Reversible inactivation of AT_2 angiotensin II receptor from cysteine–disulfide bond exchange

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Abstract Dithiothreitol (DTT) treatment of angiotensin II (Ang II) type 2 (AT_2) receptor potentiates ligand binding, but the underlying mechanism is not known. Two disulfide bonds proposed in the extracellular domain were examined in this report. Based on the analysis of ligand affinity of cysteine (Cys, C) to alanine (Ala, A) substitution mutants, we provide evidence that Cys^{35}–Cys^{290} and Cys^{117}–Cys^{195} disulfide bonds are formed in the wild-type AT_2 receptor. Disruption of the highly conserved Cys^{117}–Cys^{195} disulfide bond linking the second and third extracellular segments leads to inactivation of the receptor. The Cys^{35}–Cys^{290} bond is highly sensitive to DTT. Its breakage results in an increased binding affinity for both Ang II and the AT_2 receptor-specific antagonist PD123319. Surprisingly, in the single Cys mutants, C35A and C290A, a labile population of receptors is produced which can be re-folded to high-affinity state by DTT treatment. These results suggest that the free –SH group of Cys^{35} or Cys^{290} competes with the disulfide bond formation between Cys^{117} and Cys^{195}. This Cys–disulfide bond exchange results in production of the inactive population of the mutant receptors through formation of a non-native disulfide bond.

Key words: Angiotensin II; AT_2 receptor; Disulfide bond; Thiol potentiation; Cys–disulfide exchange; G-protein-coupled receptor

1. Introduction

Differential susceptibility to dithiothreitol (DTT) is a hallmark for distinguishing two classes of receptors for the octapeptide hormone angiotensin II (Ang II). The Ang II receptor family which includes the type 1 (AT_1) and the type 2 (AT_2) receptors is a group of heptahelical receptors. They are characterized by a single polypeptide chain containing seven transmembrane helices (TMI–TMVII) linked by four extracellular and four cytoplasmic segments [1–9]. The DTT-inactivated AT_1 receptor selectively binds biphenyl imidazole compounds such as losartan and its structural analogues. The DTT-potentiated AT_2 receptor binds imidazole pyridine carboxylic acid compounds such as PD123319. Both AT_1 and AT_2 receptors have been shown to activate G-protein-mediated intracellular signal transduction [1–7].

The covalent linking of the TMIII and the second extracellular loop through a disulfide bridge is thought to be important for generation and stabilization of the receptor structure in the entire G-protein-coupled receptor (GPCR) superfamily [10–18]. Mutation of these cysteines (Cys, C) or reduction of the disulfide linkage between them impairs the function, for instance in rhodopsin [12,13], β-adrenergic receptors [14,15] and muscarinic receptors [16] through ultimate destabilization of the TM bundle. The DTT inactivation–activation of Ang II receptor therefore must be governed by a similar structure–function principle. Through a mutagenesis study, Ohyama et al. [17] demonstrated that the rat AT_1 receptor contains two disulfide bonds in the extracellular domain, and that both the disulfide bonds are essential to maintain high affinity of AT_1 receptor for Ang II. However, the mechanism of potentiation of AT_2 receptor by DTT is not elucidated.

The AT_2 receptor presents several unusual properties. It was originally identified as a DTT-potentiated receptor that activated intracellular signal transduction pathways independent of G-protein coupling [4,18,19]. Molecular cloning led to identification of structural features of a GPCR in the deduced AT_2 receptor-polypeptide and recent studies indicate its ability to couple to G-proteins [4–6]. DTT-potentiation was a significant property of the AT_2 receptor that aided the development of a subtype-selective non-peptide antagonist well ahead of molecular cloning of the receptor cDNA [1]. Expression of this receptor is re-activated at the sites of healing skin wound, infarcted myocardium and injured vascular tissue where the redox potential may be in favor of reduction of disulfide bonds [18–20]. Hence, structure–function studies directed to understand DTT-potentiation of AT_2 receptor are clearly important. In this study, we investigated the structural basis of DTT-potentiation of the Ang II binding affinity of wild-type and mutant rat AT_2 receptors (Fig. 1) expressed in COS1 cells. We report here that disruption of interaction between Cys^{35} and Cys^{290} located in the putative extracellular domain of the AT_2 receptor is responsible for a generalized potentiation of ligand binding. When Cys^{35} and Cys^{290} residues are unpaired, reversible inactivation occurs in specific mutants through the formation of a non-native disulfide bond.

2. Materials and methods

2.1. Materials

The monoclonal antibody 1D4 was supplied by the Cell Culture Center, Endotronic Inc. (Minneapolis, MN, USA). Oligonuclides were obtained from the oligonuclide synthesis core facility of the

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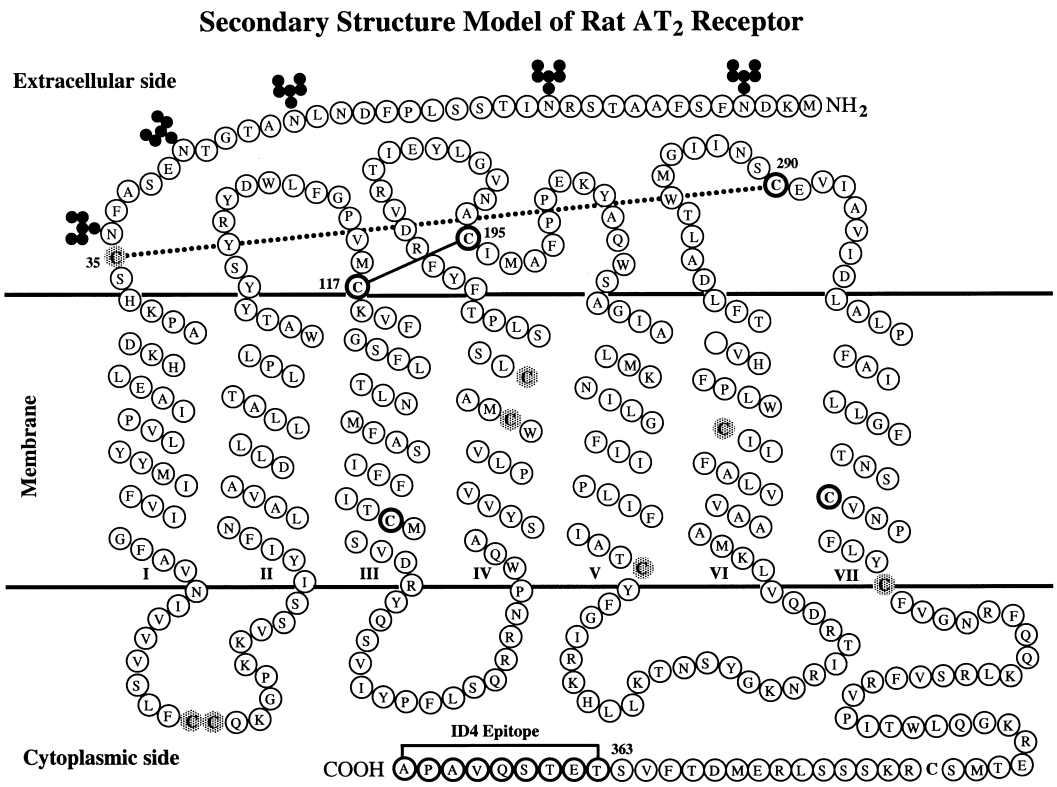


Fig. 1. A secondary structure model of the rat AT_2 receptor. The location of 14 cysteine residues is indicated in the proposed membrane topology. Five cysteines conserved between AT_1 and AT_2 receptors are shown in circles and the proposed disulfide interactions in the extracellular domain are indicated. Potential glycosylation sites and the octapeptide–epitope tag used in the immunoblotting experiments are shown.

Research Institute, The Cleveland Clinic Foundation. Ang II, [Sar^1]Ang II, [Sar^1,Ile^8]Ang II, and Ang III were obtained from Bachem. ^{125}I-[Sar^1,Ile^8]Ang II was supplied by Dr. Robert Speth, University of Washington, Pulman, WA, USA. The specific activity of the ^{125}I-[Sar^1,Ile^8]Ang II was 2200 Ci/mmol. Losartan was a gift from DuPont Merck (Wilmington, DE, USA). PD123319 was purchased from RBI, Natick, MA, USA. ^{3}H-Losartan was from Amersham. DTT and other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Cloning, mutagenesis and expression of Ang II receptors

The synthetic rat AT_1 receptor gene, cloned in the shuttle expression vector pMT3, was used for expression as described earlier [21–25]. The cDNA of AT_2 receptor was cloned from mRNA isolated from adrenal medulla of SHR rat by reverse transcriptase coupled polymerase chain reaction. The cloned cDNA was fully sequenced and modified for expression in COS1 cells by: (1) To contain a consensus Marilyn–Kozak sequence and a unique *Eco*RI site at the 5′ end and *Not*I site at the 3′ end of the gene. (2) To encode an octapeptide (ETSQVAPA) epitope for a monoclonal antibody 1D4 at the 3′ end before the stop codon. The epitope-tagged cDNA was subcloned into a shuttle vector pMT3 in which the cDNA would be transcribed from a polyoma major late promoter. Mutant AT_2 receptors were prepared by the restriction fragment replacement method and the polymerase chain reaction method. DNA sequence analysis was done to confirm each mutant construct. For expression of receptor proteins, 10 μg of CsCl-purified plasmid DNA per 10^7 cells was used in transfection. COS1 cells (American Type Culture Collection, Rockville, MD, USA), cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, were transfected by the DEAE–dextran method. Transfected cells cultured for 72 h were harvested. Cell membranes were prepared by the nitrogen par bomb disruption method and suspended in HME buffer (50 mM HEPES, pH 7.2, 12.5 mM $MgCl_2$, 1.5 mM EGTA) containing 10% glycerol as described earlier [21–25]. The receptor expression was assessed in each case by immunoblot analysis and by ^{125}I-[Sar^1,Ile^8]Ang II saturation binding analysis.

2.3. Western blot of receptors expressed in COS1 cells

Post-nuclear supernatant of COS1 cells solubilized in lysis buffer (50 mM Tris–HCl, pH 6.8, 1% CHAPS, 5 mM EDTA, pH 8.0) containing 50 μg/ml PMSF and centrifuged at 40 000 rpm was used for SDS–PAGE and Western blotting analysis. Receptor polypeptides were visualized using 2.5 μg/ml of mouse monoclonal antibody 1D4 and the alkaline phosphatase conjugated anti-mouse IgG antibody (Promega, Corp., Madison, WI, USA). The membrane was exposed to X-ray film (Kodak) at −70°C. The relative intensities of the bands were estimated by phosphorimager analysis.

2.4. DTT treatment

Membrane preparations containing receptor protein were mixed with assay buffer (50 mM sodium phosphate, pH 7.2, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.2% bovine serum albumin, and 10 μg/ml bacitracin) containing various concentrations of DTT (0–100 mM) at 22°C for 20 min. Pretreated membranes were used for equilibrium binding studies. To examine the effect of ligand occupancy, membrane preparations were preincubated with either ^{125}I-[Sar^1,Ile^8]Ang II or ^{3}H-losartan in assay buffer for 40 min at 22°C and then treated with various concentrations of DTT at 22°C for 20 min. Then binding study was performed. In recovery studies, DTT-treated membrane preparations were washed three times in cold assay buffer without DTT and then used for binding study (also see [14,15]).

2.5. Ligand binding study

The ligand binding experiments were carried out under equilibrium conditions as described before [14,21–25]. Briefly, membranes expressing receptors were incubated with 0.03–3 nM ^{125}I-[Sar^1,Ile^8]Ang II in assay buffer for competition binding study. For saturation binding study, an at least 10-fold higher concentration of ^{125}I-[Sar^1,Ile^8]Ang II than the K_d value of the receptor was used in order to get >90% bound form of added receptor. The highest concentration of ^{125}I-[Sar^1,Ile^8]Ang II is diluted by half to make 8–12 different concentrations to facilitate the Scatchard plot analysis. Non-specific binding

to the membranes was determined from ^{125}I-[Sar^1,Ile^8]Ang II binding in the presence of 10^{-5} M cold ^{127}I-[Sar^1,Ile^8]Ang II. All binding studies were performed at 22°C for 1 h unless specifically indicated. The binding reaction was stopped by filtering under vacuum (Brandel Type M-24R) on FP-200 GF/C filters (Whatman Inc., Fairfield, NJ, USA). Filter-bound ^{125}I-[Sar^1,Ile^8]Ang II was quantitated in a gamma-counter (Packard). Equilibrium binding kinetics were determined using the computer program Ligand or Scatchard plot. The K_d values represent the mean ± S.E.M. of three or more independent determinations.

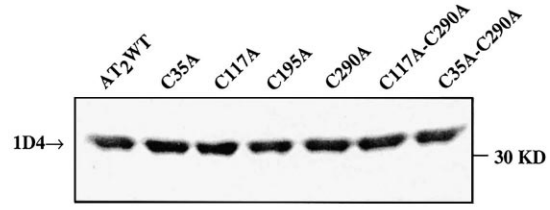
3. Results

3.1. The experimental system

The epitope-tagged AT_2 receptors were expressed in COS1 cells by transient transfection. In total membrane preparation obtained from the transfected cells, the expression of AT_2 receptor was detected by immunoblotting (Fig. 2). The expressed AT_2 receptor displayed high affinity (K_d, 1 ± 0.25 nM) binding for the peptide analogue ^{127}I-[Sar^1,Ile^8]Ang II, (Table 1). The B_{max} values measured was ≈4.2 pmol/mg. Scatchard plot analysis of the ^{125}I-[Sar^1,Ile^8]Ang II saturation binding indicated a single affinity class of receptors (not shown). Competition binding studies employing Ang II, [Sar^1]Ang II, [Sar^1,Ile^8]Ang II, Ang III, PD123319 (AT_2 selective) and Losartan (AT_1 selective), demonstrated that the receptors expressed in COS1 cells preserve the selectivity and affinity profile previously described for native tissue receptors as well as recombinantly expressed receptors. Thus, we conclude that high level expression of AT_2 receptors leads to the formation of a unique affinity state of the receptor. Expression and characterization of AT_1 receptor in COS1 cells has been described earlier [21–24]. Thus COS1 cell transient transfection is a suitable system for structure–function analysis of AT_2 receptor.

3.2. Effect of Ala substitution of extracellular Cys residues

To assign the putative disulfide interaction in the AT_2 receptor and to evaluate their contribution to receptor conformation, four single and two double Cys to Ala substitution mutants were constructed. Immunoblot analysis indicated nearly comparable levels of expression of the wild-type and all the six mutants in COS1 cells (Fig. 2). Binding affinity of the C117A and C195A mutants for all ligands was significantly reduced (> 10 000-fold, see Table 1). The double mutant, C117A–C290A also produced a > 10 000-fold decrease in ligand binding that is consistent with the phenotype of C117A mutant but not that of C290A implying that these residues are not partners in a disulfide bond. In sharp contrast



Bmax, (pmol/mg) without DTT	4.2± 0.35	1.5± 0.12	ud	ud	2.8± 0.26	ud	3.6± 0.33
Bmax, (pmol/mg) with DTT	3.9± 0.35	3.6± 0.34	ud	ud	4.7± 0.41	ud	3.3± 0.37
-Fold change	-1.08	2.43			1.76		-1.10

Fig. 2. Immunoblot analysis of the wild-type and mutant AT_2 receptors expressed in COS1 cells. All receptors were detected by utilizing a monoclonal antibody, 1D4, recognizing the epitope TETSQVAPA attached at the C-terminus in each case. The B_{max} values corresponding to the protein band are shown without DTT treatment and with DTT treatment. ud: Binding undetectable.

the C35A and C290A single mutants displayed a gain of affinity for both peptide and the non-peptide ligands. The phenotype of the C35A–C290A double mutant is consistent with the phenotype of the two single mutants.

3.3. Effect of DTT treatment of wild-type AT_2 receptor on ligand binding

Treatment with 5 mM DTT potentiated binding of ^{125}I-[Sar^1,Ile^8]Ang II to AT_2 receptors in COS1 membranes. The same treatment abolished binding to AT_1 receptor in COS1 membranes consistent with published reports on native receptors [2–7]. Fig. 3A shows the effect of various concentrations of DTT on ^{125}I-[Sar^1,Ile^8]Ang II binding to similar levels of wild-type AT_1 and AT_2 receptors in COS1 membranes. The binding to AT_2 receptors increased by 60% at 4–6 mM DTT concentration. At > 8 mM DTT concentration, the AT_2 receptor was gradually inactivated. The mid-point of the potentiation phase corresponds to ≈0.4 mM DTT and the mid-point of the inactivation phase corresponds to ≈40 mM DTT. The potentiating effect was significantly attenuated in the presence of [Sar^1,Ile^8]Ang II but not in the presence of losartan or inactive Ang II analogues. Under identical conditions DTT treatment inactivated AT_1 receptor completely at > 2 mM DTT concentration. Presence of [Sar^1,Ile^8]Ang II and losartan significantly attenuated, but non-binding analogues of Ang II and PD123319 did not protect AT_1 receptor against DTT inactivation.

The potentiation of binding to the AT_2 receptor is predom-

Table 1
Binding affinity of wild-type and mutant receptors pretreated with and without 5 mM DTT at 22°C for 20 min

	Without DTT pretreatment						With DTT pretreatment					
	[Sar^1,Ile^8]Ang II		Ang II		PD123319		[Sar^1,Ile^8]Ang II		Ang II		PD123319	
	K_i (nM)	-fold	K_i (nM)	-fold	K_i (nM)	-fold	K_i (nM)	-fold	K_i (nM)	-fold	K_i (nM)	-fold
AT_1 WT	0.3 ± 0.09	ND	3.6 ± 0.41		ND		> 1 000		> 1 000		ND	
AT_2 WT	1.0 ± 0.25		1.9 ± 0.32		17.2 ± 2.2		0.1 ± 0.02		0.7 ± 0.12		2.1 ± 0.43	
C35A	0.1 ± 0.01	−10	0.4 ± 0.11	−4.8	1.77 ± 0.5	−9.7	0.1 ± 0.01	1	0.3 ± 0.02	−2.3	1.7 ± 0.37	−1.2
C117A	> 10 000	10 000	> 10 000	1 400	> 10 000	600	> 10 000	10 000	> 10 000	10 000	> 10 000	5 000
C195A	> 10 000	10 000	> 10 000	1 400	> 10 000	600	> 10 000	10 000	> 10 0000	10 000	> 10 000	5 000
C290A	0.1 ± 0.01	−10	0.3 ± 0.08	−6.3	1.86 ± 0.5	−9.3	0.1 ± 0.01	1	0.3 ± 0.02	−2.3	1.7 ± 0.41	−1.2
C35A–C290A	0.1 ± 0.01	−10	0.3 ± 0.08	−6.3	1.51 ± 0.3	−11.4	0.1 ± 0.01	1	0.4 ± 0.06	−1.8	1.4 ± 0.36	−1.5
C117A–C290A	> 10 000	10 000	> 10 000	1 400	> 10 000	600	> 10 000	10 000	> 10 000	10 000	> 10 000	5 000

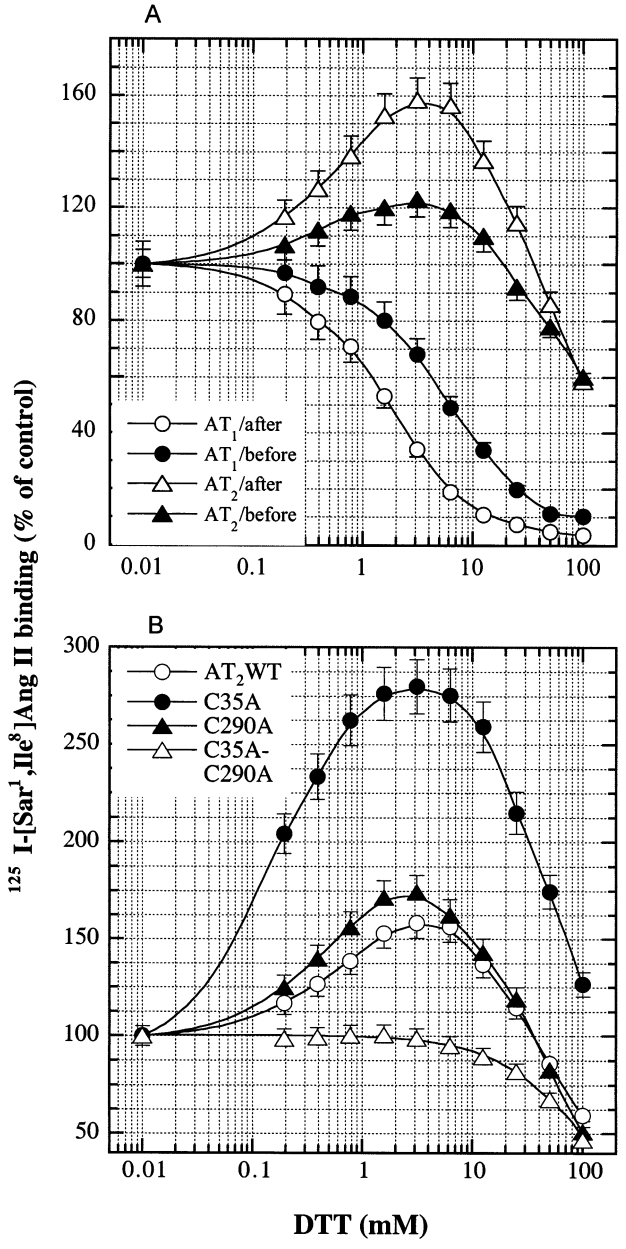


Fig. 3. Effect of varying concentrations of DTT on ^{125}I-[Sar^1,Ile^8]Ang II binding to AT_1 and AT_2 receptors. The specific binding of ^{125}I-[Sar^1,Ile^8]Ang II in each sample was adjusted to be $<10\pm1\%$ ($\approx$20 000) of total input CPM without any treatment. This value is represented as 100%. A: Binding to wild-type AT_1 and AT_2 receptors. Open symbols refer to samples exposed to DTT after 20 min exposure to ligand and closed symbol refers to samples exposed to DTT first and then ligand was added. B: Binding to wild-type and mutant AT_2 receptors in membranes pretreated with DTT.

inantly a result of a $\approx$10-fold increase in affinity for peptide ligands and $\approx$8-fold increase of affinity for PD123319 with no significant change in B_{max} (Table 1 and Fig. 2). The reversibility of the DTT-induced conformational changes was examined. The membranes were treated with DTT for 20 min and DTT was removed by washing. The recovery of the native state of the AT_2 receptor was estimated from the change in distribution from high and normal affinity receptors by Scatchard plot analysis. Removal of DTT led to nearly 100% reversal to normal affinity state (1 nM) in the AT_2 receptor. In complete contrast, the inactivation of AT_1 receptor by DTT is predominantly due to loss of binding sites (B_{max}) rather than a decrease of binding affinity. The B_{max} decreased upon DTT treatment from 5.1 to 0.72 pmol/mg. Nearly 70% of the B_{max} was recovered upon removal of DTT (data not shown). At high concentrations of DTT ($>$40 mM), AT_2 receptor inactivation involved loss of B_{max}. Thus, both AT_1 and AT_2 receptors appear to refold to normal affinity conformation upon removal of DTT.

3.4. Effect of DTT treatment on mutant AT_2 receptor on ligand binding

In order to assign the disulfide bond responsible for the DTT-induced potentiation of the AT_2 receptor, DTT-induced potentiation of ligand affinity was examined in various Cys to Ala mutants. The data presented in Table 1 indicate that the DTT-induced affinity increase is absent in mutants C35A, C290A and C35A-C290A. More importantly, these three mutants exhibit 8–10-fold higher affinity for all ligands than the wild-type receptor without DTT potentiation. This observation suggested to us that breakage of Cys^{35}–Cys^{290} disulfide bond alone is responsible for the DTT-potentiation of AT_2 receptor.

We then examined if breakage of the C35–C290 disulfide bond would change the kinetics of reduction of the C117–C195 disulfide bond. In the double mutant C35A–C290A, the DTT-potentiation was completely absent and DTT-inactivation was similar to the inactivation of wild-type AT_2 receptor (Fig. 3B). Thus, the two disulfide bonds in the AT_2 receptor have completely independent roles.

3.5. Reversible inactivation of C35A and C290A mutant receptors by Cys–disulfide bond exchange

Despite comparable levels of polypeptide expression, the measured B_{max} values for the single mutants, C35A and C290A were consistently lower than that for the wild-type and the C35A–C290A mutant. Furthermore, the B_{max} was sensitive to storage specifically in these two mutants. We systematically analyzed this phenomenon in an experiment. The mutant membranes were kept at 4°C for 24 h. The B_{max} decreased by 60 and 30% respectively in the C35A and the C290A mutants. Scatchard plot analysis revealed that the decrease of B_{max} was not due to conversion of the receptor to a low affinity state, but due to loss of binding sites. Rather surprisingly, treatment of C35A and C290A mutants with 5 mM DTT led to a dramatic increase in binding of ^{125}I-[Sar^1,Ile^8]Ang II in both C35A and C290A mutants (see Fig. 3B). In contrast, the double mutant C35A–C290A, and the wild-type AT_2 receptors did not exhibit this phenomenon. DTT treatment did not lead to regain of binding sites in the C117A–C290A defective mutant. The inactivation phase, of the binding profile is preserved in C35A and C290A mutants at $>$10 mM DTT, implying that all of the refolded receptors contain the conserved C117–C195 disulfide bond which is sensitive to a high concentration of DTT.

4. Discussion

The rat AT_2 receptor contains 14 Cys residues. 10 Cys residues located in the transmembrane and cytoplasmic do-

main are not likely to be involved in disulfide bonds (see Fig. 1). The remaining four Cys residues, Cys^{35}, Cys^{117}, Cys^{195}, and Cys^{290} are located in the extracellular domain where the redox environment may facilitate two disulfide bonds. Systematic mutation of these four Cys residues showed two different phenotypes of AT_2 receptor. The first, observed in the single C117A or C195A mutants and also in the C117A–C290A double mutant is characterized by non-detectable binding of analogues of Ang II. The polypeptide expression is not reduced in these mutants. Also, treatment with 5 mM DTT did not restore ligand binding. The observations suggest that a disulfide bond between Cys^{117} and Cys^{195} in the extracellular domain is critical for the polypeptide to gain ligand binding conformation and in its absence the polypeptide is irreversibly misfolded. The active conformation is believed to require a compact structure involving proper alignment of TM helices and the connecting loops, which is stabilized by the critical disulfide linkage [12–17]. The results also suggest that if a Cys^{35}–Cys^{195} disulfide bond is formed in the C117A–C290A mutant, then such a bond cannot substitute for the conserved disulfide bond. The inactivation of the wild-type AT_2 receptor at high concentrations of DTT, must involve reduction of this disulfide linkage.

The second phenotype is an increase in affinity for all ligands, observed in the C35A, C290A and the C35A–C290A mutants. The most characteristic feature of these three mutants is the absence of DTT-potentiation. These mutant phenotypes indicate that Cys^{35} and Cys^{290} are linked in a disulfide bond which is responsible for the DTT-potentiation phenomenon in the wild-type AT_2 receptor. Thus, the TM domain stability and strengths of the Cys^{35}–Cys^{290} and Cys^{117}–Cys^{195} S–S bonds are directly coupled. This explains the bell shape of the DTT action curve for the AT_2 receptor (Fig. 3). Attenuation of the DTT effect on both these disulfide bonds in the presence of peptide and subtype-selective non-peptide ligands suggests that occupation of the pocket protects against destabilization. In other GPCRs the bound ligands have been found to protect the receptor from reducing effects of DTT [14–16]. In β-adrenergic receptors, through reconstitution in lipid membranes, Pederson and Ross [15] demonstrated that at low concentrations DTT activated the receptor, but at higher concentrations led to inactivation. Noda et al. [14] have assigned distinct disulfide bonds responsible for each phase of DTT.

Because C35A and C290A mutant polypeptides are capable of folding to active receptor conformation, we reason that the free –SH group in either C35A or C290A mutant does not interfere with normal membrane insertion and alignment of transmembrane helices to form a binding pocket. We speculate that the unpaired –SH group in each case is the cause of destabilization of the folded conformational state of the mutant receptors through disulfide formation with either the free Cys residues in TM domain or through interference with the conserved C117–C195 disulfide bond. A non-native disulfide bond thus formed in both C35A and C290A mutant receptors could destabilize the Ang II binding state of the receptor. Since the evidence presented above indicates that C117–C195 disulfide is critical for this conformation, it seems most likely that breakage of the C117–C195 disulfide bond is the cause for gradual loss of B_{max} in the C35A and C290A mutants. This speculation is also supported by the DTT-reactivation in the inactive membrane preparations, although disulfide bond formation with a different Cys other than these two is also expected to be reversible. Thus, regain of ligand affinity from DTT treatment in both C35A and C290A mutants is consistent with a Cys disulfide bond exchange leading to reformation of the conserved disulfide bond. If the inactivation was due to interaction with a non-Cys residue, such an interaction is usually weak, hardly could be destabilizing and is not expected to be reversed by DTT. It is generally believed that the highly conserved disulfide bond is relatively stable and its breakage could lead to unfolding of the receptor. Unfolding of proteins usually results in denaturation, which is followed by an irreversible process, most often aggregation [10–15]. For instance, the irreversible inactivation of C117A, C195A and C117A–C290A mutant receptors could be the result of such unfolding. In the case of mutant C35A and C290A mutants, protection against irreversible inactivation might be afforded by the non-native S–S bond. A Cys disulfide exchange reaction has been described previously, in the generation of active rhodopsin from opsin fragments [26]. To the best of our knowledge, this is the first demonstration of rectification of a misfolded GPCR mutant by reduction of a non-native disulfide bond which prevented irreversible inactivation through unfolding reactions.

The in vivo physiological significance of DTT-mediated potentiation of AT_2 receptor is presently unknown. Indeed in many pathophysiological conditions such as vascular injury and wound healing where local redox potential may favor disulfide reduction [1–7,18–20], a regulatory mechanism for AT_2 receptor potentiation may involve disruption of the Cys^{35}–Cys^{290} bond. Recently, an intermolecular disulfide bridge formed as part of dimerization in metabotropic glutamate receptors and the calcium sensing receptor has been demonstrated [27,28]. A similar mechanism has been discovered for activation of the OxyR transcription factor by reversible disulfide bond formation [29]. Certain diseases associated with GPCR malfunction are caused by introduction of unpaired Cys residues in the extracellular domain. For instance, a disproportionate number of mutations that cause nephrogenic diabetes are single Cys substitutions in the extracellular loop 2 of V_2 vasopressin receptor. A retinitis pigmentosa mutation involves a Cys substitution in the extracellular loop 2 in human rhodopsin [10,12,13,26]. A Cys disulfide exchange reaction, such as the one described above, may be the cause of inactivation–disfunction in these mutant receptors leading to disease states.

Cys residues are found quite frequently in GPCRs, where they play quite different structure–function roles. In the transmembrane segments the Cys residues are mostly free with no particular preference in facing lipid or interior of the protein [10–17]. In the cytoplasmic tail one or more Cys residues are palmitoylated. The most conserved disulfide bridge in the extracellular domain links the TMIII with extracellular loop 2 in several GPCRs. In bovine rhodopsin, this bond helps stabilize interaction of a part of the extracellular loop with the ligand, 11-*cis*-retinal [30]. Mutational analysis has demonstrated a reciprocal relationship between stabilities of this disulfide bond and the ligand pocket formed by the TM bundle in rhodopsin, β-adrenergic receptor and the AT_1 receptor. Additional Cys residues are found in the extracellular domain of a number of other GPCRs which may form a second disulfide linkage. Substitution of these Cys residues in AT_1, chemokine CXCR1 and CCR-2 receptors seriously impairs

agonist binding, but in bradykinin B_2 receptor and NPY Y1 receptor substitution has no effect [10,11].

In summary, we report that the unpaired Cys^{35} and Cys^{290} residues in the AT_2 receptor lead to an inactive population of mutant receptors that could be rectified through a thiol sensitive step. We also show that the Cys^{35}–Cys^{290} disulfide bond confers DTT potentiation and the Cys^{117}–Cys^{195} disulfide bond confers stability in the AT_2 receptor.

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