovery (%) Analytical scale	Semi-preparative scale*
Rat liver membranes + biotinylated photoactivable probe (equilibrium binding + washings)		65	75
Photoaffinity labelling (u.v. irradiation)		28	26
Triton X-100 solubilization		70	55
Hydroxyapatite chromatography	Adsorption	94	51
	Elution	91	
Streptavidin chromatography	Adsorption	73	75
	Elution	78	
Thiopropyl-Sepharose chromatography	Adsorption	83	70
	Elution	88	

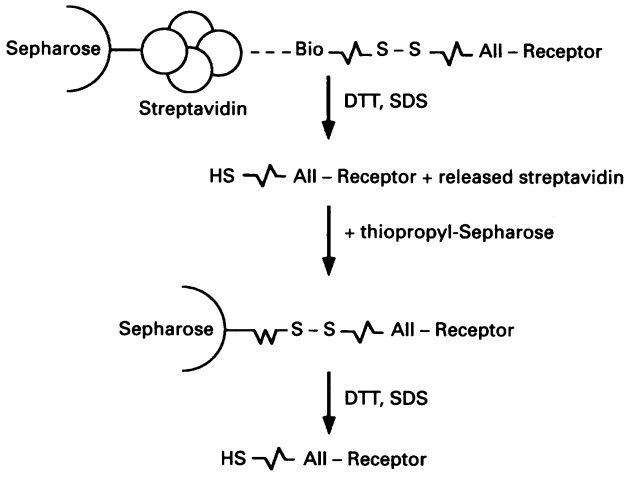

Figure 1 Principle of combined streptavidin–Sepharose and thiopropyl-Sepharose affinity steps

Probe–receptor complexes adsorbed to streptavidin–Sepharose were recovered by DTT cleavage of the disulphide bridge of the spacer arm in the presence of SDS. Released streptavidin subunits, which possess no cysteine residue, were efficiently eliminated by thiopropyl-Sepharose chromatography; eluted AII receptor was adsorbed to this gel through the thiol function of the spacer arm. Although not represented on the scheme, it cannot be excluded that receptor eluted from the streptavidin–Sepharose column can be alternatively adsorbed to thiopropyl-Sepharose through thiol functions generated by DTT cleavage of the intramolecular disulphide bridges. The thiopropyl-Sepharose-adsorbed receptor was recovered again by DTT elution in the presence of SDS.

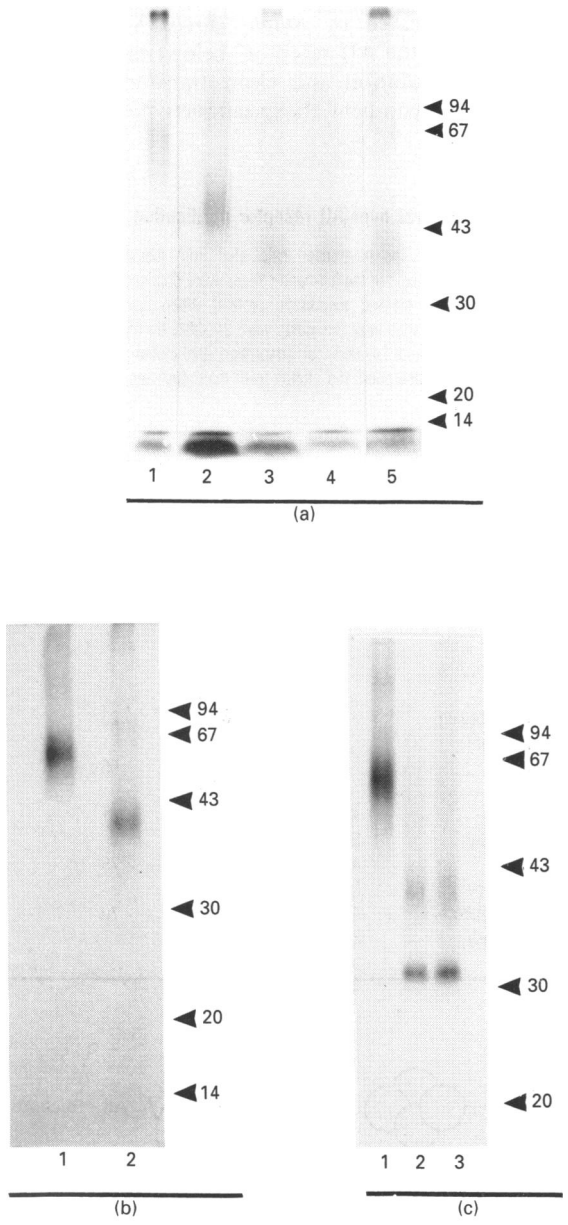

Figure 2 AII-receptor deglycosylation

(**a**) Time course of chemical deglycosylation of solubilized probe–receptor complexes. Rat liver plasma membranes were photolabelled with [^{125}I]-Bio-S-S-AII(N_3) (initial concn. 10 nM, sp. radioactivity 1800 Ci/mmol). An 80 fmol portion of Triton X-100-solubilized complexes were deglycosylated with TFMS as described by Edge et al. (1981) for various time periods before SDS/PAGE analysis (10%-acrylamide gel). Similar amounts of radioactivity were loaded on the various gel lanes; because of the iodine loss during TFMS treatment, they represented variable receptor amounts according to the reaction time. Lane 1, control receptor (TFMS omitted); lanes 2–5, TFMS treatment for 1 min, 5 min, 15 min and 30 min respectively. (**b**) Electrophoretic pattern of chemically deglycosylated purified AII receptor. Rat liver plasma membranes were photolabelled with [^{125}I]-Bio-S-S-AII(N_3) (initial concn. 8 nM, sp. radioactivity 1800 Ci/mmol) and receptors were purified as described in the Materials and methods section. A 22 fmol portion of purified receptor was deglycosylated with TFMS as described by Edge et al. (1981) before SDS/PAGE analysis (15%-acrylamide gel). Lane 1, control receptor (TFMS omitted); lane 2, TFMS treatment for 5 min. (**c**) N-glycanase deglycosylation of purified AII receptor. Rat liver plasma membranes were photolabelled with [^{125}I]-Bio-S-S-AII(N_3) (initial concn. 8 nM, sp. radioactivity 1800 Ci/mmol) and receptors were purified as described in the Materials and methods section. Then 4 fmol of purified receptor was deglycosylated with 54 units of N-glycanase/ml as described in the Materials and methods section and analysed by SDS/PAGE (15%-acrylamide gel). Lane 1, control receptor (N-glycanase omitted); lanes 2 and 3, N-glycanase treatment for 30 min and 2 h respectively. As revealed by densitometry analysis, the proportions of 40 kDa and 32 kDa entities were 47% and 53% after 30 min and 42% and 58% after 2 h. Molecular masses of protein standards are indicated in kDa.

were 885 and 3160 pmol/mg of protein respectively. As previously discussed (Marie et al., 1990), these values are probably underestimated and should be greatly improved by increasing the scale of the experiments; the relative importance of blank values in protein estimations during the later steps of the purification protocol should thus be lowered.

Deglycosylation of the AII receptor

The first interest in deglycosylating the receptor was to provide a criterion for its identification after purification. It could also be used in combination with fragmentation for future mapping studies.

The glycoprotein nature of the rat liver AII receptor was verified by the large decrease in its apparent molecular mass upon deglycosylation. Chemical deglycosylation with TFMS was first tested, as it allows easy elimination of both N- and O-linked glycosidic chains (Edge et al., 1981). The maximal extent of deglycosylation was reached after a 5 min treatment (Figures 2a and 2b); the deglycosylated receptor displayed an apparent molecular mass of 40 kDa. The similitude of deglycosylation patterns obtained for non-purified and purified samples indicates that the purified radioiodinated entity is actually the AII receptor: this is of particular importance, since the strategy does not allow its pharmacological characterization. The chemical deglycosylation conditions induced a large loss of radioactivity from covalent complexes, either through a well-known tyrosine deiodination process (Herzberg et al., 1985) or possibly by cleavage of the covalent bond between the radioactive probe and the receptor. Longer deglycosylation periods (> 1 h), which are sometimes required to remove O-linked saccharidic chains (Edge et al., 1981), could not be easily checked because of this deiodination phenomenon. However, the TFMS-treated receptor appeared to be fully deglycosylated, since its further treatment with O-glycanase (endo-α-*N*-acetylgalactosaminidase, EC 3.2.1.97) induced no additional molecular-mass decrease (results not shown). Purified receptor treatment with N-glycanase generated the same 40 kDa entity as with TFMS treatment (Figure 2c), meaning that there are few or no O-linked saccharidic chains associated with the AII receptor. Consistent with published data about other glycoproteins, a large excess of N-glycanase over receptor was required to produce extensive disappearance of the starting receptor (Tarentino et al., 1985; Hirani et al., 1987); at the same time it induced formation of a homogeneous 32 kDa entity (Figure 2c). The fact that the 40 kDa entity represents the fully deglycosylated receptor is in agreement with primary structures which have been recently reported (Sasaki et al., 1991; Murphy et al., 1991). The 32 kDa protein probably results from proteolytic cleavage: as these experiments were carried out on purified receptor, proteolysis might occur through a protease contaminating the N-glycanase preparation (Tarentino and Plummer, 1987). Attempts to inhibit this proteolysis with classical inhibitors were unsuccessful. The heterogeneity of the AII receptor, even in its deglycosylated form, can be interpreted as resulting from heterogeneous SDS fixation; in N-glycanase experiments, the contaminating proteolytic activity might cleave the receptor portion responsible for this heterogeneity.

Fragmentation of the AII receptor

The strategy of receptor purification involving its covalent labelling with iodinated probes allows highly sensitive detection and analysis of fragments associated with the hormone-binding domain(s) and is able to contribute to receptor mapping studies.

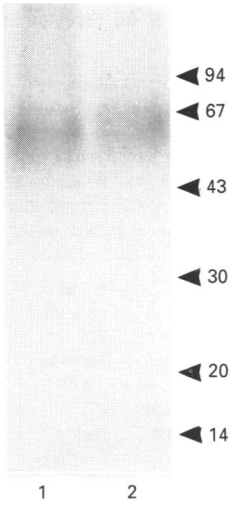

Figure 3 *Staph. aureus* V8 protease treatment of AII receptor

Rat liver plasma membranes were photolabelled with [^{125}I]-Bio-S-S-AII(N_3) (initial concn. 10 nM, sp. radioactivity 75 Ci/mmol). Then 4 pmol of purified receptor was submitted to V8-protease treatment as described in the Materials and methods section. Samples were analysed by classical SDS/PAGE (15%-acrylamide gel) and autoradiography as described in the Materials and methods section. Lane 1, control receptor (V8 protease omitted); lane 2, V8 protease treatment. Molecular masses of protein standards are indicated in kDa.

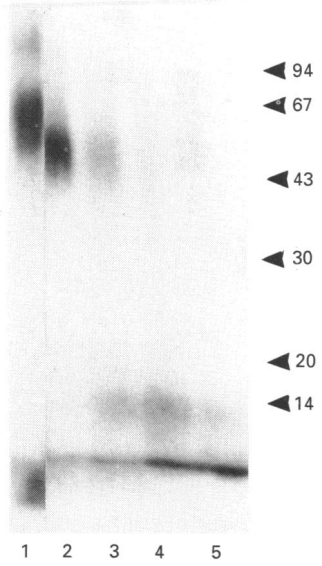

Figure 4 Tryptic proteolysis of AII receptor

Rat liver plasma membranes were photolabelled with [^{125}I]-Bio-S-S-AII(N_3) (initial concn. 8 nM, sp. radioactivity 180 Ci/mmol) and receptors were purified as described in the Materials and methods section. Then 50 fmol of purified receptor was incubated at 30 °C for 1 h with various trypsin/protein ratios. Samples were analysed by classical SDS/PAGE (15%-acrylamide gel) and autoradiography as described in the Materials and methods section. Lane 1, control receptor (1 h, trypsin omitted); lanes 2–5, trypsin/protein ratios of 1:5, 5:1, 25:1, 50:1 respectively. Molecular masses of protein standards are indicated in kDa.

The rat liver AII receptor appeared essentially insensitive to *Staph. aureus* V8 protease; this treatment did not significantly affect the electrophoretic mobility of the receptor (Figure 3), although most of the proteins were digested, as revealed by silver or Coomassie Blue staining (result not shown). This unexpected resistance led us to check various digestion conditions: when an extreme excess of protease over receptor was used, in the presence of SDS, CHAPS or n-octyl glucoside as detergents, the bulk of the receptor shifted to an apparent molecular mass of 60 kDa, whereas a minor radioactive band was observed at 15 kDa (result not shown). The resistance conditions were used as a means of further receptor purification (see 'Receptor purification' above).

Trypsin and CNBr fragmentations were carried out on analytical amounts of purified receptor; we checked the experimental conditions required for optimal production of fragments, as well as their electrophoretic separation and electroblotting on to supports consistent with automated sequencing.

Obtaining tryptic fragments was evaluated as a function of trypsin/protein ratio and digestion time. Tryptic fragments obtained after a fixed period of time (1 h) were analysed by classical SDS/PAGE gels, in accordance with Laemmli (1970). A weak trypsin/protein ration (1:5) led essentially to the formation of a 45 kDa radioactive fragment (Figure 4, lane 2); much higher ratios (5:1 and 25:1) caused proteolysis of the 45 kDa fragment and appearance of a 12 kDa entity (Figure 4, lanes 3 and 4). This latter was further degraded when the trypsin/protein ratio was increased up to 50:1 (Figure 4, lane 5).

Analysis of smaller fragments required the use of Tricine/SDS/PAGE gels, providing improved resolution of low-molecular-mass proteins (Schägger and von Jagow, 1987). Trypsin treatment of the receptor with a 5:1 enzyme/protein ratio for various digestion times (Figure 5a) generated radiolabelled fragments of apparent molecular masses 45, 28 and 12 kDa and three major low-molecular-mass fragments of 8, 7 and 6 kDa respectively. Increasing the digestion time up to 24 h induced almost complete disappearance of the starting receptor, the 45 kDa and 12 kDa entities; the 28 kDa fragments, the appearance of which was somewhat delayed, remained almost constant; at the same time, the amount of the 6 kDa fragment increased. This evolution was confirmed when using an enzyme/protein ratio of 50:1 (Figure 5b): the striking feature is the accumulation of the 6 kDa fragment (see the densitometry analysis of the autoradiogram). Very high enzyme/protein ratios have also been used for the fragmentation of other receptors (Leeb-Lundberg et al., 1987).

Control experiments were performed to check whether previous alkylation of the receptor thiol functions was able to modify the fragmentation pattern; as shown in Figure 5(c), iodoacetate treatment of thiopropyl-Sepharose eluates did not significantly alter fragment generation under our experimental conditions. This could mean that the DTT remaining after Centricon P30 ultrafiltration steps protects receptor thiol functions against reoxidation; indeed, increasing the number of DTT dialysis steps caused significant alteration of the fragmentation pattern (results not shown).

The optimal yields of the various fragments obtained are shown in Table 2, together with the corresponding experimental conditions: the best yields were obtained for the 6 kDa fragment (33%, high trypsin) and the 45 kDa fragment (29%, low trypsin).

CNBr fragmentation was classically carried out in 70% formic acid. Formic acid alone induced aggregation of the receptor, as revealed by the two bands in the autoradiogram at the entry of the stacking gel and spacer gel respectively (Figure 6, lanes 2 and 3). Moreover, in control experiments, formic acid caused cleavage of about 50% of the covalent bonds created upon photoaffinity labelling; this maximal extent of cleavage was obtained after a few hours of treatment and was no longer increased with time (evaluation of cleaved radioactive ligand by gel-filtration experiments; results not shown), thus indicating that covalent bonds between the probe and the receptor are heterogeneous.

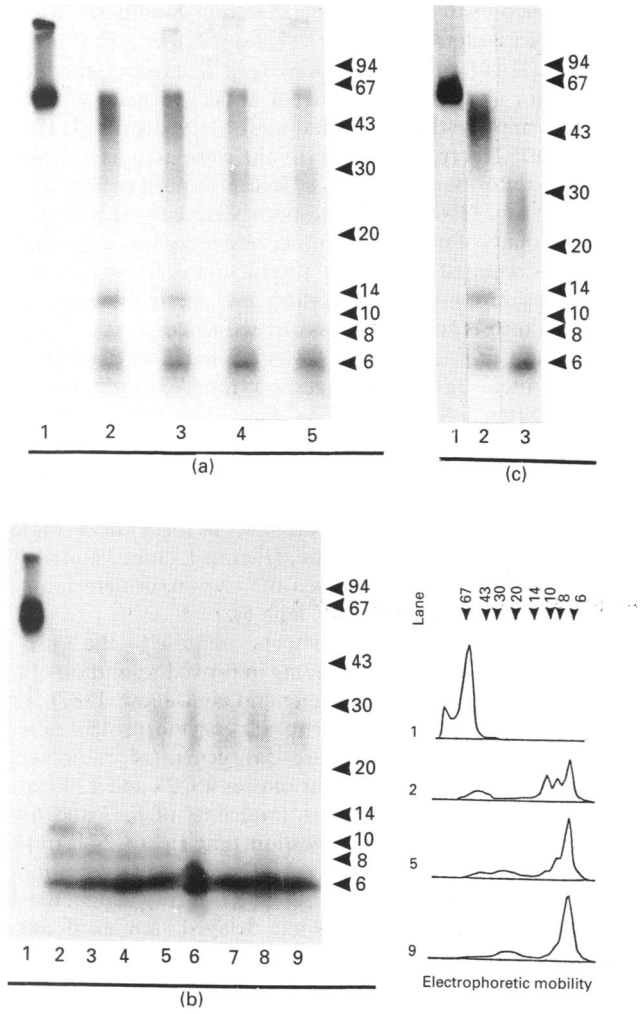

Figure 5 Time course of tryptic proteolysis of AII receptor

Rat liver plasma membranes were photolabelled with [^{125}I]-Bio-S-S-AII(N_3) (initial concn. 8 nM, sp. radioactivity 180 Ci/mmol) and receptors were purified as described in the Materials and methods section. Then 30 fmol of purified receptor was digested by using two trypsin/protein ratios and for various time periods. Control experiments were performed on alkylated receptor. Samples were analysed by Tricine/SDS/PAGE and autoradiography as described in the Materials and methods section. (**a**) Trypsin/protein ratio 5:1. Lane 1, control receptor (24 h, trypsin omitted); lanes 2–5 trypsin digestion for 3 h, 6 h, 12 h or 24 h respectively. (**b**) Trypsin/protein ratio 50:1. Lane 1, control (24 h, trypsin omitted); lanes 2–9, trypsin digestion for 5, 15 or 30 min and 1, 3, 6, 12 or 24 h respectively. A densitometric analysis of lanes 1, 2, 5 and 9 is also shown. (**c**) Tryptic proteolysis of alkylated AII receptor. Lane 1, control (24 h, trypsin omitted); lane 2, trypsin digestion for 3 h at trypsin/protein ratio 5:1; lane 3, trypsin digestion for 24 h at trypsin/protein ratio 50:1. Molecular masses of protein standards are indicated in kDa.

Table 2 Trypsin digestion of AII receptor and electroblotting of tryptic fragments

Rat liver plasma membranes were photolabelled with [^{125}I]-Bio-S-S-AII(N_3) (initial concn. 6–8 nM, sp. radioactivity 180 Ci/mmol) and purified as described in the Materials and methods section. A 30–50 fmol portion of receptor was digested by trypsin under conditions indicated in the Table. Proteolysis products were separated by Tricine/SDS/PAGE. The intact receptor or receptor fragments were electroblotted from the appropriate areas of the gels (detected by the associated radioactivities) on to Immobilon P membranes as described in the Materials and methods section, by using a Biotrap electroeluter apparatus.

Fragment	Conditions: Digestion time	Conditions: Trypsin:protein ratio	Receptor conversion into fragments (yield: % of starting receptor)	Electroblotting yield (%)	Overall yield (%)
45 kDa	1 h	1:5	29	63	18
28 kDa	12 h	50:1	7	41	3
12 kDa	3 h	5:1	10	36	4
8 kDa	5 min	50:1	11	57	6
7 kDa	12 h	5:1	4	ND	ND
6 kDa	24 h	50:1	33	67	22

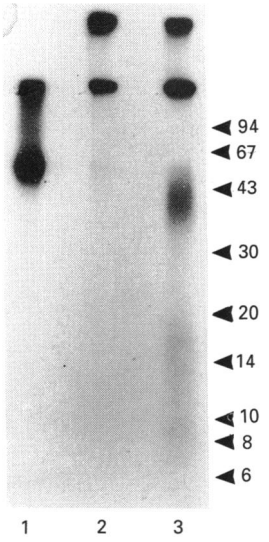

Figure 6 CNBr treatment of AII receptor

Rat liver plasma membranes were photolabelled with [^{125}I]-Bio-S-S-AII(N_3) (initial concn. 8 nM, sp. radioactivity 180 Ci/mmol) and receptors were purified as described in the Materials and methods section. Then 265 fmol of purified receptor was submitted to CNBr cleavage and analysed by Tricine/SDS/PAGE and autoradiography as described in the Materials and methods section. Lane 1, untreated receptor; lane 2, control receptor (incubation for 24 h with 70% formic acid without CNBr); lane 3, CNBr cleavage. Molecular masses of protein standards are indicated in kDa.

Analysis by Tricine/SDS/PAGE (Figure 6) indicated that CNBr action led to the formation of three radiolabelled bands of apparent molecular masses 42, 15 and 9 kDa, the last two being surprisingly heterogeneous. It is noticeable that CNBr fragmentation could occur in spite of receptor aggregation. The yields of fragment production are collected in Table 3.

Electroelution of intact receptor or fragments was carried out in a Biotrap apparatus as described in the Materials and methods section. Receptor and fragments were eluted from the selected portions of electrophoresis gels with excellent yields (> 95%) within periods of time ranging from 80 min for small fragments to 150 min for the intact receptor.

Electroblotting was performed with a Biotrap electroeluter modified as described in the Materials and methods section. Under these conditions, electroblotting of the intact receptor was always greater than 90%; no significant delay was observed between optimal times for electroelution and electroblotting.

Electroblotting of tryptic or CNBr fragments from

Table 3 CNBr cleavage of AII receptor and electroblotting of CNBr fragments

Rat liver plasma membranes were photolabelled with [^{125}I]-Bio-S-S-AII(N_3) (initial concn. 8 nM, sp. radioactivity 180 Ci/mmol); 265 fmol of purified receptor was submitted to CNBr cleavage as described in the Materials and methods section. Proteolysis products were separated and electroblotting on to Immobilon P membranes as mentioned in the Materials and methods section. The values indicated in the Table take into account the partial cleavage of probe–receptor complexes, which was estimated to be about 50% in control experiments (see the text).

Fragment	Receptor conversion into fragments (yield: % of starting receptor)	Electroblotting yield (%)	Overall yield (%)
42 kDa	18	73	13
15 kDa	12	56	7
9 kDa	13	51	7

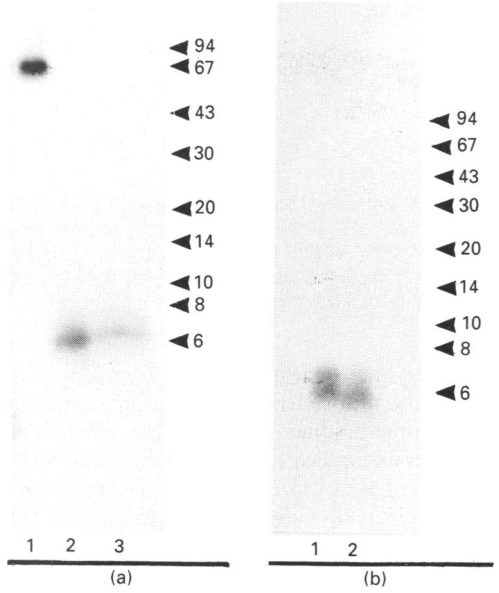

Figure 7 Deglycosylation and CNBr treatment of the 6 kDa probe–tryptic-fragment complex

Rat liver receptor was purified after initial labelling with the radioiodinated biotinylated photoactivatable probe (180 Ci/mmol). The 6 kDa probe–tryptic-fragment complex, obtained as described in the Materials and methods section, was electroeluted from Tricine/SDS/PAGE gels, then treated with TFMS or CNBr and analysed again by Tricine/SDS/PAGE and autoradiography. (**a**) Chemical deglycosylation: lane 1, intact receptor; lane 2, control fragment (16 fmol); lane 3, TFMS-treated fragment (16 fmol). (**b**) CNBr treatment: lane 1, control formic acid-treated fragment (4 fmol); lane 2, CNBr-treated fragment (4 fmol).

electrophoresis gels, carried out in the same conditions, was performed with quite satisfactory yields (Tables 2 and 3), although somewhat lowered compared with the intact receptor.

When scaled up at a semi-preparative or preparative level, these electroblotting experiments should provide a way of evaluating fragment purity by measuring the associated radioactivity and amino acid contents of the hydrolysed electroblotted proteins, as already performed for the intact receptor (Marie et al., 1990). From our fragmentation and electroblotting data it will be possible to evaluate accurately the difficulty in obtaining sequenceable fragment amounts.

Elements of AII receptor mapping

Taking into account the molecular mass of the truncated probe, the 6 kDa probe–tryptic fragment complex involves a receptor fragment of 5 kDa. The experimental verification that this small fragment is not glycosylated is presented in Figure 7(a): it was not significantly affected upon treatment by the deglycosylating agent TFMS. The unusual presence of two potential glycosylation sites on the second extracellular loop (Asn176 and Asn188) restricts the number of possible localizations of the 5 kDa fragment in the rat AT_1 receptor sequence (Murphy et al., 1991); six main localizations can be delineated, irrespective of any prediction of Arginine or lysine accessibilities to trypsin action (see Figure 8). Each of these possibilities involves one transmembrane domain and portions of loops or N- or C-tails. Further fragmentation of the 6 kDa probe–receptor-fragment complex was carried out in order to predict which of these localizations should be favoured: CNBr treatment of this entity induced a very slight decrease in its size (0.3–0.4 kDa; Figure 7b). Considering the positions of methionine residues, the most likely interpretation is that the 5 kDa tryptic fragment corresponds to the Trp^{94}–Arg^{137} sequence, comprising the third transmembrane domain and portions of the first extracellular and second intracellular loops (Figure 8).

Several groups (Iwai and Inagami, 1992; Kakar et al., 1992; Elton et al., 1992; Sandberg et al., 1992; Ye and Healy, 1992) have now reported the existence of a new rat AT_1 receptor subtype, named AT_{1B} (or AT_3 according to Sandberg et al., 1992) and highly similar (95% amino acid identity) to the previously cloned AT_{1A} subtype (Murphy et al., 1991). Obviously our mapping interpretations must take into account the presence of the two subtypes in rat liver (Iwai and Inagami, 1992; Sandberg et al., 1992). Comparison of the two receptor sequences points out minor differences in trypsin cleavage sites potentially involved in 5 kDa fragment production: Glu^{227} and Lys^{232}, located in the third intracellular loop of the AT_{1A} sequence, are replaced by Lys and Thr residues respectively in the AT_{1B} receptor; His^{272} and Lys^{275}, located in the third extracellular loop of the AT_{1A} sequence, are replaced by Arg and Glu residues respectively in the AT_{1B} subtype. As these changes involve close positions, they have no incidence on the theoretical possibilities to generate a 5 kDa fragment upon trypsin treatment. Moreover, the additional methionine, reported by two groups to be present in position 40 of the AT_{1B} receptor (Kakar et al., 1992; Elton et al., 1992), does not change the interpretation of the CNBr-fragmentation data. Finally, the sequence Trp^{95}–Arg^{137}, which represents the most likely localization of our 5 kDa tryptic fragments, is strictly identical in $AT_{1A}$and AT_{1B} receptors. Taken together, these comparisons establish that our mapping data are valid for both subtypes. It seems unlikely that a yet unknown receptor might be predominant in rat liver. Extension of our mapping studies will of course require further experiments on a defined subtype selectively expressed in a heterologous cell system; we possess preliminary evidence that the tryptic fragmentation pattern of the receptor purified from CHO cells transfected with the cDNA encoding the AT_{1A} receptor is similar to that described for rat liver receptor (results not shown).

DISCUSSION

We have previously reported an original protocol for the purification of the rat liver AII receptor (Marie et al., 1990), which belongs to the AT_1 subclass. The present work reports further investigations, at the analytical level, on deglycosylation and fragmentation of this purified rat liver receptor. The recent molecular cloning of the rat AT_1 receptor (Murphy et al., 1991)

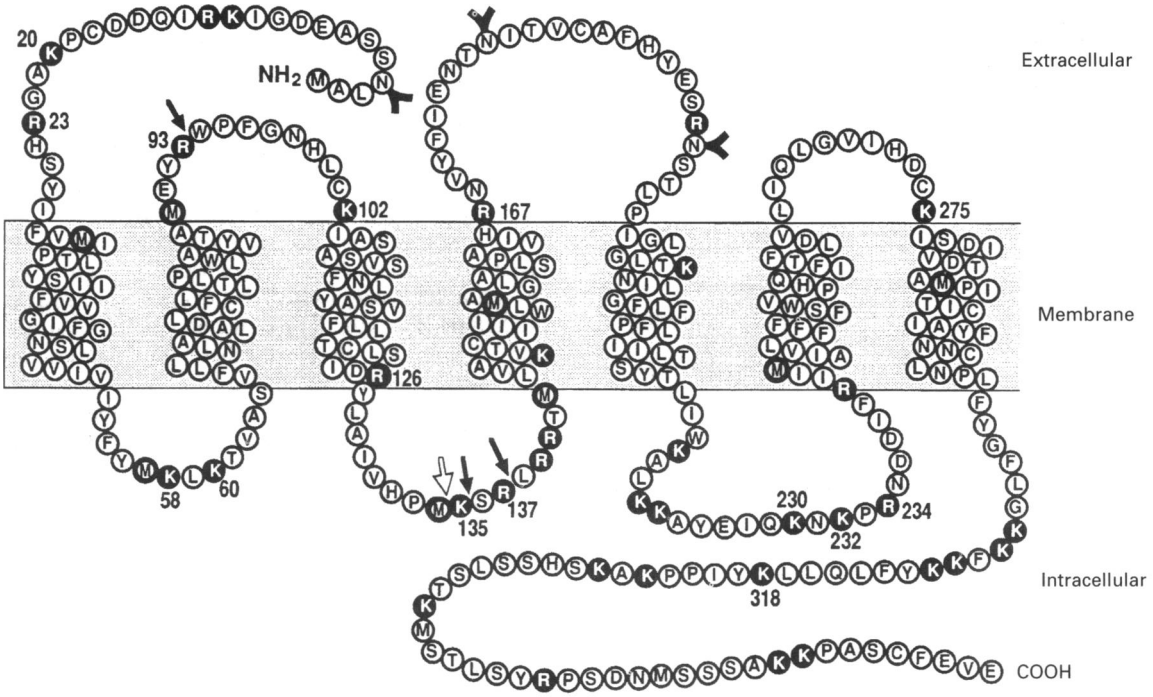

Figure 8 Localization of a rat AT_1 receptor sequence involved in AII binding

Rat liver AII receptor (AT_1 subtype) was photolabelled by the biotinylated derivative possessing a C-terminal phenylalanine, and then purified; trypsin digestion led to a 6 kDa entity which involves a receptor fragment of about 5 kDa. The figure represents the rat AT_1 receptor sequence deduced from cloning data (Murphy et al., 1991); the 5 kDa tryptic fragment is consistent with the following combinations of cleavage sites: K20 or R23 with K58 or K60; K58 or K60 with K102; R93 with K135 or R137; R126 with R167; K230 or K232 or K234 with K275; K275 with K318. The small decrease in the size of this fragment (0.3–0.4 kDa) upon CNBr treatment is consistent with the involvement of M134 in this further cleavage and the identity of the tryptic fragment with the W94–R137 sequence. Arrows indicate cleavage sites by trypsin (black symbols) and CNBr (white symbols) which give the best fit with experimental data.

allows us to propose a preliminary interpretation concerning the involvement of specific receptor sequences in AII binding.

The protocol of receptor purification is based on photoaffinity labelling of the membrane receptor with a biotinylated photoactivatable probe, which precedes solubilization of covalent probe–receptor complexes. These complexes are then purified by chromatography on hydroxyapatite, streptavidin–Sepharose and thiopropyl-Sepharose (Marie et al., 1990). This procedure was easily applied to analytical amounts, with no loss of yield at any of the purification steps. The purified entity displayed a 65 kDa apparent molecular mass in SDS/PAGE, in agreement with previous receptor photolabelling with non-biotinylated AII derivatives (Escher et al., 1978; Guillemette and Escher, 1983; Guillemette et al., 1986).

Deglycosylation and fragmentation experiments were carried out on the purified rat liver AII receptor, thus minimizing possible side reactions involving endogenous enzymes which might contaminate the preparations; it facilitates interpretations and also provides a reliable evaluation before future scale-up.

Chemical deglycosylation of the rat liver AII receptor led to a 40 kDa entity, which represents the fully deglycosylated receptor on the basis of enzymic deglycosylation using N- and O-glycanases. This value is in close agreement with the mass of protein cores calculated from the primary structures of cloned receptors [41093 Da for bovine adrenal receptor (Sasaki et al., 1991); 40889 Da for rat vascular smooth-muscle receptor (Murphy et al., 1991)]. In previous deglycosylation studies, Carson et al. (1987) have pointed out important variations of the saccharidic portion of the receptor with species and tissues.

Fragmentation was monitored by SDS/PAGE analysis of radioactive fragments. This mode of detection implies that, after chemical or enzymic treatment, only fragments which are associated with the radioactive probe, and hence include or are part of the hormone-binding domain(s), can be analysed. Fragmentation studies first indicated the resistance of the rat liver AII receptor to proteolysis by *Staph. aureus* V8 protease, whereas most of the proteins were digested. Taking advantage of this resistance, we introduced an additional purification step; in previously reported experiments (Marie et al., 1990), thiopropyl-Sepharose eluates were treated with V8 protease before electrophoresis and electrotransfer of the receptor to an Immobilon membrane, resulting in a 3–4-fold additional purification.

Trypsin fragmentation was extensively studied. We were able to find conditions under which a 6 kDa probe–tryptic-fragment complex was obtained with a rather high yield (about 30 % for the production itself; 20 % after electrotransfer to Immobilon P). This 6 kDa entity appears to be a good candidate for future studies because of its homogeneity and low molecular mass. If one refers to the structure of the rat AT_1 receptor (AT_{1A} subtype; Murphy et al., 1990), the receptor portion of this complex (about 5 kDa after subtraction of the probe contribution) might display six main localizations. The very small decrease in the size of this fragment upon CNBr treatment is consistent with the following hypothesis: the sequence Arg^{93}–Met^{134}, which comprises the third transmembrane domain and part of the adjacent extra- and the intra-cellular loops, is involved in the receptor interaction with the C-terminal phenylalanine of AII. Careful sequence comparison shows that this interpretation is also valid for the recently cloned AT_{1B} subtype (Iwai and Inagami, 1992; Kakar et al., 1992; Elton et al., 1992; Sandberg et al., 1992; Ye and Healy, 1992), which displays an extremely high similarity with the

previously cloned AT_{1A} receptor. Confirmation of our first mapping evidence should be provided by: (i) the ability of antibodies directed against synthetic selected receptor sequences to recognize this fragment; and (ii) selective chemical modification of an amino acid in the receptor and evaluation of its effect on fragment properties. The most straightforward information should result from fragment sequencing. However, it requires a scale-up of purification and fragmentation experiments; it is tedious work, essentially because of the large loss of receptor at the photoaffinity-labelling step and the difficulty to find a tissue allowing purification from crude membrane preparations. We are now trying to over-express the AT_1 receptor from its cDNA in heterologous cell systems in order to facilitate obtaining large amounts of homogeneous protein; such systems are not at present available.

One particular interest is the comparison of molecular features of the interaction of AII with its various receptor subtypes, the existence of which has recently been demonstrated pharmacologically (Whitebread et al., 1989; Chiu et al., 1989; Duncia et al., 1990; Dudley et al., 1990). At an analytical level, we demonstrated that the various steps of the purification procedure could be applied to the AT_2 receptor subtype. This receptor might display unexpected structural properties, since its does not appear to be coupled to G-proteins (Bottari et al., 1990, 1992; Sumners and Myers, 1991; Pucell et al., 1991); it reinforces the interest of its mapping by using the specific tools we have described.

The approach involving covalent labelling with specific ligands has provided structural information for adrenergic (Dohlman et al., 1988; Wong et al., 1988; Eshdat et al., 1989; Matsui et al., 1989; Strader et al., 1989) or muscarinic cholinergic (Curtis et al., 1989; Hulme et al., 1989) receptors. Examples of topography of peptide-hormone interaction with their receptors have not yet been reported. Similarities and differences between recognition of peptide hormones and small non-peptide ligands with receptors belonging to the same structural superfamily obviously constitute an interesting challenge.

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