Determinants of spironolactone binding specificity in the mineralocorticoid receptor

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Abstract

Spironolactone is a mineralocorticoid receptor (MR) antagonist in clinical use. The compound has a very low affinity for the glucocorticoid receptor (GR). Determinants of binding specificity of spironolactone to the MR were investigated using chimeras created between the ligand-binding domains (LBDs) of the MR and the GR. These chimeras had previously been used to investigate aldosterone binding specificity to the MR. Spironolactone was able to compete strongly for [³H]-aldosterone and [³H]-dexamethasone binding to a chimera containing amino acids 804–874 of the MR, and weakly for [³H]-dexamethasone binding to a chimera containing amino acids 672–803 of the MR. Amino acids 804–874 were also critical for aldosterone binding specificity. Models of the MR LBD bound to aldosterone and spironolactone were created based on the crystal structure of the progesterone receptor LBD. The ligand-binding pocket of the MR LBD model consisted of 23 amino acids and was predominantly hydrophobic in nature. Analysis of this model in light of the experimental data suggested that spironolactone binding specificity is not governed by amino acids in the ligand-binding pocket.

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Introduction

Spironolactone is an antagonist of the mineralocorticoid receptor (MR) used in the treatment of hypertension due to primary aldosteronism and in conditions of secondary hyperaldosteronism such as cardiac or hepatic failure (Doggrell & Brown 2001). The clinical use of spironolactone was placed in the spotlight with the results of the RALES trial (Pitt *et al.* 1999), which demonstrated that the addition of spironolactone to the treatment regimen of patients with severe heart failure led to a 30% decrease in mortality. Unfortunately, spironolactone has side-effects due to its cross-reactivity with the related androgen receptor, which have limited its clinical usefulness as an anti-mineralocorticoid. There is a need for better, more specific MR antagonists, and therefore a need to better understand ligand binding to the MR.

The MR belongs to the nuclear hormone receptor superfamily of ligand-dependent transcription factors. As with other members of the family, the receptor structure can be divided into three

major functional domains (Rogerson & Fuller 2000). The first of these is the N-terminal domain which contains an 'activation function' (AF-1) involved in transcriptional activation (Rupprecht *et al.* 1993a, Govindan & Warriar 1998, Fuse *et al.* 2000). In the middle of the protein is the DNA-binding domain (DBD) which binds to specific DNA sequences on target genes and is also responsible for receptor homodimerisation (Liu *et al.* 1996). C-terminal to the DBD is the ligand-binding domain (LBD) which has sequences involved in ligand binding (Fagart *et al.* 1998), transcriptional activation (AF-2) (Rupprecht *et al.* 1993a) and heat-shock protein (HSP) binding (Eisen & Harmon 1986, Nemoto *et al.* 1993). The AF-2 region of the MR, like other nuclear hormone receptors, functions by interacting with coactivator proteins (Hong *et al.* 1997, Knutti *et al.* 2000).

The critical function of the LBD is the binding of ligand. In the absence of ligand, the MR exists predominantly in the cytoplasm in a complex with a number of HSPs (Nemoto *et al.* 1993). The binding of HSPs keeps the receptor in an inactive

state, and also maintains the LBD in a conformation that promotes high-affinity ligand binding (Eisen & Harmon 1986). The binding of ligand alters the conformation of the receptor and displaces the HSPs, which exposes sequences involved in receptor dimerisation, nuclear localisation and DNA binding. These conformational changes are also required for the activation of the AF-2 region.

Of the steroid hormone receptors, the MR has the most sequence identity with the glucocorticoid receptor (GR) (Arriza *et al.* 1987). This is reflected in the overlapping binding specificities of the two receptors. Both receptors bind cortisol and corticosterone with high affinity, whereas only the MR binds aldosterone with high affinity (Funder *et al.* 1988). The synthetic GR agonist dexamethasone also binds to the MR with relatively high affinity (Arriza *et al.* 1987, Rupprecht *et al.* 1993b) although it does not activate the MR with the same potency as it does the GR (Arriza 1991, Rupprecht *et al.* 1993b). Spironolactone has a very low affinity for the GR (Couette *et al.* 1992b). The structures of aldosterone and spironolactone are shown in Fig. 1. Apart from the steroid backbone, the two structures are quite dissimilar: the aldosterone molecule contains a hemiketal side-chain between C₁₁ and C₁₈, whereas spironolactone contains a lactone ring at C₁₇ and a sulfhydryl ester side-chain at C₇. In previous work we investigated structural determinants of aldosterone binding specificity using chimeras created between the LBDs of the MR and GR (Rogerson *et al.* 1999). We identified a region in the MR LBD, between amino acids 804 and 874, that is critical for the specificity of aldosterone binding to the MR. In the present study, we have used the same chimeras to investigate the binding of spironolactone to the MR. We have found that spironolactone binds strongly to a chimera containing amino acids 804–874 of the MR, and weakly to a chimera containing amino acids 672–803 of the MR. These results are discussed in relation to a model of the MR LBD structure created from the crystal structure of the progesterone receptor (PR) LBD.

Materials and methods

Expression and reporter constructs

Creation of the MR:GR LBD chimeras is described in detail elsewhere (Rogerson *et al.* 1999).

ALDOSTERONE

SPIRONOLACTONE

Figure 1 The structures of aldosterone and spironolactone. The positions of various backbone carbons mentioned in the text are labelled on the aldosterone structure using the conventional nomenclature, as are the identities of the A, B, C and D rings.

The chimeras were created by dividing the LBD into four segments. Each chimera is designated by the identity of each particular section where 'M' indicates MR sequence and 'G' indicates GR sequence. In all cases the LBD was placed into an expression vector containing the N-terminal domain and the DNA-binding domain of the GR. The full-length receptor sequences were ligated into the pcDNA3.1(+) mammalian expression

vector (Invitrogen Life Technologies, Carlsbad, CA, USA).

Ligand-binding competition assays

Ligand-binding assays were performed in transfected COS-1 cells using either [1,2,4,6,7-³H]-dexamethasone (Amersham Biosciences, Amersham, Bucks, UK) or [1,2,6,7-³H]-aldosterone (Amersham Biosciences). Cells were grown at 37 °C in Dulbecco's modified Eagles' medium (DMEM) supplemented with 1 mM glutamine, non-essential amino acids, 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). The cells were trypsinised and replated in six-well plates at a density of 2×10^5 cells/well. After 20–24 h, the cells were transfected with the expression plasmid using Fugene 6 (Roche Molecular Biochemicals, Indianapolis, IN, USA) and then incubated overnight in DMEM+2% FBS. The medium was replaced with DMEM 1 h before the ligand-binding assay. The cells were washed three times with ice-cold phosphate-buffered saline (PBS) then ³H-labelled steroid was added in DMEM. Non-specific binding was assessed by adding a 500-fold excess of non-radioactive steroid. The cells were incubated at 37 °C for 1 h. Cells were then washed three times with ice-cold PBS and then lysed using 1 M NaOH. The suspension was added to scintillant and radioactivity was measured in a Packard 2500 TR liquid scintillation counter. Scatchard analysis of [³H]-dexamethasone binding was performed using 25, 10, 4, 1.6, 0.64 and 0.26 nM radioligand. The ability of spironolactone to compete for [³H]-dexamethasone binding was assessed using 1-, 5-, 10- and 50-fold molar excesses of the concentration of radioligand. The ability of spironolactone to compete for [³H]-aldosterone binding was assessed using 10-, 100- and 500-fold molar excesses of the concentration of radioligand. Statistical significance was measured by the Games-Howell analysis of variance test (heterogeneous variances) using the GB-STAT software (Dynamic Microsystems Inc., Houston, TX, USA).

Model of the MR LBD structure based on the PR LBD crystal structure

A model of the MR LBD dimer was generated using the homology package within the InsightII suite from Molecular Simulations Inc., San Diego,

CA, USA (1997), based on the structure of the human PR LBD dimer (Protein Data Base (PDB) identification code 1a28 (Williams & Sigler 1998)). This structure was subsequently subjected to minimisation using the Discover 2.98 program (Accelrys Inc., San Diego, CA, USA). The CFF91 force field was applied, using an electrostatic cut-off distance of 12.0 Å. Minimisation followed a protocol of initial steepest descents until the maximum derivative was less than 10⁻⁶, followed by conjugate gradients until the maximum derivative was less than 0.001 (kcal/molÅ). During this procedure, the side-chain heavy atoms of those amino acids conserved between PR and MR were held fixed in their positions in the X-ray crystal structure of PR. These constraints (rigid) were applied to prevent the model structure deviating significantly from the PR template. In addition, a water molecule in the ligand-binding site was also fixed. The ligands, aldosterone and spironolactone, were constructed from the progesterone bound in PR, and were not constrained during the minimisation.

Results

Spironolactone competition for [³H]-dexamethasone binding

To examine ligand binding to the MR, we created a series of chimeras between the MR and GR LBDs (Rogerson *et al.* 1999). The chimeras were designed by dividing the LBDs into four equivalent sections. They were named using a four letter code, corresponding to the four regions, in which 'M' denotes MR sequence and 'G' denotes GR sequence. To examine spironolactone binding, the chimeras MGGG (MR amino acids 672–803), GMGG (MR amino acids 804–874), GGMG (MR amino acids 875–932) and GGGM (MR amino acids 933–984) were used. Spironolactone binding to these chimeras was examined by its ability to compete for the binding of [³H]-dexamethasone, as [³H]-dexamethasone binds to all the chimeras, whereas [³H]-aldosterone only binds to the GMGG chimera (Rogerson *et al.* 1999). The binding experiments were performed in transfected COS-1 cells that lack endogenous MR and GR.

Before analysis of spironolactone binding, the affinity (K_d) of [³H]-dexamethasone binding to the GR, MR LBD, MGGG, GMGG, GGMG and GGGM chimeras was determined (Table 1). Only

Figure 2 Spirolactone competition for [³H]-dexamethasone binding to MR LBD, GR, and the chimeras MGGG, GMGG, GGGM and GGGM. Ligand-binding studies were performed in COS-1 transformed monkey kidney fibroblast cells using a concentration of 20 nM [³H]-dexamethasone for all constructs except GGGM, where 50 nM [³H]-dexamethasone was used. The concentrations of spironolactone used were defined by the concentration of [³H]-dexamethasone. Non-specific binding was assessed using a 500-fold excess of dexamethasone. The results are expressed as a percentage of the binding in the absence of spironolactone. The graphs show the mean ± S.E.M. from the results of two independent experiments (*n*=6). **P*<0.05, relative to assay containing no spironolactone; ***P*<0.01, relative to assay containing no spironolactone.

construct	IC ₅₀ (nM)
MR LBD	3
MGGG	750
GMGG	7

Figure 3 Estimation of IC₅₀ values for [³H]-dexamethasone binding to the MR LBD and MGGG and GMGG chimeras.

one binding site was identified in each of the Scatchard analyses. Table 1 also shows B_{max} values for [³H]-dexamethasone binding to the constructs, demonstrating that all are expressed at high levels. A K_d value of 11 ± 3 nM was obtained for [³H]-dexamethasone binding to the GR in the whole-cell binding assay. This value compares with K_d values of 2–3 nM obtained in studies using cytosols derived from transfected COS cells (Roux *et al.* 1996, Giannoukos *et al.* 1999), suggesting that the COS-1 cells have only a minor effect on the access of the radioligand to the receptor. The K_d values for [³H]-dexamethasone binding to the GR

Table 1 K_d and B_{max} measurements of [³H]-dexamethasone binding to constructs used in the study. Each measurement was performed twice. Values are means ± S.E.M.

Construct	K _d (nM)	B _{max} (pmol/2 × 10 ⁵ cells)
GR	11 ± 3	60 ± 10
MR LBD	8 ± 1	220 ± 20
MGGG	21 ± 1	40 ± 40
GMGG	15 ± 5	70 ± 35
GGMG	10 ± 1	200 ± 140
GGGM	54 ± 4	60 ± 40

and the MR LBD were very similar, which is consistent with a study examining the ability of dexamethasone to compete for [³H]-aldosterone binding to the MR and [³H]-dexamethasone binding to the GR (Rupprecht *et al.* 1993b). In all cases, the Hill coefficients were very close to 1, suggesting no co-operativity in binding. On the basis of these results, the spironolactone competition experiments were performed using 20 nM [³H]-dexamethasone for the GR, the MR LBD and the MGGG, GMGG and GGMG chimeras, and 50 nM [³H]-dexamethasone for the GGGM chimera. Spironolactone was added in 1-, 5-, 10- and 50-fold molar excesses of the concentration of radioligand used for each construct.

Figure 2 shows spironolactone competition for [³H]-dexamethasone binding to the MR LBD, the GR and the MGGG, GMGG, GGMG and GGGM chimeras. As expected, spironolactone competed strongly for [³H]-dexamethasone binding to the MR LBD. In contrast, spironolactone competed very weakly for [³H]-dexamethasone binding to the GR. One micromolar spironolactone reduced [³H]-dexamethasone binding to the GR by approximately 25%. A similar result was obtained in another study examining the ability of spironolactone to compete for [³H]-dexamethasone binding to the GR (Couette *et al.* 1992b). Spironolactone competed strongly for [³H]-dexamethasone binding to the GMGG chimera, which contains amino acids 804–874 of the MR. Spironolactone competed weakly, but significantly, for [³H]-dexamethasone binding to the MGGG chimera, which contains amino acids 672–803 of the MR. In contrast, spironolactone did not appear to bind to the GGMG and GGGM chimeras at the concentrations used. The approximate IC₅₀ values of spironolactone competition for [³H]-dexamethasone binding to the MR LBD and MGGG and GMGG chimeras were determined by plotting the percentage binding against the log of the concentration of spironolactone (Fig. 3). The IC₅₀ values shown in Fig. 3 are derived from the equations of the lines. The IC₅₀ value of 3 nM obtained for the MR LBD was in very good agreement with the K_d value of 3 nM calculated for [³H]-spironolactone binding to the MR in chicken intestine cytosols (Couette *et al.* 1992a). This also suggested that COS-1 cells do not affect access of spironolactone to the receptor in the binding assay. The IC₅₀ value of spironolactone binding

Figure 4 Spironolactone competition for [^3H]-aldosterone binding to the MR LBD and GMGG chimera. Ligand-binding studies were performed in COS-1 transformed monkey kidney fibroblast cells using a concentration of 2 nM [^3H]-aldosterone for the MR LBD and 10 nM [^3H]-aldosterone for the GMGG chimera. The concentrations of spironolactone used were defined by the concentration of [^3H]-aldosterone. Non-specific binding was assessed using a 500-fold excess of aldosterone. The results are expressed as a percentage of the binding in the absence of spironolactone. The graphs show the mean $\pm$ S.E.M. from the results of two independent experiments ($n=6$). ** $P<0.01$, relative to assay containing no spironolactone.

to the GMGG chimera was 7 nM, very similar to the value obtained for the MR LBD. The IC_{50} value of spironolactone binding to the GMGG was two orders of magnitude lower than that to the MR LBD, confirming the weak binding of spironolactone to this chimera.

The ability of spironolactone to bind to the GMGG chimera was then tested by its ability to compete for [^3H]-aldosterone binding. The K_d value of aldosterone binding to the GMGG chimera is 10-fold greater than for the wild-type MR LBD (Rogerson *et al.* 1999). To assess spironolactone binding to the wild-type MR LBD, 2 nM [^3H]-aldosterone was used and, for the GMGG chimera, 10 nM [^3H]-aldosterone was used. As shown in Fig. 4, spironolactone was able to compete for [^3H]-aldosterone binding to both constructs. The IC_{50} values of spironolactone competition for [^3H]-aldosterone binding were calculated as described above (results not shown). The IC_{50} values of the MR LBD and GMGG chimera were 13 nM and 25 nM respectively. These values were higher than the corresponding IC_{50} values of spironolactone competition for [^3H]-dexamethasone binding, which reflects the higher affinity of aldosterone for the MR. They were consistent with the above results, showing that spironolactone had a similar affinity for the MR LBD and the GMGG chimera.

MR LBD model

Using the PR crystal structure, a model of aldosterone-bound MR LBD was generated (Fig. 5a). The structure consists of 11 α -helices (although to be consistent with the PR crystal structure the helices are labelled H1, followed by H3–H12) and a β -turn between helices 5 and 6. The binding pocket was predicted to consist of 23 amino acids. There are 16 hydrophobic residues in the binding pocket: Leu⁷⁶⁶, Leu⁷⁶⁹, Leu⁷⁷² and Ala⁷⁷³ in helix 3, Trp⁸⁰⁶, Met⁸⁰⁷ and Leu⁸¹⁴ in helix 5, Phe⁸²⁹ in the β -turn, Met⁸⁴⁵ and Leu⁸⁴⁸ between helices 6 and 7, Met⁸⁵² in helix 7, Leu⁹³⁸ and Phe⁹⁴¹ in helix 11, Val⁹⁵⁴ and Phe⁹⁵⁶ between helices 11 and 12, and Leu⁹⁶⁰ in helix 12. There are six polar residues in the binding pocket: Asn⁷⁷⁰ and Gln⁷⁷⁶ in helix 3, Ser⁸¹⁰ and Ser⁸¹¹ in helix 5, and Cys⁹⁴² and Thr⁹⁴⁵ in helix 11. Finally, there is one positively charged residue in the binding pocket: Arg⁸¹⁷ in helix 5. Figure 5b shows the positions of residues critical for ligand binding. Helix 3 (H3) lies behind the aldosterone molecule in this representation. In this helix, Asn⁷⁷⁰ is positioned to make hydrogen bonds with the 18- and 21-hydroxyl groups of aldosterone, and Gln⁷⁷⁶ is positioned to make a hydrogen bond with the 3-keto group of aldosterone as well as a water molecule. Helix 5 (H5) lies in front of aldosterone. In this helix, Arg⁸¹⁷ is also positioned

Figure 5 MR LBD model based on the crystal structure of the PR LBD (Williams & Sigler 1998). Shown are (a) the overall structural elements of the model and the position of aldosterone (amino acids 804–874 are coloured in grey), (b) a close-up of aldosterone binding in the model with the positions of amino acids known to be important for ligand binding and (c) a close-up of spironolactone binding in the model with the positions of amino acids that may interact with the molecule. The helices are numbered as per the published crystal structure of the PR LBD. The figures were prepared using the MOLSCRIPT (Kraulis 1991), Raster3D (Merrit & Bacon 1997) and InsightII programs.

to make a hydrogen bond with the 3-keto group of aldosterone and the same water molecule. Mutation of these residues has been shown to dramatically decrease the binding affinity of aldosterone (Fagart *et al.* 1998).

We fitted spironolactone into this model (Fig. 5c). It was assumed that spironolactone binds in the same orientation as aldosterone and that the 3-keto

group of spironolactone forms hydrogen bonds with Gln⁷⁷⁶ and Arg⁸¹⁷. Compared with aldosterone, the steroid backbone structure of spironolactone was displaced approximately 0.7 Å away from helices 3 (H3) and 7 (H7). In this position, the C₁₇ lactone ring of spironolactone fits easily into the binding pocket and the carbonyl group is positioned to make contacts with the side-chains of

Asn⁷⁷⁰ in helix 3 and Thr⁹⁴⁵ in helix 11. The C₇ sulfhydryl ester side-chain projects down from the plane of the page, and fits in between helices 3 and 7. It can make hydrophobic contacts with the side-chains of Leu⁷⁶⁹ from helix 3, and Leu⁸⁴⁸ and Met⁸⁵² from helix 7 without significant distortion.

Aldosterone and spironolactone binding to the MR LBD was also modelled using the co-ordinates of the more recently published GR LBD crystal structure (Bledsoe *et al.* 2002) (results not shown). The results did not differ significantly from the modelling based on the PR LBD.

Discussion

The MR antagonist spironolactone has a much higher affinity for the MR than it does for the GR (Couette *et al.* 1992*b*). This has allowed us to use chimeras created between the MR and GR ligand-binding domains to determine sequences in the MR that are important for spironolactone binding specificity. We have previously used the same approach to investigate determinants of aldosterone binding specificity to the MR (Rogerson *et al.* 1999). Spironolactone competed potently for both [³H]-dexamethasone and [³H]-aldosterone binding to the GMGG chimera, demonstrating that the difference in binding affinity of the antagonist to the MR and GR is conferred by amino acids 804–874 of the MR LBD. Interestingly, the same region of the MR contains sequences critical for aldosterone binding specificity (Rogerson *et al.* 1999). The other three chimeras tested in this study bind dexamethasone but not aldosterone (Rogerson *et al.* 1999). The MGGG chimera also binds spironolactone, although with an IC₅₀ value two orders of magnitude lower than that of the MR LBD. This result suggested that amino acids 672–803 of the MR LBD can make a weak, but still significant, contribution to spironolactone binding specificity. In contrast, the lack of binding of spironolactone to the GGMG and GGGM chimeras suggested that amino acids 875–984 of the MR LBD are not involved in the difference in spironolactone binding affinity between the MR and GR.

To investigate the interaction of the MR LBD with spironolactone we created models of the MR LBD bound to both spironolactone and aldosterone, based on the published crystal structure of the PR LBD bound to progesterone (Williams & Sigler

1998). In the model, amino acids 804–874 encompass α -helices 5–8, although helix 8 does not form part of the binding pocket. Helix 5 and the β -turn lie on opposite sides of the steroid A ring and helix 7 abuts the steroid D-ring (Fig. 5*b* and *c*). In the binding pocket of both the aldosterone- and spironolactone-bound molecules there are two residues that differ between the human MR and GR in this region (corresponding GR amino acids in brackets), Ser⁸¹⁰ (Met⁶⁰⁴) and Leu⁸⁴⁸ (Gln⁶⁴²). Serine⁸¹⁰ can be mutated with no effect on the affinity of aldosterone binding (Fagart *et al.* 1998). Interestingly, a Ser⁸¹⁰→Leu mutation in the MR has been found in eight individuals from the same family with severe early onset hypertension (Geller *et al.* 2000). One consequence of this mutation is the conversion of spironolactone into an MR agonist in *in vitro* transactivation assays, but with no apparent effect on binding affinity (Geller *et al.* 2000). It was concluded that the leucine mutation stabilises the active conformation of LBD by forming a hydrophobic interaction with Ala⁷⁷³ on helix 3 of the protein (Geller *et al.* 2000). Leucine⁸⁴⁸ of the MR corresponds to Gln⁶⁴² of the GR. In the crystal structure of the GR LBD, this residue interacts with the 17 α -hydroxyl group of dexamethasone (Bledsoe *et al.* 2002). It was proposed from the GR crystal structure that steroids lacking a 17 α -hydroxyl, such as spironolactone and aldosterone, will bind with low affinity to the GR because of the absence of this hydrogen bond (Bledsoe *et al.* 2002). However, corticosterone has the same affinity for the human GR as cortisol (Lind *et al.* 2000), but also lacks the 17 α -hydroxyl group, arguing against this hypothesis. In the spironolactone-bound structure, Leu⁸⁴⁸ is positioned to interact with the C₇ sulfhydryl-ester side-chain. It is unlikely, however, that Leu⁸⁴⁸ is critical for the binding specificity of spironolactone, as a glutamine residue appears in this position in the AR, which has a high affinity for spironolactone (Corvol *et al.* 1975).

The inability of the MR LBD model to identify residues involved in binding specificity suggested that the difference in the affinity of spironolactone for the MR and GR is conferred by amino acids in the (804–874) region of the MR LBD that do not interact directly with the ligand. There are precedents for this type of effect in other steroid hormone receptors. The specificity of dexamethasone binding to the GR involves two residues, Asp⁶⁴¹ and Leu⁶⁴⁷ (Robin-Jagerschmidt *et al.* 2000),

that do not interact directly with the ligand in the GR LBD crystal structure (Bledsoe *et al.* 2002). Similarly, the binding of the progestin RU27987 to the PR is conferred, in part, by a serine residue that lies on the surface of the receptor (Robin-Jagerschmidt *et al.* 2000). The guinea pig GR has a decreased affinity for glucocorticoids, due to amino acid differences with the human sequence that occur in a region that again does not form part of the ligand-binding pocket (Keightley *et al.* 1998). It is, of course, possible that binding specificity is conferred by amino acids in the MR ligand binding pocket that were not identified in the model, which was based on the PR LBD crystal structure. Because of this possibility, we subsequently modelled the MR LBD using the recently available co-ordinates of the GR LBD crystal structure (Bledsoe *et al.* 2002). It is very likely that the MR LBD is closest in structure to the GR LBD, given that the two proteins share 57% amino acid identity in this domain. The amino acids that form the binding pocket in this model are identical to those that form the binding pocket in the model based on the PR LBD (results not shown).

The result of the MGGG chimera has demonstrated that amino acids 673–803 of the MR make a weak contribution to spironolactone binding specificity. In the MR LBD model, amino acids 672–803 encompass α -helices 1, 3 and 4, although only helix 3 contributes to the ligand-binding pocket. Helix 3 forms one face of the pocket and is positioned to make interaction with the A, C and D rings of aldosterone and spironolactone, as well as the lactone ring of spironolactone (Fig. 5). There are two residues that differ between the human MR and GR in this region (corresponding GR amino acids in brackets), Leu⁷⁶⁶ (Met⁵⁶⁰) and Ala⁷⁷³ (Gly⁵⁶⁷). Mutagenesis and molecular modelling studies demonstrate that Ala⁷⁷³ sterically inhibits the binding of 11 β -substituted spirolactones to the MR (Auzou *et al.* 2000). Spironolactone itself, however, contains no substituent at C₁₁, so Ala⁷⁷³ is unlikely to be involved in binding specificity of the antagonist. Interestingly, Leu⁷⁶⁶ is conserved in the AR, but not in the GR. In the MR LBD model, Leu⁷⁶⁶ lies in close proximity to the lactone ring of spironolactone (results not shown). It is therefore possible that Leu⁷⁶⁶ contributes to the binding specificity of spironolactone, although its contribution will be small, due to the weak nature of spironolactone binding to the MGGG chimera.

A feature of MR antagonists is the presence of a C₇ side-chain. *In vivo*, spironolactone is converted to canrenone by the cleavage of this side-chain. Canrenone has a 10-fold lower affinity than spironolactone for the MR (Corvol *et al.* 1981). Therefore, hydrophobic interactions of the C₇ sulfhydryl-ester side-chain with Leu⁷⁶⁹, Leu⁸⁴⁸ and Met⁸⁵², predicted from the model, appear to be important for the affinity of spironolactone for the MR, although they do not account for binding specificity. *In vivo* canrenone is in equilibrium with the open E-ring metabolite canrenoate, in which the lactone ring has been cleaved to form a carboxylic acid. Canrenoate has a very low affinity for the MR (Corvol *et al.* 1981). Modelling of canrenoate binding (results not shown) suggests that the carboxylate oxygens could form hydrogen bonds with the side-chain atoms of Thr⁹⁴⁵ and Asn⁷⁷⁰. Binding of canrenoate, however, is unlikely to be energetically favourable, as there is no compensatory positive charge in the protein (i.e. an arginine or lysine) in the vicinity to match the negative charge of the carboxylate. This may explain the very low affinity of canrenoate binding to the receptor.

In summary, the same region of the MR LBD, encompassing amino acids 804–874, is critical for the binding specificity of the synthetic antagonist spironolactone and the natural ligand aldosterone. Modelling of the MR LBD bound to the two ligands suggests that this region forms part of the ligand-binding pocket; however, we were unable to identify amino acids in this region that may be important for binding specificity. Further work using MR:GR chimeras will be needed to identify the individual amino acids that confer ligand binding specificity in the MR. The results of the RALES trial (Pitt *et al.* 1999), using spironolactone, and the recently completed EPHEsus trial (Pitt *et al.* 2003), using the related MR antagonist eplerenone, demonstrate the therapeutic benefit of MR antagonists in the treatment of heart failure. An understanding of the binding of spironolactone to the MR may prove to be very useful in the development of new receptor antagonists for use in the treatment of heart failure and hypertension.

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