Development of Affinity Resins for Isolation of Angiotensin Receptors

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The development of affinity resins for the isolation of angiotensin II receptors from adrenal fasciculata cells is described. The approach is based on the avidin-biotin interaction. The advantages of the technique are delineated.

INTRODUCTION

It has been recognized for some time that the production of aldosterone by adrenal glomerulose cells is regulated by adrenocorticotropic hormone (ACTH) and angiotensin II. More recently it has become clear that glucocorticoid biosynthesis in fasciculata cells is also stimulated by angiotensins [1].

Bovine adrenal fasciculata cells respond to ACTH, angiotensin I (AI), angiotensin II (AII), and angiotensin III (AIII) with formation of corticosteroids [2]. While the response to ACTH is mediated by cAMP, the stimulation by the angiotensins is not accompanied by an increase in the cyclic nucleotide [3]. Accordingly, fasciculata cells contain two classes of receptors that mediate steroid production via different pathways.

Hormonal stimulation of aldosterone in the glomerulosa cell requires extracellular potassium [4] and calcium [5,6]. Canine glomerulosa cells contain both high affinity ($K_a = 3.3 \times 10^8M^{-1}$) and lower affinity ($K_a = 2.5 \times 10^8M^{-1}$) binding sites for $^{125}$I AII [7] with the high affinity sites accounting for 25–33 percent of the total receptor population.

AII stimulation of bovine fasciculata cells to produce corticosteroids is less well understood than are similar events in glomerulosa cells. Although Peytreman et al. [1] reported that cAMP production is elicited by AII, more recent evidence supports the view that cAMP is not a second messenger in AII stimulation of adrenal cells [3]. Hormonal stimulation of steroid production in fasciculata cells requires extracellular calcium [3]. Whereas potassium is essential for steroidogenesis in the glomerulosa cells, it does not affect steroid production in the fasciculata cell [3].

Fasciculata cells contain specific angiotensin receptors which bind $^{125}$I labeled AII saturably and with high affinity. Binding studies have identified a single class of binding sites with $K_a$ values of $0.2 \times 10^8M^{-1}$ for AII and $0.7 \times 10^8M^{-1}$ for AIII [3]. ACTH does not displace labeled AII or AIII from the cells, providing evidence for the specificity of the angiotensin receptors.

Several attempts have been made to identify AII receptors in various tissues by

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cross-linking radiolabeled photoaffinity derivatives of AII to receptors or by cross-linking $^{125}$I AII using disuccinimidyl suberate. Solubilization of the covalently linked complexes followed by separation of the materials by SDS-PAGE under reducing conditions has produced two estimates for the molecular weight of the receptor. An $M_r$ of 116,000 was reported for the cross-linked receptor from rat adrenal[8], while $M_r$ 68,000 was found for the photoaffinity labeled receptor from dog adrenal and uterus [9] and for the cross-linked preparation from rabbit liver [10].

Although some functional properties of the AII receptors have been characterized with isolated cells and particulates, little is known regarding their molecular characteristics. This appears to be partly due to their instability. Apparently intact solubilized receptor capable of specifically binding angiotensin ligands has been obtained from rat adrenal glomerulosa particles using the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-l-propane-sulfonate ("Chaps") [11]. Using 5 mM "Chaps," 45 percent of membrane proteins and 50 percent of the AII receptors could be solubilized. Binding assays were performed at a "Chaps" concentration of 2 mM since there was inhibition of binding at higher detergent concentrations. The solubilized receptor preparation was very unstable, losing 50 percent of its binding activity when stored at –70°C for ten days. Loss of activity at 40°C was more rapid. The solubilized receptors retained the properties of the particulate receptor as concerns binding of AII and congeners.

In connection with our long-standing interest in hormones that are involved in the stimulation of steroidogenesis in adrenal fasciculata cells, we have initiated an investigation aimed at the isolation of AII receptors from bovine fasciculata cells, using affinity chromatography based on the avidin-biotin approach [12]. In this paper, we describe studies relating to the preparation of an affinity column for the purification of these receptors.

To establish the feasibility of this approach, several fundamental criteria have to be met. The biotin-containing analogs of AII must be able to interact with AII receptors; they must form strong complexes with suc-avidin; and they must be capable of interacting simultaneously with suc-avidin and AII receptors in order to be suitable ligands for affinity chromatography. In this paper, we describe the synthesis and characterization of two potential ligands.

**EXPERIMENTAL PREPARATION OF COMPOUNDS**

Avidin was prepared from hens’ egg white by the method of Orr [13] and was converted to suc-avidin in the manner described previously [14].

$N^\alpha$-tert-butoxycarbonyl-$N^\alpha$-biotinyl-L-lysine (N$^\alpha$-tert-butoxycarbonyl-biocytin) was prepared from N$^\alpha$-tert-butoxycarbonyl-L-lysine and the N-hydroxysuccinimido ester of biotin. The material was recrystallized from methanol-water; m.p. 136–138°C; [α]$_D^{28}$ +39.6° (c 0.975, MeOH).

**Anal. Calcd. for C$_21$H$_{38}$N$_4$O$_8$S**

**Found**

C, 53.37; H, 7.68; N, 11.86

C, 53.47; H, 7.88; N, 11.87

The N-hydroxysuccinimido ester of the title compound was prepared in the usual manner and was recrystallized from isopropanol/acetone/ethyl acetate; m.p. 111–113°C;

**Anal. Calcd. for C$_{25}$H$_{39}$N$_3$O$_8$S**

**Found**

C, 52.71; H, 6.90; N, 12.29

C, 52.56; H, 7.01; N, 12.14
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\[ \text{N}^{\text{a}}\text{-tert-butoxycarbonyl-N}'\text{-6-(biotinylamido)hexyl-L-lysine} \]
This compound was prepared from \( \text{N}^{\text{a}}\text{-tert-butoxycarbonyl-L-lysine} \) and the N-hydroxysuccinimido ester of 6-(biotinylamido)hexanoic acid and was recrystallized from methanol/water; m.p. 123–125°C; \([\alpha]_D^{27} + 31.4^\circ\) (c 1.04, MeOH).

Anal. Calcd. for C\(_{27}\)H\(_{47}\)N\(_5\)O\(_7\)S
C, 55.36; H, 8.09; N, 11.96
Found
C, 55.35; H, 8.26; N, 12.02

The N-hydroxysuccinimido ester of the title compound was prepared in the usual manner and was recrystallized from isopropanol/acetone/ethyl acetate; m.p. 112–114°C;

Anal. Calcd. for C\(_{31}\)H\(_{50}\)N\(_6\)O\(_9\)S
C, 54.53; H, 7.38; N, 12.31
Found
C, 54.43; H, 7.51; N, 12.21

\[ \text{[Biocytin']}\text{angiotensin II} \]
This compound was prepared from H-Arg-Val-Tyr-Val-His-Pro-Phe-OH(AIII) [15] and N-hydroxysuccinimido \( \text{N}^\text{a}-\text{tert-butoxycarbonyl-biocytin} \) followed by deprotection with trifluoroacetic acid; homogeneous on high performance liquid chromatography (HPLC); amino acid ratios in acid hydrolysate; Lys\(_{0.98}\)Arg\(_{0.10}\)Val\(_{2.04}\)Tyr\(_{1.03}\)His\(_{0.88}\)Pro\(_{1.04}\)Phe\(_{1.00}\).

\[ \text{[N}'\text{-6-(biotinylamido)hexyl-L-lysine'}\] \text{angiotensin II} \]
This compound was prepared from (AIII) and N-hydroxysuccinimido \( \text{N}^\text{a}-\text{tert-butoxycarbonyl-N}'\text{-6-(biotinylamido)hexyl-L-lysine} \) followed by deblocking with trifluoroacetic acid; homogeneous on HPLC; amino acid ratios in acid hydrolysate; Lys\(_{0.93}\)Arg\(_{1.03}\)Val\(_{2.02}\)Tyr\(_{1.03}\)His\(_{0.93}\)Pro\(_{1.03}\)Phe\(_{1.03}\).

**ANALYTICAL METHODS AND BIOASSAYS**

Peptides were hydrolyzed for 20 hours at 110°C in 6 N hydrochloric acid containing 1 percent (v/v) phenol. Amino acids in the hydrolysates were determined in a Waters instrument by the Pico-Tag method. HPLC of the peptides was conducted with the same instrument using a Bondapak C\(_{18}\) column with the following solvent systems: (pump A) 0.1 percent phosphoric acid; (pump B) 0.1 percent phosphoric acid:acetonitrile (1:1). The linear gradient was 10–60 percent pump B over 25 minutes with a pumping speed of 2 ml/minute. Samples of approximately 10 \( \mu \)g were injected. The rates of dissociation of the suc-avidin-angiotensin analog complexes were determined as described previously [16]. The preparation of calf adrenal fasciculata cells and the steroid bioassays were conducted as described previously [17].

**RESULTS**

We have synthesized two biotinylated derivatives of AII: i.e., [biocytin'] angiotensin II and [N'-6-(biotinylamido)hexyllysine'], angiotensin II. In these analogs, the N-terminal aspartic acid of AII is replaced by a lysine residue whose \( \varepsilon \)-amino group is acylated with either biotin or 6-(biotinylamido)hexanoic acid. These analogs were selected because the extensive literature [18] on the biological activity of AII derivatives indicates that the N-terminal amino group is important for biological activity. The analogs differ with respect to the position of biotin, which in analog I is attached directly to the \( \varepsilon \)-amino group of the N-terminal lysine, whereas in analog II an aminohexanoic acid spacer is interspaced between the \( \varepsilon \)-amino group of lysine and the biotin. The structures of AII, AIII, and the two analogs are illustrated in Fig. 1. The two peptides were synthesized by coupling \( \text{N}^\text{a}-\text{tert-butoxycarbonyl-biocytin} \) or
H-Arg-Val-Tyr-Val-His-Pro-Phe-OH

Val^5 Angiotensin III

H-Asp-Arg-Val-Tyr-Val-His-Pro-Phe-OH

Val^5 Angiotensin II

H-Bct-Arg-Val-Tyr-Val-His-Pro-Phe-OH

Bct^1, Val^5 Angiotensin II

H-Lys(BAH)-Arg-Val-Tyr-Val-His-Pro-Phe-OH

Lys(BAH)^1, Val^5 Angiotensin II

N^o-tert-butoxycarbonyl-N^4-6-(biotinylamido)hexyllysine to AIII, using the N-hydroxysuccinimide ester procedure. The protected intermediates were deprotected with trifluoroacetic acid. The structures of biocytin and N^4-6-(biotinylamido)hexyllysine are illustrated in Fig. 2. The biotinylated peptides were characterized by elemental analysis, high performance liquid chromatography (results not shown), and amino acid analyses of their acid hydrolysates. Judged by these criteria, the analogs were homogeneous.

A comparison of the steroidogenic activity in bovine fasciculata cells of the two analogs with that of AII is illustrated in Fig. 3. Both analogs elicit essentially the same maximal stimulation as AII but when compared at the level of 50 percent stimulation are approximately 10 percent as active as AII.

We have determined the ability of the analogs to bind to succinoylavidin (succavidin) and have assessed the dissociation rate of the analog-suc-avidin complexes. The k^-1s^−1 values are 2.6 × 10^−7 for [biocytin^1] AII and 2.2 × 10^−7 for [N^4-6-
(biotinylamido)hexyllysine') AII. These values correspond to a $t_{1/2}$ of 30 and 36 days, respectively, and demonstrate that the analogs bind firmly to suc-avidin. The effect of suc-avidin on the steroidogenic activity of the analogs (Fig. 3) shows that while the [biocytin'] AII is significantly inhibited at a suc-avidin-analog ratio of 2:1, the activity of the analog embodying the aminohexyl spacer arm is not affected by suc-avidin even at a 5:1 ratio.

**DISCUSSION**

We are attempting the isolation of AII receptors from bovine fasciculata cells by the avidin-biotin technology, which we have developed and applied successfully to the isolation of highly purified insulin receptors from human placenta [19]. The avidin-biotin approach to the isolation of hormone receptors is based on the very strong non-covalent interaction ($K_d \approx 10^{-13}$M) between the egg-white protein avidin and biotin. The principle of the procedure is illustrated in Fig. 4. Two components—i.e., Sepharose-immobilized succinoyl avidin (I) and a biotinylated hormone (II)—are mixed to form the affinity column (III). It is of importance to eliminate, as much as possible, nonspecific interactions between the affinity resin and constituents in extracts containing solubilized receptors. We have observed that avidin, a basic protein, binds avidly and nonsaturably to rat liver plasma membranes, which constitutes an undesirable property. Extensive succinoylation of avidin to form succinoylavidin (suc-avidin) markedly reduces nonspecific interactions, and, as a consequence, we employ suc-avidin in our studies [14]. Succinoylation of avidin reduces the half time of dissociation of the avidin-biotin complex from 200 to 127 days [20].

A series of fundamental questions have to be answered prior to attempting the actual isolation of receptors by the avidin-biotin approach. These are (1) Is it possible to prepare biotinylated analogs of the hormone that exhibit high specificity and affinity for the receptor? (2) Do such analogs bind to suc-avidin, and what is the dissociation rate of the biotinylhormone-suc-avidin complexes? (3) Can the analogs bind simultaneously to suc-avidin and AII receptors? Studies designed to answer these questions are discussed below.

As can be seen in Fig. 3, [biocytin'] angiotensin II and [N'-6-(biotinylamido)hexyl-
lysine\(^1\) angiotensin II stimulate corticoid biosynthesis of fasciculata cells and thus are capable of binding to cell surface receptors. The dissociation rates of the suc-avidin complexes of the two analogs demonstrate that they bind very strongly to suc-avidin. The structural differences between the analogs are not reflected in the dissociation behavior of their complexes with suc-avidin.

From the point of view of affinity chromatography, it is important to assess the ability of the analogs to bind simultaneously to both the AII receptors and to suc-avidin. Once we establish that stable complexes are formed between the biotin-containing analogs and suc-avidin, we measure the ability of the analogs to stimulate steroidogenesis in the presence of an excess of suc-avidin. Since such experiments are performed in the presence of an excess of suc-avidin, it is assumed that under these conditions the assay solutions do not contain free analog: i.e., analog that is not non-covalently attached to suc-avidin. As can be seen in Fig. 3, the addition of suc-avidin to the assay solutions exerts a different effect on the steroidogenic response of the two analogs. At a molar ratio of 2:1 of suc-avidin to [biocytin\(^1\)] AII, steroidogenesis is lowered to 1 percent that of the AII control, but under the same conditions, the activity of \([N'-6-(\text{biotinylamido})\text{hexyllysine}^1]\) AII is not decreased. Increasing the suc-avidin concentration to a molar ratio of 5:1 does not potentiate the inhibition with either analog, indicating that no free ligand is present in the assay mixture at the 2:1 ratio. Since suc-avidin contains four biotin binding sites per mol, the mol ratios, in terms of binding sites, are actually 8 and 20:1, respectively. These results show that both analogs have the ability to interact simultaneously with suc-avidin and the AII cell receptors. We interpret the difference in the behavior of the two analogs in steric terms. The greater distance between the cell surface receptors and the suc-avidin in \([N'^-6-(\text{biotinylamido})\text{hexyllysine}^1]\) angiotensin II eliminates the steric impediment that appears to exist with [biocytin\(^1\)] angiotensin II. We have observed similar effects with biotinylated insulins [21]. Steroidogenesis induced by underivatized AII is not inhibited by suc-avidin, in agreement with observations with insulin and ACTH, where the bioeffect of the underivatized hormones was likewise unaffected by suc-avidin.

On the basis of the above observations, the construction of an avidin-biotin based affinity resin for AII receptor isolation (Fig. 4) simply involves mixing of immobilized suc-avidin [19] (I) with the \([N'^-6-(\text{biotinylamido})\text{hexyllysine}^1]\) AII (II), resulting in the non-covalent attachment of the ligand to the biotin binding sites on the suc-avidin. The observation that \([N'^-6-(\text{biotinylamido})\text{hexyllysine}^1]\) AII retains biological activity in the presence of an excess of suc-avidin (Fig. 3) suggests that affinity resins in which this ligand is attached to immobilized suc-avidin will bind solubilized AII receptors. The availability of functional, soluble angiotensin receptors from adrenal glomerulosa cells [11] and of AII specific affinity columns provides the tools for experiments
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FIG. 5. Application of the avidin-biotin system to the isolation of hormone receptors. Dashed lines represent non-covalent bonds.

designed to isolate angiotensin II receptors. Feasible isolation protocols are illustrated in Fig. 5. In method A, soluble receptor (R) is percolated through an affinity column of the type described to form the complex shown in the center of Fig. 5. The column is then exhaustively washed to remove contaminating materials. Alternatively, the ligand [N'-6-(biotinylamido)hexyllysine] AII can be added to a solution of solubilized receptor to form the soluble complex BHR. Percolating a solution containing this complex through a column of immobilized suc-avidin as in B will result in the formation of the same complex obtained by method A. Both these schemes have been used for the isolation of insulin receptors from human placenta [19; unpublished results]. Removal of functional, purified insulin receptor from affinity resins was achieved by eluting the column with pH 5.0 acetate buffer containing 1 M sodium chloride [22]. The replacement of biotin in the ligand BHR by a biotin analog such as dethiobiotin that exhibits weaker affinity for suc-avidin than biotin provides a route to the isolation of receptor-ligand complexes as illustrated in D. Such an approach would permit dissociation of the receptor-ligand complex from the column by 20 mM biotin which will occupy the open sites on the suc-avidin.

The development of affinity resins by the avidin-biotin approach offers the following advantages:

1. Attachment of biotin to the hormone is targeted and, thus, provides a uniform site for receptor binding.
2. Hormone ligands are prepared by simple, well-defined solution methods which can be monitored accurately.
3. Formation of the affinity resin is highly specific (avidin-biotin interaction) and is achieved by simply mixing immobilized avidin derivatives with the ligand.
4. The amount of ligand on the column can be varied at will to achieve optimal operation, since the interaction of the components is quantitative.
5. The technique can be readily scaled up for production of larger quantities of receptors.
6. Once the technique is worked out for a particular situation, it is highly reproducible.
7. In theory, the technique is applicable to any receptor whose affector can be biotinylated without destruction of biological function.

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