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Chemical characterization of ligand binding site fragments from turkey β-adrenergic receptor

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Affinity-labeled β-adrenergic receptor from turkey erythrocyte membranes was specifically cleaved near cysteine residues after *S*-cyanylation. Analysis of the labeled polypeptide fragments suggests that iodocyanopindolol diazirine reacted with an amino acid residue which is located in the non-glycosylated region containing the sixth and seventh transmembrane domains of the receptor. However, the possibility cannot be excluded that a second residue, located between the third and fifth transmembrane domains, was also labeled. Since treatment with either hydroxylamine or triethylamine resulted in removal of the affinity label from the protein, the present study suggests that aspartic or glutamic acid residues are present in the adrenergic-binding site which is located in the above-mentioned domains. The procedure for specific chemical cleavage of the affinity-labeled adrenergic receptor should also be useful for future structural and comparative studies of other adrenergic receptors.

Adrenergic receptor, β-; Affinity labeling; Cysteine cleavage; Receptor-binding site

1. INTRODUCTION

Among the numerous proteins belonging to the family of membrane receptors coupled to proteins which bind GTP, the β-adrenergic receptors have been subjected to the most thorough investigation of both their physiological and structural properties (review [1]). The turkey β-adrenergic receptor (tβAR), isolated from erythrocyte plasma membranes, was one of the first receptors of this type for which the amino acid sequence was determined [2]. Although a putative model of its folding in the membrane was deduced from sequence homology and resemblance to the light receptor rhodopsin, the exact nature and location of the catecholamine-binding site require further investigation. Recent studies employing site-directed mutagenesis [1,3,4], selective proteolysis [5], affinity labeling [6] and other methods [1] demonstrated that the binding site comprises residues belonging to several of the seven transmembrane domains and established the importance of some amino acids, such as aspartates located in these domains, for ligand binding.

Here, we have used photoaffinity labeling together with chemical and enzymatic approaches to characterize further the location and chemical nature of the residues which are involved in the ligand-binding site of tβAR. The procedure for specific chemical cleavage of the affinity-labeled adrenergic receptor described here should be useful for future structural and comparative studies of other adrenergic receptors.

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Abbreviations: tβAR, turkey β-adrenergic receptor; ICD, [^{125}I]iodocyanopindolol diazirine; buffer A, 0.2 M Tris-HCl (pH 8) containing 1 mM EDTA; NTCB, 2-nitro-5-thiocyanobenzoic acid

2. EXPERIMENTAL

2.1. *Affinity labeling of tβAR and gel electrophoresis*

Turkey erythrocyte plasma membranes were prepared as described [7]. Affinity labeling of the membranes with

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[^{125}I]iodocyanopindolol diazirine (ICD) [8] was performed as reported [9] except that the ICD concentration was 100 pM, and the labeled membranes were stored at −70°C at a protein concentration of 6 mg/ml. Electrophoresis on 15% polyacrylamide gels in the presence of SDS and autoradiography were performed as in [9]. All procedures described in this and subsequent sections were carried out at 0°C unless otherwise indicated.

2.2. *S-cyanylation of affinity-labeled membranes*

Affinity-labeled membranes (100 μl) were suspended in 10 mM Tris-HCl (pH 8) and centrifuged at 20000 × *g* for 20 min. The pellet was suspended in 100 μl of 0.2 M Tris-HCl and 1 mM EDTA (pH 8, buffer A), and distributed equally into five tubes (samples a–e). Following incubation of (a) and (b) at 37°C for 2 h, all tubes were recentrifuged as above. Samples (a)–(d) were each suspended in 40 μl buffer A containing 8 M urea (BRL, ultra pure grade), 10 mM dithiothreitol and 1% SDS, and incubated at 37°C for 1 h; sample (e) was resuspended in buffer A (40 μl). 2 μl of 1 M 2-nitro-5-thiocyanobenzoic acid (NTCB, Fluka) [10] in ethanol was then added to samples (a), (c) and (e). After incubation of all samples at 37°C for 30 min, the pH was raised to 9 by addition of 3 M Tris base (~3.5 μl), followed by further incubation at 37°C for 20 h. Electrophoresis sample buffer (5 ×) was then added and electrophoresis and autoradiography were performed as described.

2.3. *Enzymatic treatments with* Staphylococcus aureus *V8 protease and endoglycosidase F*

Affinity-labeled membranes (200 μl) were treated with NTCB as described above [section 2.2, sample (c)]. The resulting reaction mixture was applied to two wells (100 μl in each) of a polyacrylamide gel. Following electrophoresis, the gel was autoradiographed at −70°C for 15 h and areas on the gel corresponding to labeled polypeptide fragments (see section 3.2) were excised from the two equivalent lanes. The individual fragments of one lane were treated with *S. aureus* V8 protease [11], run in parallel with the untreated fragments on a second gel and autoradiographed as described.

NTCB-treated membranes [section 2.2, sample (c)] and affinity-labeled membranes (100 μl of each) were separately dialyzed against 0.1 M NaH_2PO_4 (pH 6.1) containing 50 mM EDTA, 0.1% SDS and 0.1% β-mercaptoethanol. Nonidet P-40 (Sigma, 20% in water) was then added to give a final concentration of 1% (final volume 140 μl). Endoglycosidase F (NEN, 700 U/ml, 0.5 μl) was added to half the volume of each sample, and the four samples were incubated at 37°C for 15 h. After addition of electrophoresis sample buffer (5 ×) the samples were subjected to electrophoresis and autoradiography.

2.4. *Reaction with hydroxylamine and triethylamine*

Affinity-labeled membranes (100 μl) were suspended in 10 mM Tris-HCl (pH 8) and centrifuged at 20000 × *g* for 20 min. The pellet was suspended in 100 μl buffer A containing 8 M urea and 1% SDS, incubated at 37°C for 1 h and divided equally into five aliquots. Each aliquot of the solubilized labeled membranes was mixed with 20 μl of: (a) 2 M hydroxylamine hydrochloride in 0.2 M Na_2CO_3, 1% SDS and 8 M urea (pH 8); (b) same as (a) but without hydroxylamine hydrochloride; (c) buffer A saturated with triethylamine at 23°C; (d) buffer A titrated with NaOH to pH 12; (e) buffer A. After all samples were incubated at 37°C for 1 h, they were each mixed with 10 μl electrophoresis sample buffer (5 ×) and subjected to gel electrophoresis and autoradiography.

3. RESULTS AND DISCUSSION

3.1. *Affinity labeling of tβAR*

Affinity labeling of turkey erythrocyte membranes with ICD yielded two specifically labeled polypeptides of 42 and 50 kDa (fig.1, lane 1) corresponding to the tβAR [12]. When the affinity-labeled membranes were incubated at 37°C for 1 h, prior to their solubilization in the gel electrophoresis sample buffer, only the 42 kDa band was obtained (fig.1, lane 2); this polypeptide has been shown to represent a proteolytic degradation product of the 50 kDa polypeptide, lacking the glycosylated N-terminal segment of the receptor [12].

3.2. *Cleavage of tβAR by NTCB*

Following cleavage of the solubilized affinity-labeled tβAR by NTCB, three labeled polypeptide fragments of 18, 23 and 28 kDa (F18, F23 and F28, respectively) were revealed after gel electrophoresis and autoradiography (fig.1, lane 3). Complete cleavage of the receptor was achieved when both modification and cleavage were carried out in the presence of 1% SDS, 8 M urea and 10 mM dithiothreitol; no further cleavage into smaller fragments could be observed when higher concentrations of the reagents or longer reaction times were employed. Incomplete cleavage of the labeled receptor resulted when urea, or reduction with dithiothreitol prior to reaction with NTCB, were omitted (not shown). Cleavage of the solubilized 42 kDa affinity-labeled polypeptide (see section 3.1) by NTCB resulted in three labeled fragments (18, 23 and 28 kDa) identical to those obtained after cleavage of the affinity-labeled tβAR (fig.1, lane 4), suggesting that these fragments lack the N-terminal region of the receptor.

As shown in fig.1 (lane 5), two additional labeled fragments were obtained (F21 and F37 corresponding to 21 and 37 kDa) when intact affinity-labeled turkey membranes were treated with NTCB in the absence of SDS and urea.

Previous attempts to cleave specifically the affinity-labeled receptor by other chemical means, such as treatment with CNBr, resulted in cleavage

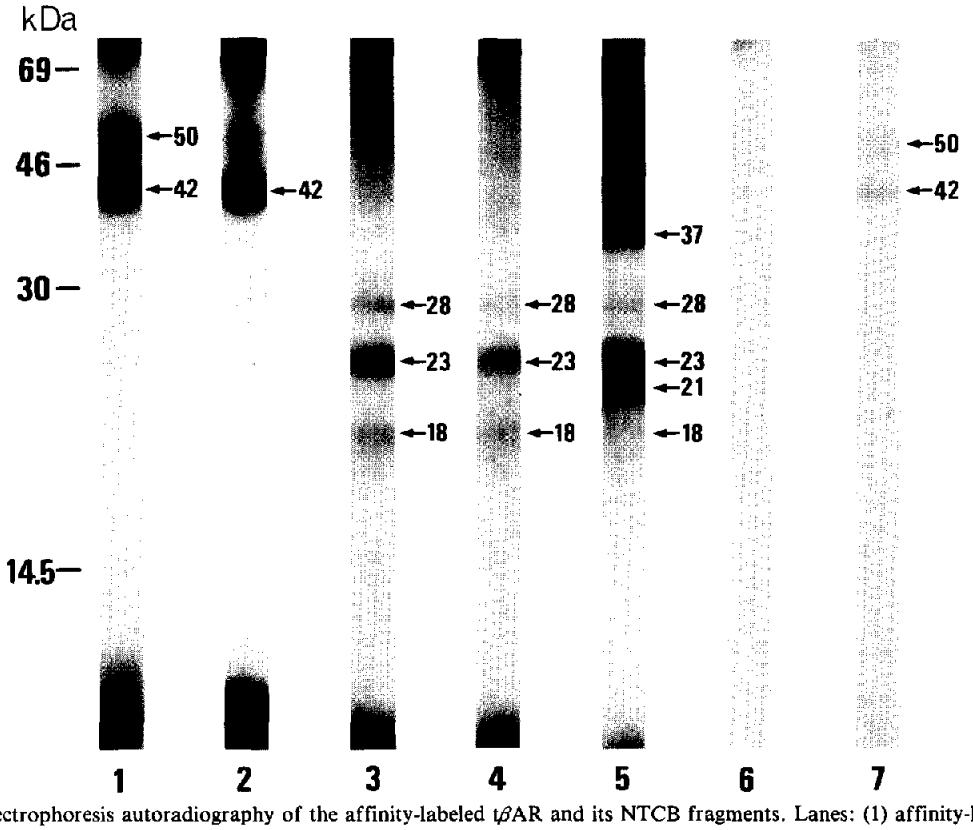

Fig.1. Gel electrophoresis autoradiography of the affinity-labeled tβAR and its NTCB fragments. Lanes: (1) affinity-labeled turkey erythrocyte membranes; (2) as lane 1 but after incubation at 37°C for 1 h prior to electrophoresis; (3) solubilized affinity-labeled membranes treated with NTCB; (4) NTCB treatment of solubilized labeled membranes after incubation at 37°C for 1 h; (5) NTCB treatment of intact labeled membranes; (6,7) labeled membranes reacted with triethylamine or hydroxylamine, respectively; left lane, molecular mass markers. Arrows indicate polypeptides of interest mentioned in section 3; for experimental details see text.

and loss of the label due to the reaction conditions (unpublished and [6]). The mild conditions used in our procedure did not lead to removal of the label from the protein, and enabled specific cleavage by NTCB to yield labeled fragments that could be further analyzed and characterized.

3.3. *Enzymatic treatments of NTCB-labeled fragments*

Incubation of the affinity-labeled 50 kDa receptor with endoglycosidase F resulted in a change in its electrophoretic migration corresponding to a molecular mass of 45 kDa (not shown), as described before [12], indicating that the carbohydrate moiety located in the N-terminal region [2,7] of the affinity-labeled tβAR was removed by the glycosidase. Upon treatment of the NTCB-labeled fragments (F18, F23, F28) with endoglycosidase F, an electrophoretic pattern identical to that of the untreated fragments (fig.1, lane 3) was observed. This finding supports our previous conclusion (see section 3.2) that these fragments originated from the non-glycosylated region of the affinity-labeled receptor.

When labeled fragments F18, F23 and F28 were subjected, separately or as a mixture, to treatment with *S. aureus* V8 protease, all three yielded a polypeptide with an identical molecular mass of 17 kDa (fig.2, lanes 4–6). A polypeptide of 17 kDa was also the main product obtained when the solubilized affinity-labeled receptor was subjected to digestion by this protease (not shown).

3.4. *Localization of the labeled fragments in the receptor polypeptide chain*

Reaction of the affinity-labeled receptor with NTCB under the various conditions used did not apparently result in cleavage of all 19 cysteine

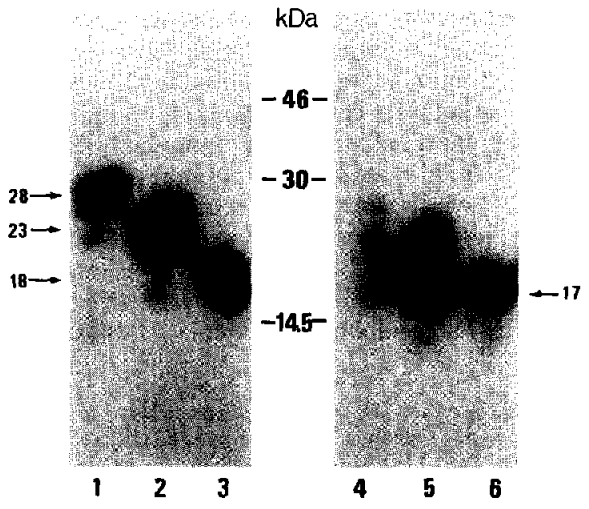

Fig.2. Gel electrophoresis autoradiography of affinity-labeled NTCB fragments of affinity-labeled tβAR treated with *S. aureus* V8 protease according to [11]. Lanes: (1–3) NTCB fragments of 28, 23 and 18 kDa, respectively, separated previously by gel electrophoresis; (4–6) same fragments as in lanes 1–3, treated with the V8 protease; middle lane, molecular mass markers. Arrows denote fragments of interest discussed in the text.

residues located along the polypeptide chain [2]; indeed, the smallest labeled polypeptide observed after cleavage was still twice the size (15 kDa) of the longest segment between two neighbouring cysteine residues present in the protein (Cys^{10}–Cys^{85}). It has been previously established that integral membrane proteins, such as the human erythrocyte band 3 [13–16], are not susceptible to cleavage by NTCB in 1% SDS at cysteine residues located in hydrophobic domains (usually embedded in the lipid bilayer). Based on this knowledge, we assumed that the cysteine residues present in the transmembrane domains of the tβAR [2] were not cleaved by NTCB. Since F18, F23 and F28 do not contain the carbohydrate moiety of tβAR, they should be located between Cys^{114} and the C-terminal end. The 17 kDa labeled polypeptide resulting from cleavage of these fragments by *S. aureus* V8 is suggested to be common to all of them. Analysis of the possible overlap between the molecular masses of the NTCB fragments and those of polypeptides located between possible cleavage sites in the primary structure of the receptor led to the localization of these affinity-labeled fragments proposed in fig.3A.

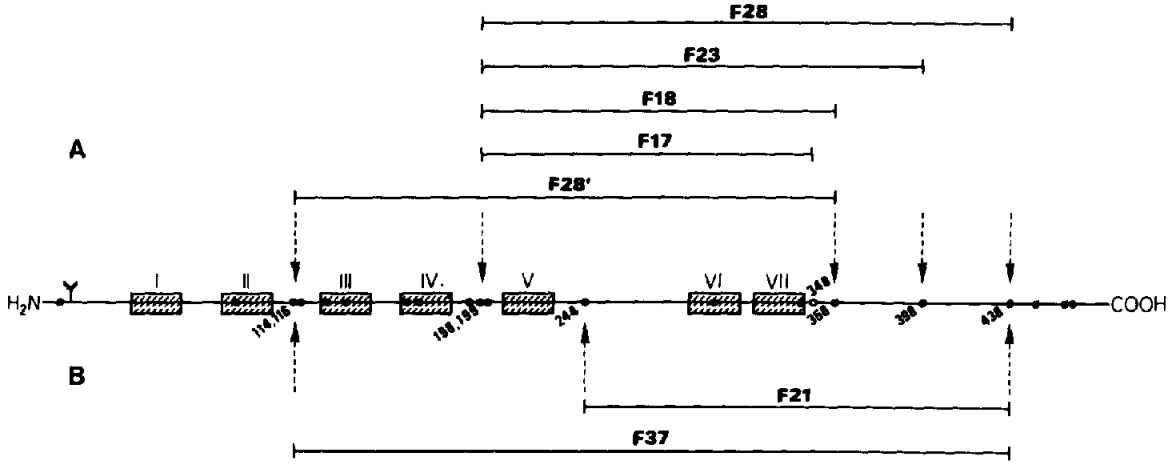

Fig.3. Schematic representation of the localization of the NTCB affinity-labeled fragments in the primary structure of tβAR. (A,B) Fragments obtained from cleavage of solubilized or intact affinity-labeled membranes, respectively: F18, F24 and F28 are proposed to have Cys^{198} (or Cys^{199}) as their N-terminal and Leu^{357}, Asp^{397} and Tyr^{437} as their respective C-terminals, based on their molecular mass and those of the domains between the cysteine residues in the primary structure. However, it is possible that F28 is also located between Cys^{114} (or Cys^{116}) and Leu^{357} (designated F28'). This alignment supports the suggestion of a 17 kDa polypeptide common to these fragments (F17, between Cys^{198}-Phe^{201} and Asp^{348}), obtained after their cleavage by V8 protease. F37 and F21, obtained following NTCB cleavage of intact labeled membranes, are proposed to have Tyr^{437} as their C-terminal and Cys^{114} (or Cys^{116}) and Cys^{244} as their respective N-terminals. Arrows indicate proposed cleavage sites of cysteine residues; numbers indicate the position in the primary structure; I-VII represent the transmembrane domains of the receptor [2]; (●) Cys residues; (○) Asp residue; (Y) carbohydrate moiety.

The observation that cleavage by NTCB of solubilized or intact affinity-labeled membranes resulted in more than one labeled fragment in each case (fig.1, lanes 3,5) implies incomplete modification or cleavage due to side reactions such as β-elimination [10]. It is worth noting that the cytoplasmic Cys^{244} probably reacted with NTCB in the intact membranes to yield F21 (fig.3B), but not in membranes solubilized in SDS. The proposed alignment of all the fragments obtained (fig.3) clearly suggests that the affinity label is attached to the receptor in the region located between Cys^{244} and Asp^{348}, containing part of the third cytoplasmic loop and the sixth and seventh transmembrane domains.

As shown in fig.3A, F28 could also be matched with the domain located between Cys^{114} and Leu^{357}. We have observed that when cleavage with NTCB was performed in the presence of 10 mM dithiothreitol on intact membranes which were affinity labeled with higher concentrations of ICD (500 pM), a labeled fragment of 15 kDa which can overlap with the sequence between Cys^{114} and Arg^{243} was also observed (not shown). Therefore, the possibility that the affinity label can also react, under certain conditions, with an amino acid located between the third and fifth transmembrane regions cannot be excluded.

3.5. *Cleavage with hydroxylamine and triethylamine*

Reaction of the solubilized affinity-labeled tβAR, or of the NTCB-treated receptor, with either hydroxylamine or triethylamine, resulted in almost complete (>95%) removal of the label from both the intact receptor (fig.1, lanes 6,7) and its NTCB fragments. In the control experiment, neither the labeled receptor nor the labeled fragments revealed any loss of radioactivity or change in their mobility, when incubated under the same conditions but in the absence of hydroxylamine or triethylamine. These results imply that the affinity label ICD is covalently bound to the turkey adrenergic receptor via an ester bond, i.e. to the carboxylate side chain of either aspartic or glutamic acid residues which reside in the binding site.

3.6. *Concluding remarks*

The results described here suggest that the affinity labeling with ICD occurs on an aspartic or glutamic acid residue located in or near the sixth or seventh transmembrane domain of tβAR. An additional carboxylate is possibly labeled in the region between the third and fifth transmembrane domains. These results are in support of a recent study [6] which demonstrated that Trp^{330}, located in the seventh transmembrane domain, and an unidentified residue, located between the third and fifth transmembrane domains, were affinity labeled by iodoazidobenzylpindolol.

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