Purification and properties of active atrial-natriuretic-peptide receptor (type C) from bovine lung

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Atrial-natriuretic-peptide (ANP) receptor, previously identified as a 140 kDa protein with a disulphide-linked homodimeric structure, was purified from bovine lung by $(\text{NH}_4)_2\text{SO}_4$ fractionation and affinity chromatography on ANP–Affi-Gel 10. The purified receptor had a binding capacity of 4.2 nmol of ANP/mg of protein and an affinity constant of 6.5 pM. The isoelectric point of the receptor was 5.8, consistent with the acidic nature of the protein (amino acid analysis revealed a predominance of glutamic acid and aspartic acid residues). Treatment with endoglycosidase H and glycopeptidase F revealed that the receptor has three complex types of oligosaccharide chains per 70 kDa subunit. Deglycosylation of the receptor did not affect its binding activity. Reduction with dithiothreitol and reoxidation by dialysis revealed a strong tendency of the receptor subunits to dimerize via disulphide cross-linking; however, carboxymethylation of the reduced receptor indicated that the intersubunit disulphide bond is not necessary for the ligand-binding activity.

INTRODUCTION

Atrial natriuretic peptide (ANP) represents a novel cardiac endocrine system regulating blood pressure and water and electrolyte balance. In response to altered fluid and volume status, it is released from the atria and exerts actions on many key organs of the circulatory system such as the kidney, adrenal gland and vasculature to produce natriuresis, suppression of aldosterone secretion and vasorelaxation [1–6]. To understand the molecular mechanism of ANP action, it is essential to characterize the ANP receptor that initiates the cascade of cellular responses when it interacts with ANP, leading to a fall in blood pressure and extracellular fluid volume.

There is strong evidence indicating that the ANP receptor is heterogeneous. Pharmacological and biochemical studies have indicated the presence of two classes of receptor subtype which differ markedly in their subunit structures: one is a 140 kDa homodimer comprised of two identical 70 kDa subunits [7–10] and the other is comprised of a single polypeptide chain of 135 kDa [11–13]. The former dimeric form is further divided into two subclasses (type I and type II) [14]. The latter single-chain receptor is unique in that it has both ANP-binding and guanylate cyclase activities within the same molecule. These structural features deduced from biochemical data have been confirmed by cell-free translation of receptor mRNA [15] and by cloning, sequencing and expression of cDNAs for both types [16–18]. They are also supposed to differ functionally. Since the ANP-binding site of the single-chain receptor is covalently linked to the cytosolic guanylate cyclase domain [17], it is considered to be a biologically relevant receptor (B-

type receptor) [19], mediating ANP-induced accumulation of intracellular cyclic GMP [11–13]. The major physiological role of the dimer-type receptor is supposed to mediate the sequestration and metabolic clearance of ANP and is therefore termed 'C-type receptor' to denote its role in the clearance of ANP [18,19]. However, it is not known whether the clearance of ANP is the sole function of the dimer-type receptor or if it is also involved in signal transduction. In any case, these multiple receptors may be used to generate a greater diversity of ANP responses than could be achieved with a single subtype as commonly seen in other signalling systems. Our efforts have been focused on the study of the dimeric form of ANP receptor, since (i) it is the major form in many target tissues, and (ii) we have recently obtained evidence that it is also coupled to the effector enzyme guanylate cyclase [20].

In a previous report [9] we showed that bovine lung is a relatively rich source of type I dimeric ANP receptor and suitable for use in receptor purification. In the present study we purified active ANP receptor (dimer type or type C) from bovine lung plasma membranes and characterized it in terms of stability, ion requirement, ligand specificity, isoelectric point, effects of deglycosylation and carboxymethylation on the ligand-binding activity and stoichiometry of ANP binding. The results indicated that, although the native receptor has a disulphide-linked homodimeric structure, the disulphide bridge is not necessary for the binding activity, nor are the carbohydrate residues required. The receptor was resistant to mild acid treatment, freezing and thawing, and freeze-drying. In the absence of univalent ions, however, the receptor lost its ANP-binding activity.

Abbreviations used: ANP, atrial natriuretic peptide; PBS, phosphate-buffered saline (20 mM-sodium phosphate/0.12 M-NaCl, pH 7.4); PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis.

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EXPERIMENTAL

Materials

Fresh bovine lungs were obtained from Shibaurazoki Co., Tokyo, Japan. Rat ANP-(99–126)-peptide, pepstatin A and leupeptin were purchased from Peptide Institute, Osaka, Japan (the numbering of amino acids follows the numbering system of rat pro-ANP [21]); atriopeptin I [ANP-(103–123)-peptide] and III [ANP-(103–126)-peptide] and phenylmethanesulphonyl fluoride (PMSF) were from Sigma, St. Louis, MO, U.S.A.; ANP-(111–126)-peptide was from Cambridge Research Biochemicals, New York, NY, U.S.A.; endoglycosidase H, glycopeptidase F and neuraminidase were from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Affi-Gel 10, Bio-Gel P-6DG and Econo-Columns were purchased from Bio-Rad, Richmond, CA, U.S.A.; Pharmalyte pH 3–10 and Superose 12 were from Pharmacia, Uppsala, Sweden. BCA protein-assay reagents were obtained from Pierce Chemical Company, Rockford, IL, U.S.A.; silver-stain kits were from Kanto Chemical Co., Tokyo, Japan. ^{125}I -ANP (2000 Ci/mmol) was from Amersham, International, Bucks., U.K. *N*-Terminal-truncated ANP-(101–126)-peptide was synthesized by a solid-phase peptide synthesizer and purified by gel-filtration, cation-exchange and partition chromatography. All other reagents were of analytical grade.

Purification of active ANP receptor (dimer type or type C) from bovine lung plasma membranes

ANP receptor was purified by a modification of our previous method [9]. In the modified procedure, elution from the affinity column was achieved with acetic acid instead of SDS to obtain the active receptor as detailed below. Preparation of bovine lung plasma membranes, extraction with 0.5% (w/v) Triton X-100, fractionation with $(\text{NH}_4)_2\text{SO}_4$ [25–70% (w/v) satn.] and dialysis against phosphate-buffered saline (PBS) were carried out as previously described [9]. The diffusate (1.45 litres) was loaded on to an ANP affinity column (1 cm $\times$ 3 cm) prepared by coupling ANP-(101–126)-peptide to Affi-Gel 10 [9]. The flow rate was 30 ml/h. After washing the column with 100 ml each of (i) PBS containing 0.2% Triton X-100, (ii) 10 mM-Hepes, pH 7.4, containing 5 mM-EDTA, 0.5 M-NaCl and 0.5% Triton X-100, and (iii) 10 mM-Hepes, pH 7.4, containing 5 mM-EDTA, 0.1 M-NaCl and 0.1% CHAPS, the column was eluted with 0.1 M-acetate, pH 5.0, containing 0.1 M-NaCl and 0.2% Lubrol at a flow rate of 1.5 ml/h, and 3 ml fractions were collected. The first two fractions containing ANP-binding activity were used for characterization, either directly or after neutralization with 2 M-Tris/HCl, pH 7.5. All steps of purification were carried out at 4 °C in the presence of 5 mM-EDTA, 0.5 mM-PMSF, leupeptin (5 $\mu\text{g}/\text{ml}$) and pepstatin (5 $\mu\text{g}/\text{ml}$).

^{125}I -ANP binding assays

Samples containing ANP receptor were diluted with assay buffer (PBS containing 0.2% Triton X-100 and the proteinase inhibitors) and aliquots (100 μl) were incubated with 5 fmol (10 μl) of ^{125}I -ANP at 30 °C. After a 30 min incubation, bound and free radioligands were separated by gel filtration on a series of Bio-Rad Econo-Columns (0.7 cm $\times$ 10 cm) packed with 4 ml of Bio-Gel P-6DG. The columns were developed with the assay

buffer, and flow-through fractions (1.8 ml) containing the ^{125}I -ANP-receptor complex were counted for radioactivity. Specific binding was defined as the difference between the amount of radioligand bound in the absence and in the presence of 1 μM unlabelled ANP; non-specific binding was less than 5%.

To determine the affinities of various ANP analogues, competitive binding assay was used, in which purified ANP receptor (diluted 1:20000 with the assay buffer) was incubated with ^{125}I -ANP in the presence of different concentrations of unlabelled ANP analogues. Subsequent steps for determining the specific binding were the same as above.

For experiments requiring pretreatment of receptor such as examination of pH stability and effects of chemical modifications on its binding stability, the purified ANP receptor was treated with various buffers, enzymes or reagents (details are given below) and assayed for its ^{125}I -ANP-binding activity as described above.

Enzymic deglycosylation

Purified ANP receptor was denatured by heating for 10 min in the presence of 0.05% (w/v) SDS. The denatured receptor (5 μg) was then treated with (i) 40 munits of endoglycosidase H in 100 μl of 60 mM-sodium acetate, pH 5.0, containing 1 mM-PMSF, 0.025% SDS, 0.5% (v/v) 2-mercaptoethanol and 0.35% Triton X-100 or (ii) 5 units of glycopeptidase F in 50 mM-phosphate buffer, pH 7.4, containing 5 mM-EDTA, 10 mM-2-mercaptoethanol and 0.1% SDS for 16 h at 37 °C. Partial digestion was achieved by treating non-denatured receptor with 1 unit of glycopeptidase F in the absence of 2-mercaptoethanol and SDS. The reaction was terminated by adding the sample buffer for SDS/polyacrylamide-gel electrophoresis (SDS/PAGE).

Deglycosylated receptors retaining ligand-binding activity were prepared by treating purified ANP receptor (5 μg) with 5 units of glycopeptidase F in 50 mM-phosphate buffer, pH 7.4, containing 5 mM-EDTA and 0.2% Lubrol, or 50 munits of neuraminidase in 0.1 M-sodium acetate, pH 5.0, containing 0.1 M-NaCl, 5 mM-EDTA and 0.2% Lubrol at 37 °C for 16 h, and used for assessing the contribution of sugar residues to the ^{125}I -ANP-binding activity and pI.

Carboxymethylation

Disulphide bonds were first cleaved by treating 150 ng of the purified receptor with 5 mM-dithiothreitol in 100 μl of 20 mM-Tris/HCl, pH 7.4, containing 10 mM-EDTA, 120 mM-NaCl and 0.2% Triton X-100 at room temperature for 1 h. The reduced receptor was then carboxymethylated with 10 mM-iodoacetamide. At the end of a 40 min incubation at room temperature, excess reagents were removed by dialysis against PBS containing 0.2% Triton X-100.

Isoelectric focusing

Focusing was done with an LKB 8100 column (110 ml) according to the manufacturer's instructions. The column was filled with (i) dense cathode solution [1.7% (w/v) phosphoric acid in 50% (w/v) sucrose], (ii) focusing solution containing 1% CHAPS and 1% (w/v) Pharmalyte (pH 3–10) in a linear 50–0% sucrose gradient and (iii) light anode solution [2% (w/v) NaOH]. After electrofocusing at 4 °C for 18 h at 1000 V, 1 ml fractions were collected and the pH gradient was

measured. Receptor activity in each fraction was determined by the ^{125}I -ANP-binding assay after dilution with 0.2 M-Tris/HCl, pH 7.4, containing 0.2% Triton X-100.

Amino acid analysis and sequencing

For the determination of amino acid composition, the purified receptor (20 μg) was dialysed overnight against water, freeze-dried and hydrolysed in 6 M-HCl containing 0.1% phenol and 0.01% dithiothreitol at 110 °C for 24 h. Analysis was performed by Dr. Jun Kondo at Mitsubishi Chemical Industries Limited Research Center (Yokohama, Japan) using an automatic amino acid analyser.

Purified receptor was first cleaved with CNBr in 70% (v/v) formic acid at 30 °C for 16 h. After freeze-drying, the reaction products were dissolved in 0.2 M- NH_4HCO_3 and left at 40 °C for 24 h. The CNBr-cleaved fragments were precipitated with acetone/triethylamine (99:1, v/v) and further digested with a lysyl endopeptidase (*Achromobacter lyticus* protease I). Conditions for h.p.l.c. to separate the resulting peptide fragments are described in the legend to Fig. 8 (below). Amino acid sequences of the peptide fragments were determined by using an Applied Biosystems gas-phase protein sequencer.

Protein assay and SDS/PAGE

Protein concentration was determined using a Pierce BCA or a micro BCA protein assay kit, which consists of a CuSO_4 solution and a solution of the copper (I)-chelating reagent bicinchoninic acid. Bovine serum albumin was used as standard.

SDS/PAGE was carried out under non-reducing and reducing conditions with the Laemmli buffer system [22]. Protein bands in the gel were revealed with a Kanto silver-stain kit.

RESULTS AND DISCUSSION

Purification of the dimer-type ANP receptor

Purification of bovine lung ANP receptor was carried out as described in the Experimental section. The methodology is essentially a modification of our previous procedure [9] and involves three steps: Triton extraction, $(\text{NH}_4)_2\text{SO}_4$ fractionation and affinity chromatography on ANP-Affi-Gel 10. A summary of the purification data from 5 kg of bovine lung is shown in Table 1.

Establishing suitable conditions to elute the bound receptors from the affinity column was a critical step for isolating the active receptor. To establish mild elution conditions, we examined the effect of acid treatment on the dissociation of the ^{125}I -ANP-receptor complex. As

Fig. 1. Acid-induced dissociation of receptor-ligand complex

The receptor-ligand complex was prepared by incubating ^{125}I -ANP (5 fmol, 20000 c.p.m.) and purified receptor (1 ng) in 10 μl of PBS, pH 7.4, containing 0.2% Triton X-100 at 30 °C for 30 min. Stability of the receptor-ligand complex at different pH values was examined by transferring the preformed complex into 90 μl of various buffered solutions. After 1 h incubation at 30 °C, the amounts of ^{125}I -ANP-receptor complexes remaining were determined by gel filtration as described in the Experimental section. The following buffers (0.1 M) containing 0.2% Triton X-100 were used: acetate (pH 4.5–5.5); Mes (pH 5.5–6.5); Hepes (pH 6.5–7.5); Tris (pH 7.5–8.5); and 2-(cyclohexylamino)ethanesulphonic acid (pH 8.5–10.0).

shown in Fig. 1, exposure to acid (< pH 6.0) caused rapid dissociation of the complex. As a result, acetate buffer (pH 5.0) was used to elute the bound receptors from the column to ensure high recovery (Fig. 2). An unusual property of the ANP receptor that we noticed at this step of purification was that the receptor itself is quite resistant to mild acid, and the ANP-binding activity of the acid eluate from the affinity column can be restored on neutralization, even after prolonged storage (5 weeks) at pH 5.0.

The purity of the ANP receptor was assessed by SDS/PAGE, and the result is shown in Fig. 2 (inset). The affinity-purified receptor gave a major band with an M_r of about 140000. Analysis of an overloaded sample demonstrated the presence of higher- M_r species in addition to the predominant 140 kDa band. We first hypothesized that the higher- M_r species were minor

Table 1. Summary of ANP-receptor purification from bovine lung plasma membranes

Step	Volume (ml)	Protein (mg)	^{125}I -ANP-binding activity (pmol/mg of protein)	Purification (fold)	Yield (%)
Triton extraction	20000	75700	0.066	1	100
$(\text{NH}_4)_2\text{SO}_4$ fractionation	1500	31000	0.114	1.7	71
Affinity chromatography	6	0.5	4200	64000	42

Fig. 2. Affinity chromatography and purity (inset) of ANP receptor

Bovine lung ANP receptor, partially purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation, was applied to an ANP-(101–126)-peptide–Affi-Gel 10 column and eluted with 0.1 M-acetate, pH 5.0, containing 0.1 M-NaCl and 0.2% Lubrol as described in the Experimental section. The inset shows SDS/PAGE profiles of affinity-purified ANP receptor. Amounts of samples loaded were: lanes a and b, 40 ng; lane c, 250 ng. Abbreviation: 2-ME, 2-mercaptoethanol. Molecular masses of ANP receptors were estimated by using the following molecular-mass standards (given in kDa); myosin (200), β -galactosidase (116), phosphorylase b (97), bovine serum albumin (68) and ovalbumin (46).

contaminating proteins and tried to remove them by gel filtration, ion-exchange chromatography and wheat-germ agglutinin–agarose chromatography. In spite of our efforts, no significant separation was obtained. The reason for this failure was later provided by immunoblot analysis, which revealed that the higher- M_r species were oligomers of the ANP receptor (type C). These results indicate that the ANP receptor has a strong tendency to aggregate.

Molecular architecture and stability

The purified receptor gave a band of 140 000 M_r under the non-reduced state in SDS/PAGE and of M_r 70 000 in the reduced state. This is consistent with our previous observation [9] and indicates that the bovine lung ANP receptor is a 140 kDa dimer composed of two disulphide-linked 70 kDa subunits of similar sequence. Lewicki *et al.* [10,16] have also shown, by analysing the structures of the affinity-purified receptor and its cloned cDNA, that the ANP receptor expressed on the cultured bovine aortic smooth-muscle cells has a similar dimeric structure.

The purified ANP receptor was very stable. It could be stored at -20°C with no substantial loss of binding activity for more than 6 months. The ANP-binding activity was not affected by repeated freezing and thawing. Storage of the purified receptor in freeze-dried-

Fig. 3. Effect of pH on the stability of the ANP receptor in the presence or absence of NaCl

Purified ANP receptor (30 ng) was exposed to acidic and alkaline conditions at 4°C for 16 h in the presence of various concentrations of NaCl. The buffers used were the same as those in Fig. 1, except their concentrations were 20 mM. Receptor solutions were then neutralized by dilution to 20-fold of its original volume with PBS, pH 7.4, containing 0.2% Triton X-100 and assayed for remaining ^{125}I -ANP-binding activity.

powder form at 4°C prolonged its stability for at least 1 year. In contrast with the stability under these conditions, the ligand-binding activity of the ANP receptor was found to be highly dependent on the ionic strength of the solvent. When stored in solutions with low salt concentrations, the receptor showed a drastic loss of binding activity, even at physiological pH values (Fig. 3). Taken together, the data presented in Figs. 3 and 1 suggest that the ANP receptor undergoes a pH-dependent conformational transition from an inactive to active state and that this process is dependent on the ionic strength (i.e. salt concentration) of the medium. It is therefore important to store the ANP receptor in neutral buffers containing 0.1 M or higher concentrations of salt or in mild acid (pH 5–6).

Pharmacological properties

Fig. 4 shows the ligand-binding specificity of the purified bovine lung ANP receptor established by competitive binding assay. ^{125}I -ANP binding was competitively inhibited by unlabelled ANP and its analogues, atriopeptins I and III, and atriopeptin I was relatively less active. The N-terminal-deleted fragment, ANP-(111–126)-peptide, reacted only weakly, and unrelated peptides, such as angiotensin II and substance P, were ineffective as competitors. The analogue specificity summarized in Fig. 4 is very similar to those reported for ANP-clearance receptors in cultured vascular endothelial [23] and smooth-muscle cells [24] and bovine adrenal cortex [13].

Recently, De Lean [25], Meloche *et al.* [26] and Heim *et al.* [27] have reported that the natriuretic drug amiloride potentiates the action of ANP by inducing a

Fig. 4. Ligand specificity of the bovine lung ANP receptor

Affinity-purified ANP receptor was characterized for its ability to bind ANP analogues by the competitive ^{125}I -ANP binding assay. Specific binding in the absence of unlabelled ligands was taken as 100%. Competitors used were ANP (●), ANP-(101–126)-peptide (◆), atriopeptin I (▲), atriopeptin III (▼) and ANP-(111–126)-peptide (■). Open symbols represent displacement curves obtained with ANP (○) and atriopeptin I (△) in the presence of 10^{-4} M-amiloride. The results show that amiloride has no direct effect on the binding.

shift of the dose–response curves to the left. We were therefore interested to know whether the effect of amiloride observed using crude membrane preparations could also be detected in the purified receptor preparation. As shown in Fig. 4, no modulating effect of amiloride on ligand binding was observed. This result is consistent with the suggestion by Meloche *et al.* [26] who proposed that, in the membranes, the ANP receptor exists as a complex with an amiloride-sensitive effector protein.

Carbohydrate chains

To determine the type and number of *N*-linked oligosaccharides attached to the ANP receptor, we carried out enzymic deglycosylation of the receptor using glycosidases with different specificities. The purified receptor was resistant to digestion with endoglycosidase H, which cleaves only high-mannose or hybrid structures, but does not cleave other complex structures. Partial digestion with glycopeptidase F (*N*-glycanase) yielded three staggered bands with lower M_r (Fig. 5, lane c) than the intact receptor (lane b) when analysed by SDS/PAGE under reducing conditions; similarly, non-reduced samples containing disulphide-linked homodimeric receptors gave six new bands at the positions corresponding to 110–130 kDa (results not shown) when compared with the untreated control (lane a), indicating that the products differ by one oligosaccharide chain each. Taken together, these results suggest that the ANP receptor contains three asparagine-linked complex-type carbohydrate chains per subunit. This finding agrees with the number of potential *N*-glycosylation sites predicted from cDNA sequence analysis [16].

Fig. 5. SDS/PAGE profiles of deglycosylated ANP receptor

Purified receptor was treated with endoglycosidases as described in the Experimental section and analysed by SDS/PAGE under non-reducing (a) and reducing (b–d) conditions. Lanes a and b, control; lane c, partial digestion with glycopeptidase F (GP-F); and lane d, complete digestion with glycopeptidase F. Molecular masses of standard proteins in kDa are: β -galactosidase (116), phosphorylase *b* (97), bovine serum albumin (68) and ovalbumin (46). Abbreviation: 2-ME, 2-mercaptoethanol.

Fig. 6. Effect of deglycosylation and carboxymethylation of the ANP receptor on its ligand-binding activity

Receptor activities of native (○, ●), deglycosylated (△, ▲) and carboxymethylated (□, ■) ANP receptor were examined by competitive binding assay. The amount of receptor contained in one assay tube was adjusted by Scatchard analysis. Open and closed symbols designate the competition curves obtained when ANP and atriopeptin I were used as competitors respectively.

We further examined whether deglycosylation affects the ANP-binding activity of the receptor by comparing the abilities of the native and deglycosylated (Fig. 5, lane d) receptors to bind ^{125}I -ANP. No significant difference was observed (Fig. 6), indicating that the sugar residues are not directly involved in the receptor activity. However, this result does not necessarily exclude the possibility that the carbohydrate chains play some roles in signal transduction, as in the case of the β_2 -adrenoceptor [28].

Amino acid composition, pI and partial sequence

The amino acid composition of the purified bovine lung ANP receptor, calculated on the basis of an M_r of 56000 for the deglycosylated subunit, was (single-letter amino acid symbolism): D, 43.1 (D+N=42); E, 58.3 (E+Q=55); S, 32.1 (35); G, 39.4 (39); H, 9.8 (15); R, 23.1 (35); T, 18.7 (22); A, 46.1 (46); P, 23.5 (15); Y, 15.1 (19); V, 29.7 (33); M, 12.1 (13); I, 19.4 (22); L, 42.9 (48); F, 21.8 (26); and K, 24.8 (21) [C and W were not determined]; for comparison, the predicted values from the cDNA sequence [16] are shown in parentheses. Noteworthy is the predominance of glutamic and aspartic acid residues in the receptor. The values agree well, within experimental error, with those predicted by cDNA analysis [16]. The isoelectric point of the receptor was 5.8 (Fig. 7).

Partial amino acid sequence of the purified receptor was determined by sequencing the peptide fragments generated by cleavage with CNBr and *Achromobacter lyticus* proteinase I. Fig. 8 shows the h.p.l.c. separation of the peptide fragment (a) and the corresponding sequences of well-separated peaks (b). The amino acid sequences determined are in complete agreement with that predicted for the homodimeric ANP receptor by Fuller *et al.* [16] from the cDNA cloned from cultured bovine aortic smooth-muscle cells. This result is consistent with our recent finding that bovine lung and cultured vascular cells have the same type of ANP receptor [14].

Fig. 7. Isoelectric focusing of native (●) and neuraminidase-treated (○) ANP receptor

Samples were prepared and electrofocused as described in the Experimental section to determine their isoelectric points. Fractions containing the receptor were identified by the ^{125}I -ANP binding assay.

Fig. 8. Reverse-phase h.p.l.c. of peptide fragments of ANP receptor (a) and amino acid sequences determined (b)

Purified receptor was reduced and resulting 70 kDa receptor subunits were fragmented with CNBr and *A. lyticus* proteinase I and separated on a Bakerbond butyl column (0.46 cm $\times$ 25 cm). The solvent system used was 0.1% trifluoroacetic acid (TFA) (A) and 60% acetonitrile/propan-2-ol (3:7, v/v) in 0.082% TFA (B); elution was by a linear gradient from (A) to (B) at a flow rate of 1 ml/min. Peaks 1-4 were sequenced, and their sequences are presented in (b). The residue numbers given indicate the corresponding regions of the receptor sequence predicted from cDNA by Fuller *et al.* [16].

Disulphide bridge between the subunits

Treatment with the reducing agent 2-mercaptoethanol or dithiothreitol cleaved the disulphide bond linking the subunits (Fig. 9, lane a). Upon removal of the reducing agents by dialysis, the disulphide-linked dimeric structure was readily restored (lane b), indicating that the receptor subunit has a strong potential for dimerization and disulphide cross-linking. This might explain the trace amount of the 140 kDa band observed even when the reduced receptor was analysed by SDS/PAGE (Fig. 2, lane b). Since the stacking and separation gels did not contain reducing agents, dimerization by disulphide bonding of the 70 kDa subunits could occur during electrophoresis. In fact, the reduced subunits recovered by excising the 70 kDa band from SDS/polyacrylamide gels gave a faint 140 kDa band in addition to the major 70 kDa band when re-electrophoresed under the same reducing conditions.

To determine whether the disulphide-linked dimeric structure is essential for the binding activity, we next prepared carboxymethylated subunits that lack the ability to form the intersubunit disulphide bond (Fig. 9, lane d) and assayed their ANP-binding activity. As shown in Fig. 6, the carboxymethylated ANP receptor bound ANP equally as well as the native receptor. This

Fig. 9. Regeneration of the ANP-receptor intersubunit disulphide bond using the native reduced receptor (b) and lack of such product in the case of the carboxymethylated ANP receptor (d)

The purified ANP receptor was reduced by incubating the protein in the presence of 5 mM-dithiothreitol, and a portion of the reduced material was carboxymethylated with 10 mM-iodoacetamide. After dialysis to remove the remaining reagents, native non-carboxymethylated (lanes a and b) and carboxymethylated (lanes c and d) samples were analysed by SDS/PAGE under reducing (a and c) and non-reducing (reoxidized; b and d) conditions.

result suggests that the disulphide linkage is not required for the ligand-binding activity of the receptor. However, it is conceivable that the disulphide-linked dimeric structure is necessary for signal transduction. Although the dimeric form of the ANP receptor is commonly thought to serve merely as a clearance receptor [16,18,19], the above possibility could be a intriguing question that deserves further experimentation using, for example, reconstituted systems composed of the native or chemically modified receptor and the effector protein(s).

Characterization of the ligand-binding site of ANP receptor by Scatchard analysis

The fact that the disulphide-linked subunit structure is not necessary for the ANP-binding activity of the receptor (Fig. 6) suggests that each 70 kDa subunit has its own binding activity. To examine this possibility, we tried to isolate the carboxymethylated monomers by gel filtration on Superose 12. However, the attempt was not rewarding, since the carboxymethylated forms of the ANP receptor were eluted as a dimer (140 kDa) or higher- M_r aggregates. It is therefore unclear whether the 70 kDa monomer has the ability to bind ANP or the ANP-binding activity is acquired only after the interaction with another subunit. Unique approaches would be necessary for determining the minimal functional size responsible for the ANP binding (i.e. binding stoichiometry).

Scatchard analysis of the binding data is shown in Fig.

Fig. 10 Scatchard analysis of ANP binding to the purified receptor

The purified ANP receptor (1 ng) was incubated with increasing concentration of ^{125}I -labelled ANP in 100 μl of PBS containing 0.5% Triton X-100. After 1 h incubation, bound ^{125}I -ANP was separated from free radioligand and counted for radioactivity. Non-specific binding was determined in the presence of 1 μM non-radioactive ANP. Data were analysed by non-linear curve-fitting using the LIGAND computer program [33].

10, together with the calculated dissociation constant (K_D) and number of binding sites (B_{max}). The ANP-binding capacity of the purified homodimeric receptor was 4.2 nmol/mg, compared with an expected value of 7.1 nmol/mg for a binding stoichiometry of 1:1. The discrepancy between the experimental and theoretical values might be attributable to (i) losses of binding activity during purification, (ii) limitations of the Scatchard analysis of binding data for determining precise stoichiometry [29] or (iii) masking or lowering of the affinity of the binding sites as a result of receptor aggregation, which was inevitable even in the presence of 1% Triton X-100, CHAPS or Lubrol. Although further investigations are needed to determine which rationale is the best, the third explanation seems to be the most likely, since ANP receptor was found to be very stable.

The dimer-type receptor is the major class of ANP receptors in vascular endothelial [8,23] and smooth-muscle cells [10,24]. These locations are quite reasonable with respect to its proposed functional role in the clearance and modulation of plasma levels of ANP [19]. This view was strengthened by the recent cloning work by Chinkers *et al.* [17], which has definitely demonstrated the existence of a second class of ANP receptor that contains a constitutive guanylate cyclase activity sensitive to ANP. Although this guanylate cyclase-associated single-chain receptor may play a central role in mediating

the variety of physiological actions of ANP, it is not clear whether the dimer-type receptor functions only as a clearance receptor or is also actively involved in causing cellular responses. Our recent study using an antiserum specific for the dimer-type ANP receptor suggested the possible involvement of the receptor in the signal transduction [20]. Localization of the dimer-type receptor in the specialized non-vascular cells such as the podocytes of the renal glomeruli [30] and the alveoli of the lung [31,32] is also suggestive of its direct involvement in functions other than the sequestration and metabolic clearance of ANP. Therefore detailed characterization of the dimer-type receptor as well as the single-chain receptor/cyclase will remain to be an important subject of continuing research.

In summary, the ANP receptor having a disulphide-linked homodimeric structure was purified 62000-fold with an overall yield of 42% by $(\text{NH}_4)_2\text{SO}_4$ fractionation and affinity chromatography from bovine lung. The purified receptor was surprisingly stable under many different conditions. Enzymic deglycosylation indicated that the receptor has three *N*-linked oligosaccharides per one 70 kDa subunit, which are not essential for the ligand-binding activity. The receptor had a strong propensity for dimerization and disulphide cross-linking. However, the intersubunit disulphide bond was not necessary for the receptor activity. The ligand-binding domain of the receptor was characterized by Scatchard analysis. The purification procedure developed will provide a means of isolating sufficient amounts of ANP receptor for reconstitution experiments or producing monoclonal antibodies, and the structural information gained will form an important basis for elucidating the mechanism of action of ANP.

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