New probes for angiotensin II receptors

Synthesis, radioiodination and biological properties of biotinylated and haptened angiotensin derivatives

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The present work delineates the basis for chemical modifications which can be introduced on the angiotensin II (AII) molecule to design probes suitable for indirect affinity techniques, especially for receptor purification. Using the solid-phase synthesis strategy, biotin or dinitrophenyl moieties have been added at the *N*-terminus of AII, with aminohexanoic acid as spacer arm. The resulting probes, (6-biotinylamido)hexanoyl-AII (Bio-Ahx-AII) and dinitrophenylaminohexanoyl-AII (Dnp-Ahx-AII), were prepared in their monoiodinated and highly labelled radioiodinated forms, with possible sulphoxidation of biotin. In addition to their ability to interact with streptavidin and anti-Dnp antibodies respectively, the two ligands displayed almost unchanged affinities for hepatic AII receptors as compared with AII. Bio-Ahx-AII and Dnp-Ahx-AII behaved as agonists on several AII-sensitive systems. The potential applications of these probes, receptor purification, cell labelling and sorting and histochemical receptor visualization, are discussed.

INTRODUCTION

The development of the avidin–biotin system, based on the high affinity of the vitamin biotin for the tetrameric glycoprotein avidin, has given rise to important applications for studying interactions between biological molecules such as the interactions of membrane determinants with specific antibodies or lectins (reviewed by Boorsma, 1983; Wilchek & Bayer, 1984). Biotinylation of specific ligands could allow, among many other possible applications, receptor visualization [with possible amplified detection sensitivity (Hsu *et al.*, 1981)], or analysis and sorting of discrete cell subpopulations bearing the corresponding receptors (Wilchek & Bayer, 1984; Berenson *et al.*, 1986). These approaches have been achieved or at least attempted with biotinylated derivatives of hormones, which might also represent valuable tools for the purification of their specific receptors either by direct (Finn *et al.*, 1984; Hazum *et al.*, 1986) or indirect (Redeuilh *et al.*, 1985) affinity chromatography strategies.

Indirect affinity chromatography, involving adsorption of stable biotin-tagged hormone–receptor complexes to immobilized avidin, should be especially useful when membrane receptors do not recognize the hormone any longer once solubilized. This holds true for angiotensin II receptors (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe:AII): while fairly stable complexes can be obtained after solubilization of receptors occupied by ligands such as Sar¹-AII (Guillemette *et al.*, 1985, 1986), high-yield solubilization, easily achieved with non-ionic detergents for the receptor–ligand complex (Guillemette *et al.*,

1985), does not yield a preparation of unoccupied solubilized receptor able to bind the hormone. Solubilization procedures using CHAPS (Capponi *et al.*, 1983; Chansel *et al.*, 1984), or digitonin (Sen *et al.*, 1983, 1984), which have been claimed to fulfil this latter requirement, led, in our hands, to low yield or imperfectly solubilized preparations.

In the present study, which constitutes the prerequisite to these applications, we explored the possibility of the introduction of haptens (biotin or dinitrophenyl) into the AII molecule through chemical modifications allowing preserved affinities for the receptors and biological properties, as well as recognition by the secondary acceptors avidin or anti-Dnp antibodies. We report the complete solid-phase synthesis, properties, and radioiodination of (6-biotinylamido)hexanoyl-AII (Bio-Ahx-AII) and dinitrophenylaminohexanoyl-AII (Dnp-Ahx-AII), the design of which was based on the following facts and predictions. (1) Structural modifications introduced at the *N*-terminus of the molecule usually preserve its biological potency and activity (Regoli *et al.*, 1974). (2) The usefulness of bifunctional molecules critically depends on their ability to be simultaneously recognized by their two acceptors. For this reason an aminohexanoyl (Ahx) group, consistent with the solid-phase synthesis, has been introduced as spacer arm between the two specific domains of the molecule. (3) Two different haptens, biotin and Dnp, were selected. This takes into account the possibility that the interaction of Dnp with appropriate antibodies (monoclonal anti-Dnp) should display steric characteristics different from those of avidin–biotin and especially ensure easier reversibility.

Abbreviations used: AII, angiotensin II; Sar¹-AII, [sarcosine-1]-AII; TFA, trifluoroacetic acid; Ahx, aminohexanoic acid; Dnp, 2,4-dinitrophenyl; Me₂SO, dimethyl sulphoxide; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; Bio, biotinyl; InsP, inositol phosphate; InsP₂, inositol bisphosphate; InsP₃, inositol trisphosphate; OBzl, benzyloxy.

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MATERIALS AND METHODS

Materials

The chemicals used in the present study were purchased from the following sources: streptavidin from Sigma; acetonitrile (h.p.l.c. grade) from Rathburn (Walkerburn, Scotland); Iodogen (1,3,4,6-tetrachloro-3,6-diphenylglycouril) from Pierce; amino acids for peptide synthesis, Ahx, *N*-2,4-Dnp-aminohexanoic acid (Dnp-Ahx), AII and Sar¹-AII, from Bachem (Bubendorf, Switzerland).

Carrier-free Na¹²⁵I (1800 Ci/mmol) was obtained from Amersham, and *myo*[³H]inositol (16.5 Ci/mmol) from New England Nuclear. (1-Sarcosine, [³H₅]-[phenylalanine-8]AII (130 Ci/mmol) was a generous gift of Dr. E. Escher (Sherbrooke, Canada). Monoclonal anti-Dnp antibodies were a generous gift of Sanofi Research Center (Montpellier, France).

C₁₈ reverse-phase h.p.l.c. columns (μ Bondapak TM/C₁₈, 0.4 cm $\times$ 30 cm and 0.8 cm $\times$ 30 cm) were purchased from Waters Associates, and TSK 4000 SW and 3000 SW gel filtration columns (0.75 cm $\times$ 60 cm) from LKB (Bromma, Sweden).

Synthesis and characterization of biotinylated and dinitrophenylated angiotensin derivatives

The two ligands were synthesized manually by solid-phase methodology using chloromethylated resin (Pierce; 1–2 equivalent of Cl/g) with *t*-butoxycarbonyl as temporary protecting group, benzotriazoloxyltris(dimethylamino)phosphonium hexafluorophosphate (Castro *et al.*, 1975) as coupling reagent and 2 equivalents of each added molecule. The protected forms of the peptides were: Bio-Ahx-Asp(OBzl)-Arg(tosyl)-Val-Tyr-2,6-dichlorobenzyl-Ile-His(Dnp)-Pro-Phe-O-CH₂-polymer, and Dnp-Ahx-Asp(OBzl)-Arg(tosyl)-Val-Tyr-2,6-dichlorobenzyl-Ile-His(Dnp)-Pro-Phe-O-CH₂-polymer.

At this stage the histidine residue was deprotected by thiolysis (Shaltiel, 1967). Complete peptide deprotection and release from the resin was achieved by classical HF procedure (1 h, 0 °C, 15 % anisole). The crude lyophilized product was purified by reverse-phase h.p.l.c. (0.8 cm $\times$ 30 cm column), using acetonitrile gradients in 0.1 % aqueous trifluoroacetic acid (TFA). The pH of the TFA solution was adjusted to 3.5 with diethylamine, except for the final desalting step preceding n.m.r. analysis. The solutions of purified peptide were calibrated on the basis of their absorbance at 280 nm for Bio-Ahx-AII, and at 340 nm for Dnp-Ahx-AII.

The structures of the purified Bio-Ahx-AII and Dnp-Ahx-AII were verified by the analysis of their ¹H-n.m.r. spectra at 360 MHz [one-dimensional and two-dimensional correlation spectroscopy ('COSY') spectra] in [²H]Me₂SO.

Iodination and radioiodination of Bio-Ahx-AII and Dnp-Ahx-AII

Monoiodinated Bio-Ahx-AII and Dnp-Ahx-AII were prepared as described by McFarlane (1958). Briefly, the peptides were dissolved in 10 mM-phosphate buffer, pH 6.0. Iodine chloride (ICl) in methanol was added stepwise up to an ICl/peptide molar ratio of 0.75:1. The reaction was allowed to proceed for 5 min at 0 °C under continuous shaking and stopped by direct application to a C₁₈ reverse-phase h.p.l.c. column equilibrated with 10 % acetonitrile, in 0.1 % TFA. Separation of the iodinated from unreacted products was achieved using

two-slope acetonitrile gradients in 0.1 % aqueous TFA applied at a 1 ml/min flow rate. The gradients were built as follows: 10 % to 30 % acetonitrile in 15 min, then 30 % to 38 % acetonitrile in 30 min for Bio-Ahx-AII and its iodinated derivatives; and 10 % to 34 % acetonitrile in 20 min, then 34 % to 42 % acetonitrile in 30 min for Dnp-Ahx-AII and its iodinated derivatives.

¹²⁵I-labelled Bio-Ahx-AII and Dnp-Ahx-AII were obtained according to Fraker & Speck (1978) using Iodogen as solid-phase oxidizing agent. Na¹²⁵I (1–2 mCi) was reacted at room temperature with the peptide dissolved in acetic acid (0.25 M) in Iodogen-coated polypropylene tubes. On a molar basis, the relative proportions of peptide, Na¹²⁵I, and Iodogen were 10:1:17. The reaction was allowed to proceed for either 2 or 30 min. It was stopped by aspiration from the polypropylene tube and application to the C₁₈ reverse-phase h.p.l.c. column. The monoradioiodinated derivatives of Bio-Ahx-AII (¹²⁵I-Bio-Ahx-AII) and Dnp-Ahx-AII (¹²⁵I-Dnp-Ahx-AII) were purified as indicated above for the unlabelled iodinated compounds. Their concentrations were calculated from the ¹²⁵I content of the purified fractions assuming that the specific radioactivity of the labelled peptide was that of the carrier-free Na¹²⁵I which was used.

Interactions of Bio-Ahx-AII and Dnp-Ahx-AII with streptavidin and anti-Dnp antibodies

Radioiodinated Bio-Ahx-AII (120 pmol in 75 μ l of 100 mM-Tris/acetate, pH 7.2) was incubated with 30 pmol of streptavidin for 150 min at 4 °C in the absence or presence of a 1000-fold excess of biotin. The incubate was applied to a TSK 4000 SW column equilibrated with 100 mM-Tris/acetate, pH 7.2, and eluted at a flow rate of 0.5 ml/min. Absorbance at 254 nm was monitored and 0.5 ml fractions were collected and assayed for radioactivity.

Radioiodinated Dnp-Ahx-AII (75 pmol in 75 μ l of incubation buffer: 100 mM-Tris/acetate, pH 7.2; 100 mM-Na₂SO₄; 0.05 % (v/v) octa(ethylene glycol) mono-*n*-dodecyl ether was incubated for 90 min at 4 °C in the presence of monoclonal anti-Dnp antibody (500 pmol). The incubate was filtered at a flow rate of 0.5 ml/min through a TSK 3000 SW column equilibrated with the incubation buffer. Other conditions are as indicated above. A sample which was not incubated in the presence of anti-Dnp antibody was used as a control.

Binding assays on liver membranes

Rat liver plasma membranes were prepared from male Sprague-Dawley rats as described by Neville (1968) up to step 11. Membranes were stored in liquid N₂. After thawing, the membrane suspension (about 10 mg/ml) was diluted in a solution (binding buffer) composed of 50 mM-Tris/HCl, pH 7.4, 5 mM-MgCl₂, 1 mg of BSA/ml, and 1 mg of bacitracin/ml. The membranes were centrifuged at 27000 *g* for 10 min at 4 °C and resuspended in binding buffer.

Dose-dependent ¹²⁵I-Bio-Ahx-AII and ¹²⁵I-Dnp-Ahx-AII binding to liver membranes was measured as follows. Membranes (50 μ g/assay) were incubated for 30 min at 30 °C in a total volume of 90 μ l of binding buffer containing 0.1–60 nM of the labelled ligand tested. The reaction was stopped by addition of 3 ml of ice-cold filtration buffer (50 mM-Tris/HCl, pH 7.4, 5 mM-MgCl₂) and immediate filtration through Metriacel GA-3

Gelman filters (1.2 μm). The filters were washed with 3×3 ml of filtration buffer and radioactivity retained on the filters was counted. Non-specific binding was measured by incubating membranes in the presence of the labelled ligand and an excess (10 μM) of unlabelled ligand.

The affinity of unlabelled Bio-Ahx-AII and Dnp-Ahx-AII for liver membranes was determined from competition experiments. Membranes were incubated in the presence of a constant amount of [^3H]Sar¹-AII (0.5 nM) and increasing amounts of unlabelled ligand (0.5–500 nM). Other conditions were as indicated above.

Effects of Bio-Ahx-AII and Dnp-Ahx-AII on inositol phosphate and corticosteroids production by rat adrenal cells in primary culture

Adrenal glands were taken from male Long-Evans rats. Glomerulosa cells were isolated and cultured as described by Gallo-Payet *et al.* (1986). Inositol lipids from these cells were labelled by culturing the cells for 3 days in the presence of *myo*[^3H]inositol added to the culture medium at a concentration of 2 $\mu\text{Ci/ml}$.

The experiments were performed using cells grown in plastic Petri dishes (35 mm diameter) and at a density of about 4×10^5 cells per dish. After removal of the radioactive culture medium, the cells were incubated for 60 min in a fresh culture medium deprived of *myo*[^3H]inositol, then for 15 min in phosphate-buffered saline solution complemented with 1 mg of glucose/ml (PBS-glucose) and 10 mM-LiCl, and finally for an additional 15 min period in PBS-glucose containing 10 mM-LiCl, BSA (1 mg/ml), 1 mM-tyrosine, 0.06% Me₂SO, and Bio-Ahx-AII, Dnp-Ahx-AII, or AII (0.1 nM–0.3 μM). The incubation was terminated by rapidly removing the medium and adding 1 ml of 5% perchloric acid and 200 μl of BSA (20 mg/ml). The inositol phosphates InsP₁, InsP₂ and InsP₃ were isolated by chromatography on Dowex 1X10 (formate form) resins as described by Berridge *et al.* (1983).

The effects of Bio-Ahx-AII and Dnp-Ahx-AII on aldosterone production were tested on glomerulosa cells after 3 days in primary culture. Cells were incubated for 2 h in PBS-glucose in the presence of the agents to be tested. The incubation medium was collected and assayed for aldosterone content, as described by Gallo-Payet *et al.* (1986).

RESULTS

Synthesis and n.m.r. characterization of biotinylated and dinitrophenylated AII derivatives

The solid-phase methodology for the synthesis of AII derivatives modified at the *N*-terminus allowed the linkage of additional groups to the protected AII sequence and therefore avoided linkage in non-desired positions. The synthesized molecules had the expected structures shown in Fig. 1. Indeed, proton n.m.r. spectroscopy at 360 MHz allowed unambiguous assignment of the signals corresponding to: (1) the different amino acids of the AII sequence (with reference to previously published n.m.r. studies; Aumelas *et al.*, 1985); (2) the amide proton in the aspartyl residue, indicating that the spacer arm has been attached at the *N*-terminus of the AII molecule; (3) the amide proton of the aminohexanoyl spacer arm reflecting linkage of

biotin through a peptide bond; (4) the protons of biotin or dinitrophenyl moieties.

Iodination and radioiodination of Bio-Ahx-AII and Dnp-Ahx-AII

Unlabelled mono- and di-iodo derivatives of Bio-Ahx-AII and Dnp-Ahx-AII were classically obtained by the action of ICl at tyrosyl residues (McFarlane, 1958). They were purified by C₁₈ reverse-phase h.p.l.c. using acetonitrile gradients in 0.1% aqueous TFA. This procedure allowed a complete separation of the non-reacted, monoiodinated and di-iodinated species which were eluted in that order. The monoiodinated and di-iodinated species exhibited the classical bathochromic shift of the u.v. spectrum due to monoiodination (λ_{max} , 304 nm) and di-iodination (λ_{max} , 310 nm at basic pH) and were identified on this basis. In the experimental conditions used (see the Materials and methods section), the overall yield of monoiodo-Bio-Ahx-AII and monoiodo-Dnp-Ahx-AII production from the starting compounds was about 30%.

The radioiodinated products were prepared using Iodogen as solid-phase oxidizing agent. Within a 30 min exposure to Iodogen, Dnp-Ahx-AII gave a single [^{125}I]-labelled product which co-migrated on h.p.l.c. columns with the unlabelled monoiodo-Dnp-Ahx-AII derivative prepared by the ICl method. Quite different results were obtained with Bio-Ahx-AII. Within short (2 min) reaction times with Iodogen, Bio-Ahx-AII gave similar amounts of two radioiodinated products, one of which co-migrated with the unlabelled monoiodo derivative (Fig. 2a), the other one having a shorter retention time (respective retention times 26.5 and 23 min in the described conditions). When the reaction time was increased to 30 min, the more polar product was exclusively obtained. The latter product was tentatively identified as monoiodo-biotinyl sulphoxide-Ahx-AII [^{125}I -Bio(O)-Ahx-AII] on the following bases: (1) ICl-iodinated Bio-Ahx-AII, reacted for 30 min with Iodogen in conditions strictly similar to those of radioiodination, was completely transformed into a more polar product identical to the radioactive compound obtained in long-lasting Iodogen reaction (Fig. 2b); (2) reduction of the more polar radioiodinated compound by dimethyl sulphide (10% by vol.) in strongly acidic medium (11 M-HCl) according to Savige & Fontana (1977) restored (60% yield for a 30 min reaction time at room temperature) chromatographic co-migration of radioactivity with ICl-iodinated Bio-Ahx-AII (Fig. 2c). Monoiodo-Bio(O)-Ahx-AII and non-iodinated Bio-Ahx-AII had the same retention time. For preparing [^{125}I]-Bio(O)-Ahx-AII it was therefore necessary to select a reaction time in the presence of Iodogen which was long enough to ensure complete oxidation (and concomitant reduction of h.p.l.c. retention time) of the starting product in excess. The possible usefulness of iodinated Bio(O)-Ahx-AII will be discussed later.

Biological properties of Bio-Ahx-AII, Dnp-Ahx-AII and their monoiodinated derivatives

Bio-Ahx-AII and Dnp-Ahx-AII had a high affinity for AII receptors from rat liver membranes. They inhibited [^3H]Sar¹-AII binding in a dose-dependent manner. The mean K_d values deduced from these competition experiments were 6 and 2 nM for Bio-Ahx-AII and Dnp-Ahx-AII respectively (results not shown).

Bio-Ahx-AII				Dnp-Ahx-AII			
		NH	H _α				
		δ (p.p.m.)	³ J (Hz)			δ (p.p.m.)	
All	Phe	d 8.29 d 8.28	7.5 7.5	4.43 4.43	β 3.01 β 3.03	β' 2.93 β' 2.93	Phe
	Pro	— —	— —	4.38 4.39	β 2.05 β 2.03	β' 1.78 β' 1.80	Pro
	His	d 8.27 d 8.27	7.3 8.5	4.76 4.78	β 3.01 β 3.04	β' 2.91 β' 2.91	His
	Ile	d 7.78 d 7.78	8.6 8.5	4.17 4.13	β 1.64 β 1.65		Ile
	Tyr	d 7.97 d 7.97	8.0 8.1	4.49 4.49	β 2.82 β 2.82	β' 2.64 β' 2.64	Tyr
	Val	d 7.60 d 7.61	8.8 8.8	4.19 4.15	β 1.91 β 1.92		Val
	Arg	d 7.94 d 7.94	8.3 8.0	4.26 4.26	β 1.64 β 1.65	β' 1.46 β' 1.50	Arg
	Asp						Asp
O = C		NH d 8.12 d 8.14	7.3 7.5	4.54 4.55	β 2.64 β 2.65	β' 2.49 β' 2.47	O = C
Ahx		m 2.06			2.12		Ahx
		1.5 to 1.3					
NH		m 3.06 t 7.42	³ J 5.5		1.65 3.47		NH
C = O					t 8.81	³ J 6.0	C = O
Bio	H ₁	6.34	(CH ₂) ₇	1.61	H ₃ d 8.86,	⁴ J ₅₋₃ 2.7	Dnp
	H ₃	6.38	(CH ₂) ₈	1.45	H ₅ d ² 8.25	³ J ₅₋₆ 9.6	
	H _{3a}	4.13	(CH ₂) ₉	1.48		⁴ J ₃₋₅ 2.7	
	H ₄	3.06	(CH ₂) ₁₀	2.06	H ₆ d 7.20	³ J ₅₋₆ 9.6	
	H ₆ , H _{6'}	2.82 2.58					
	H _{6a}	4.31					

Fig. 1. Chemical structure and ¹H-n.m.r. characteristics of Bio-Ahx-AII and Dnp-Ahx-AII

¹H-N.m.r. data were obtained in [2H]Me₂SO at 305 K. Only chemical shifts of amide proton (with its ³J_{HNC_αH}), H_α and H_{ββ'} are given in the Figure. Chemical shifts of other characteristic protons are: -Bio-Ahx-AII: Phe aromatic m 7.28–7.16, Pro δm 3.64 and δ'm 3.45, His C₂H s 8.82 and C₄H s 7.33, Ile CH₃ d 0.76 and t 0.78, Tyr H₂₋₆ d 7.00 and H₃₋₅ d 6.61, Val CH₃ d 0.74 and d 0.77, Arg NH t 7.71 and d CH₂ m 3.01; -Dnp-Ahx-AII: Phe aromatic m 7.35–7.15, Pro δm 3.64 and δ' 3.47, His C₂H s 8.85 and C₄H s 7.34, Ile CH₃ d 0.75 and t 0.77, Tyr H₂₋₆ d 7.00 and H₃₋₅ d 6.60, Val CH₃ d 0.76 and d 0.74, Arg NH t 7.47 and δ CH₂ m 3.06.

These values are higher than those obtained for AII itself or Sar¹-AII (1.5 and 0.5 nM respectively; Keppens *et al.*, 1982; Guillemette *et al.*, 1986). However, it was of much interest to observe that attachment of bulky groups to the AII molecule was not accompanied by a dramatic reduction in its ability to bind to AII receptors. These results are a further illustration of the well-established fact that structural modifications introduced at the N-terminus of the AII molecule usually preserve its biological potency and activity (Regoli *et al.*, 1974).

Monoiodination of the Bio-Ahx-AII and Dnp-Ahx-AII molecules also preserved a high affinity for liver membrane AII receptors. Direct binding experiments

carried out with ¹²⁵I-Bio-Ahx-AII and ¹²⁵I-Dnp-Ahx-AII are illustrated in Fig. 3. ¹²⁵I-Bio-Ahx-AII interacted with a single class of high-affinity binding sites (Fig. 3a). The observed K_d value of 2 nM is close to that determined for Bio-Ahx-AII. The maximal binding capacity of 1.74 pmol/mg of protein was very close to that determined with the highly specific ligand for AII receptors, [³H]Sar¹-AII. It is noteworthy that the non-specific component of ¹²⁵I-Bio-Ahx-AII binding to liver membranes was very low (less than 2% of total binding at full receptor occupancy, Fig. 3a). This is indeed an essential property required from ligands designed as potential tools for affinity chromatography experiments.

Fig. 2. Reverse-phase h.p.l.c. elution profiles of iodinated and radioiodinated Bio-Ahx-AII

H.p.l.c. was carried out as described in the Materials and methods section. Absorbance of the eluate was monitored at 254 nm (—) and ^{125}I radioactivity was measured in 0.3 ml fractions (---). (a) Purified unlabelled monoiodo-Bio-Ahx-AII (5 nmol) obtained by ICI action was co-chromatographed with trace amounts of the less polar ^{125}I -labelled compound obtained after a 2 min Iodogen-catalysed radioiodination. The Figure also shows the shape of the acetonitrile gradient in 0.1% TFA which was used. (b) Unlabelled monoiodo-Bio-Ahx-AII (10 nmol) obtained by ICI action was treated for 30 min by Iodogen under experimental conditions identical to those of radioiodination except for the presence of Na^{125}I which was replaced by unlabelled NaI . The product of the reaction was co-chromatographed with trace amounts of the more polar ^{125}I -labelled product obtained after Iodogen-catalysed radioiodination of Bio-Ahx-AII. Inset: absorbance elution profile of a blank Iodogen reaction mixture showing the non-peptidic nature of the observed contaminating peaks. (c) Trace amounts of the more polar ^{125}I -labelled product obtained after Iodogen-catalysed radioiodination of Bio-Ahx-AII were submitted to reduction by 10% dimethyl sulphide in 11 M-HCl, for 30 min at room temperature and co-chromatographed with unlabelled monoiodo-Bio-Ahx-AII.

The complex formed between ^{125}I -Bio-Ahx-AII and AII receptors was highly stable since 75% remained undissociated when treated with a 1000-fold excess of AII for 90 min at 30 °C, reflecting transition into a 'quasi irreversible state' as observed with other ligands for AII receptors (Keppens *et al.*, 1982).

Dose-dependent binding of ^{125}I -Dnp-Ahx-AII appeared more complex (Fig. 3b). When non-specific binding was determined in the presence of excess unlabelled monoiodo-Dnp-Ahx-AII, several classes of specific binding sites could be evidenced, as shown by a curvilinear Scatchard plot of the data (Fig. 3b). Apparently ^{125}I -Dnp-Ahx-AII interacted with a class of high-affinity binding site and with one (or several) class(es) of low-affinity and high-capacity binding site. The estimated K_d and maximal binding capacity of the high-affinity binding sites were 1.2 nM and 1.95 pmol/mg of protein respectively. When Sar^1 -AII was used to evaluate non-specific binding, only one class of high-affinity specific binding site was evidenced. The K_d (1.1 nM) and maximal capacity (1.75 pmol/mg of protein) of these sites are almost identical to those of the high-affinity binding sites detected when using unlabelled monoiodo-Dnp-Ahx-AII to determine the non-specific component of total binding. The most likely explanation for these observations is that ^{125}I -Dnp-Ahx-AII interacted with AII receptors and 'pseudo-specific' sites which might represent sites of interaction of the Dnp moiety of the molecule with hydrophobic portions of the membrane.

To further validate our conclusion that Bio-Ahx-AII and Dnp-Ahx-AII can be considered as specific probes for AII receptors, we verified that these AII analogues were able to induce the classical responses elicited by AII in several cell types, namely adenylate cyclase inhibition (Jard *et al.*, 1981; Marie *et al.*, 1985) and stimulation of inositol lipid breakdown (Farese *et al.*, 1984; Smith *et al.*, 1984; Lynch *et al.*, 1985; Canonico & MacLeod, 1986; Gallo-Payet *et al.*, 1986). Bio-Ahx-AII and Dnp-Ahx-AII stimulated in a dose-dependent manner the accumulation of labelled inositol phosphates by *myo*[^3H]inositol-prelabelled glomerulosa adrenal cells in primary culture (Fig. 4). Bio-Ahx-AII and Dnp-Ahx-AII were almost as potent as AII itself ($[\text{A}_{50}]$ values of 6.0 and 2.4 nM respectively, as compared with 1 nM for AII). Bio-Ahx-AII and Dnp-Ahx-AII also stimulated aldosterone production by these cells (Table 1). The higher stimulation obtained with Bio-Ahx-AII compared with AII itself might be explained by differences in proteolytic resistance of the ligands, which should be critical in tests based on steroid accumulation throughout a 2 h incubation period and make difficult correlations of dose-response curves with the relative potencies of the ligands

Table 1. Steroidogenic effects of Bio-Ahx-AII, Dnp-Ahx-AII and AII

The effects of Bio-Ahx-AII and Dnp-Ahx-AII on aldosterone production were tested on glomerulosa cells after 3 days in primary culture. Cells were incubated for 2 h in PBS-glucose in the presence of the agents to be tested. The incubation medium was collected and assayed for aldosterone content, as described by Gallo-Payet *et al.* (1986).

Treatment	Aldosterone production (pg/ml)
None	60 ± 20
AII (5 nM)	260 ± 10
Bio-Ahx-AII (5 nM)	465 ± 35
Dnp-Ahx-AII (5 nM)	205 ± 15

Fig. 3. Dose-dependent binding of ^{125}I -Bio-Ahx-AII and ^{125}I -Dnp-Ahx-AII to rat liver plasma membranes

Membranes prepared according to Neville's procedure (50 μg /assay) were incubated for 30 min at 30 $^{\circ}\text{C}$ with various concentrations of the two labelled ligands used. ^{125}I -Bio-Ahx-AII and ^{125}I -Dnp-Ahx-AII were diluted with unlabelled monoiodo-Bio-Ahx-AII and -Dnp-Ahx-AII up to specific radioactivities of 72 and 76 Ci/mmol respectively. Binding was measured as described in the Materials and methods section. (a) the specific ($\bullet$) and non-specific ($\circ$) components of ^{125}I -Bio-Ahx-AII binding are shown in the left panel. Non-specific binding was measured in the presence of unlabelled monoiodo-Bio-Ahx-AII (10 μM). The corresponding Scatchard plots are shown in the right panel. The K_d and B_{max} values were calculated by linear regression analysis (K_d 2 nM, B_{max} 1.74 pmol/mg of protein). Values on the graph are means of triplicate determinations; the s.d. values never exceeded 3% of the mean values. Two experiments were performed each time which gave identical results. (b) ^{125}I -Dnp-Ahx-AII was used as the radioactive ligand. In this case non-specific binding was determined in the presence of either 12 μM unlabelled monoiodo-Dnp-Ahx-AII ($\circ$) or 12 μM unlabelled Sar¹-AII ($\blacktriangle$). The left panel shows the dose-dependent total binding ($\square$), apparent specific binding determined with monoiodo-Dnp-Ahx-AII as the unlabelled ligand ($\blacksquare$), and apparent specific binding determined with Sar¹-AII as the unlabelled ligand ($\bullet$). The corresponding Scatchard plots are shown in the right panel. In one case ($\bullet$), linear regression analysis of the plot gave K_d and B_{max} values of 1.1 nM and 1.75 pmol/mg of protein respectively. In the other case ($\blacksquare$), the plot was fitted with a two-site model: the solid line corresponds to the best fit obtained; note that it is consistent with experimental points ($\blacksquare$). The broken line is the Scatchard plot corresponding to the high-affinity binding sites. The computed K_d and B_{max} values of these sites were 1.2 nM and 1.95 pmol/mg of protein respectively. Values on the graph are means of triplicate determinations; the s.d. values never exceeded 5% of the mean values. Two experiments were performed which gave identical results.

for inositol lipid breakdown. Both probes also inhibited the adenylate cyclase activity from rat adenohypophyseal membranes (results not shown).

Interaction of Bio-Ahx-AII and Dnp-Ahx-AII with streptavidin and anti-Dnp antibodies

Bio-Ahx-AII and Dnp-Ahx-AII interacted with their putative secondary acceptors, namely avidin (or avidin derivatives) and monoclonal anti-Dnp antibodies respectively. Preincubation of ^{125}I -Bio-Ahx-AII with streptavidin (which is known to give less non-specific interactions with proteins as compared with avidin) led

to biotin specific formation of a complex which could be isolated on a high-performance size-exclusion chromatography column (Fig. 5a). Similarly, ^{125}I -Dnp-Ahx-AII and monoclonal anti-Dnp antibodies formed complexes which were stable enough to be evidenced by size-exclusion chromatography (Fig. 5b).

DISCUSSION

We have established in the present work the structural basis for chemical modifications of the AII molecule which can be used for the design of affinity probes

Fig. 4. Effects of Bio-Ahx-AII and Dnp-Ahx-AII on inositol lipid breakdown in glomerulosa adrenal cells in primary culture

Primary cultures of rat adrenal glomerulosa cells (4×10^5 cells per dish) were grown for 3 days in the presence of *myo*[^3H]inositol. The cells were incubated for 15 min in the presence of AII (●), Bio-Ahx-AII (Δ), or Dnp-Ahx-AII (○) at the indicated concentrations. Inositol phosphates ($\text{InsP} + \text{InsP}_2 + \text{InsP}_3$) were determined as described in the Materials and methods section. The plotted values represent the means $\pm$ S.D. of three determinations from separate dishes of cultured cells. The estimated $[A_{50}]$ values for AII-, Bio-Ahx-AII-, and Dnp-Ahx-AII-stimulated inositol phosphate production were 1, 6 and 2.4 nM respectively. Two experiments were performed which gave similar values.

possessing high affinity for AII receptors and two highly specific secondary acceptors, avidin and anti-Dnp antibodies. Such bifunctional molecules might represent appropriate tools for the development of indirect affinity techniques applicable to: (1) the labelling and sorting of AII-sensitive cell sub-populations; (2) amplified AII receptor visualization with classically derivatized secondary ligands avidin or anti-Dnp antibodies; or (3) purification of AII receptors by affinity chromatography using immobilized avidin or anti-Dnp antibodies.

With respect to their ability to interact with AII receptors, Bio-Ahx-AII and Dnp-Ahx-AII, in spite of their rather bulky substitutions, exhibit several of the properties which must be fulfilled in order to be used as affinity probes. They are biologically active on the two AII-responsive systems which were used, namely glomerulosa adrenal cells in primary culture and rat adenohypophyseal membranes. Bio-Ahx-AII and Dnp-Ahx-AII inhibit the binding to liver membranes of highly specific labelled ligand for AII receptors. The radioiodinated derivatives of Bio-Ahx-AII and Dnp-Ahx-AII labelled with high-affinity (K_d values in the nM range) AII receptors from rat liver membranes. At least for ^{125}I -Bio-Ahx-AII, binding to liver membranes was highly specific. The non-specific component of total binding was almost negligible (Fig. 3a). That ^{125}I -Dnp-Ahx-AII exhibits, in addition to high-affinity hormone-specific binding sites, pseudo-specific lower-affinity sites (supposed to involve interactions with hydrophobic portions of the

Fig. 5. Interaction of Bio-Ahx-AII and Dnp-Ahx-AII with streptavidin and anti-Dnp antibodies

^{125}I -Bio-Ahx-AII (120 pmol) was incubated with streptavidin (30 pmol) for 150 min at 4°C in the absence (●) or presence (○) of a 1000-fold excess of biotin. The incubation mixture was analysed by high-pressure size-exclusion chromatography at 4°C on a TSK 4000 SW column equilibrated with 100 mM-Tris/acetate, pH 7.2, at a flow rate of 0.5 ml/min. Absorbance of the eluate was monitored and ^{125}I radioactivity in 0.5 ml fractions was measured. (b) ^{125}I -Dnp-Ahx-AII (75 pmol) was incubated with a monoclonal anti-Dnp antibody (500 pmol) for 90 min at room temperature in a total volume of 75 μl . The incubate (●) and a portion of ^{125}I -Dnp-Ahx-AII (Δ) were applied separately and filtered at 40°C on the TSK 3000 SW column with 100 mM-Tris/acetate, pH 7.2, 100 mM- Na_2SO_4 , and 0.05% octa(ethylene glycol) monon-dodecyl ether. Other conditions are as indicated above.

membrane), does not necessarily preclude its usefulness in all experimental situations.

The radioiodinated forms of Bio-Ahx-AII and Dnp-Ahx-AII were able to interact with their respective secondary specific acceptors, streptavidin and monoclonal anti-Dnp antibodies. However the effectiveness of these bifunctional ligands, which implies simultaneous

dual recognition of AII receptors and avidin or antibodies, can be critically dependent on the properties (length, hydrophobicity, rigidity) of the spacer arm between the two specific domains of the molecule; furthermore, the steric requirements for effective bifunctionality might vary with the biological system or application considered. In this respect, the use of the solid-phase synthesis methodology offers the possibility to modify, from the same starting protected peptidic segments, the nature of the spacer arm without expected drastic reduction in affinity of the resulting probes for the receptors.

Moreover homologous probes possessing a C-terminal azidophenylalanine should allow, when required, the attainment of covalent biotinylated hormone-receptor complexes, as already suggested for ACTH-derived biotinylated photoactivable probes (Hofmann *et al.*, 1986). Indeed high-yield photoaffinity labelling procedures for AII receptors have been recently described (Guillemette *et al.*, 1985, 1986).

The observation that biotin sulphoxide was produced during the course of iodination of Bio-Ahx-AII deserves a special comment: the very high affinity of biotin for avidin constitutes an expected difficulty for the development of affinity chromatography techniques using biotinylated bifunctional ligands. Chemically modified biotin molecules with reduced affinity for avidin have been proposed (dethiobiotin, iminobiotin) (Finn & Hofmann, 1985; Orr *et al.*, 1986). Sulphoxidation, which can be easily obtained, might be an alternative possibility to modulate the affinity of biotin for avidin.

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REFERENCES

- Aumelas, A., Sakarellos, C., Lintner, K., Fermandjian, S., Khosla, M. C., Smeby, R. P. & Bumpus, F. M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1881-1885
- Berenson, R. J., Bensinger, N. I. & Kalmasz, D. (1986) *J. Immunol. Methods* **91**, 11-19
- Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473-482
- Boorsma, D. M. (1983) in *Techniques in Immunocytochemistry* (Bullock, G. R. & Petrusz, P., eds.), vol. 2, pp. 155-174, Academic Press, London
- Canonica, P. L. & MacLeod, R. (1986) *Endocrinology* (Baltimore) **118**, 233-238
- Capponi, A. M., Birabeau, M. A. & Vallotton, M. B. (1983) *J. Recept. Res.* **3**, 289-299
- Castro, B., Dormoy, J. R., Evin, G. & Selve, C. (1975) *Tetrahedron Lett.* **14**, 1219-1222
- Chansel, D., Ardaillou, N. & Ardaillou, R. (1984) *FEBS Lett.* **170**, 243-246
- Farese, R. V., Larson, R. & Davis, J. S. (1984) *Endocrinology* (Baltimore) **114**, 302-304
- Finn, F. M., Titus, G., Morstman, D. & Hofmann, K. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7328-7332
- Finn, F. M. & Hofmann, K. (1985) *Methods Enzymol.* **109**, 418-445
- Fraker, P. J. & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849-857
- Gallo-Payet, N., Guillon, G., Balestre, M. N. & Jard, S. (1986) *Endocrinology* (Baltimore) **119**, 1042-1047
- 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
- Hazum, E., Sehvarz, I., Waksman, Y. & Keinan, D. (1986) *J. Biol. Chem.* **261**, 13043-13048
- Hofmann, K., Romovacek, M., Stehle, C. J. & Finn, F. M. (1986) *Biochemistry* **25**, 1339-1346
- Hsu, S. M., Raine, L. & Fanger, H. (1981) *J. Histochem. Cytochem.* **29**, 577-580
- Jard, S., Cantau, B. & Jakobs, K. H. (1981) *J. Biol. Chem.* **256**, 2603-2605
- Keppens, S., De Wulf, H., Clauser, P., Jard, S. & Morgat, J. L. (1982) *Biochem. J.* **208**, 809-817
- Lynch, C. J., Blackmore, P. F., Charest, R. & Exton, J. H. (1985) *Mol. Pharmacol.* **28**, 93-99
- Marie, J., Gaillard, R. C., Schoenenberg, P., Jard, S. & Bockaert, J. (1985) *Endocrinology* (Baltimore) **116**, 1044-1050
- McFarlane, A. S. (1958) *Nature* (London) **182**, 53
- Neville, D. M. (1968) *Biochim. Biophys. Acta* **154**, 540-552
- Orr, G. A., Heney, G. C. & Zeheb, R. (1986) *Methods Enzymol.* **122**, 83-94
- Redeuilh, G., Secco, C. & Baulieu, E. (1985) *J. Biol. Chem.* **260**, 3996-4002
- Regoli, D., Rioux, F., Park, W. K. & Choi, C. (1974) *Can. J. Physiol. Pharmacol.* **52**, 39-49
- Savigne, W. & Fontana, A. (1977) *Methods Enzymol.* **47**, 453-459
- Sen, I., Jim, K. F. & Soffer, R. L. (1983) *Eur. J. Biochem.* **136**, 41-49
- Sen, I., Bull, H. G. & Soffer, R. L. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1679-1683
- Shaltiel, S. (1967) *Biochem. Biophys. Res. Commun.* **29**, 178-183
- Smith, J. B., Smith, L., Brown, E. R., Barnes, D., Sabir, M. A., Davis, J. S. & Farese, R. V. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7812-7816
- Wilchek, M. & Bayer, E. A. (1984) *Immunol. Today* **5**, 39-43