Structural analysis of the DNA-binding domain of alternatively spliced steroid receptors

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Abstract

We have generated 24 DNA-binding domain structure models of alternatively spliced or mutated steroid receptor variants by homology-based modeling. Members of the steroid receptor family dispose of a DNA-binding domain which is built from two zinc fingers with a preserved sequence homology of about 90%. Data from crystallographic analysis of the glucocorticoid receptor DNA-binding domain are therefore appropriate to serve as a template structure. We inserted or deleted amino acid residues in order to study the structural details of the glucocorticoid, mineralocorticoid, and androgen receptor splice variants. The receptor variants are created by QUANTA- and MODELLER-based modeling. Subsequently, the resulting energy-minimized models were compared with each other and with the wild-type receptor respectively. A prediction for the receptor function based mainly on the preservation or destruction of secondary structures has been carried out. The simulations

showed that amino acid insertions of one, four or nine additional residues of existing steroid receptor splice variants were tolerated without destruction of the secondary structure. In contrast, a deletion of four amino acids at the splice site junction leads to modifications in the secondary structure of the DNA-recognition helix which apparently disturb the receptor–DNA interaction. Furthermore, an insertion of 23 amino acid residues between the zinc finger of the androgen receptor leads to a large loop with an additional α -helical structure which seems to disconnect a specific contact from its hormone response element. Thereafter, the prediction of receptor function based on the molecular models was compared with the available experimental results from the *in vitro* function tests. We obtained a close correspondence between the molecular modeling-based predictions and the conclusions of receptor function drawn from *in vitro* studies.

Journal of Endocrinology (2002) **173**, 429–436

Introduction

The members of the steroid receptor family (glucocorticoid, mineralocorticoid, progesterone, androgen and estrogen receptors) act as ligand-dependent transcription factors. All receptors are provided with a highly conserved DNA-binding domain (DBD) consisting of two zinc fingers each, which are expressed by separate exons (Evans 1988). One zinc finger establishes the receptor–DNA contact by binding a specific hormone response element (HRE), the other is involved in receptor dimerization (Fig. 1). For example, the important amino acid residues responsible for the DNA–receptor interaction in the wild-type glucocorticoid receptor (GR) are localized in the P-box of the first zinc finger (C457–C460) and in the interfinger region (V462–Y474). Near the P-box, a transition in the secondary structure from a β -strand (C450–L455) to an α -helix (G458–E469) can be observed; this

was termed the DNA-recognition helix by Luisi *et al.* (1991). As well as the adjacent exon junction (exon 3 (G470) to exon 4 (Q471)), the D-box of the second zinc finger up to the amino acid D485 builds an extended coil, which is followed by a second helix (P493–L504). The molecular shape of the zinc fingers is determined by the tetrameric co-ordinative binding of four cysteines with a central Zn^{2+} ion (Luisi *et al.* 1991). Thus, the conserved amino acid sequence and the resulting 3D structure provide for an optimal, specific DNA binding and are a prerequisite for regulating target gene expression by steroid hormone receptors.

Alternative splicing of exons 3 and 4 of the mineralocorticoid receptor (MR) and GR leads to mRNAs with an additional in-frame insertion in the interfinger region between the two zinc fingers of the DBD (Fig. 1). Here, the alternative used donor splice site at the end of exon 3/intron C rejoins with the conserved acceptor splice

Figure 1 Organization and structure of the DNA binding domain of the MR, GR and AR genes. White boxes, untranslated exons; grey boxes, translated exons. Amino acid sequence in one letter code: grey letters, DNA identification sequence. Amino acids marked with a plus sign form α helices. ZF, zinc finger.

site of intron C/exon 4 (Bloem *et al.* 1995). Corticoid receptors (MR and GR) from different species with an insertion of one (GR+1 in primate), four (MR+4 in human, rat and *Xenopus laevis*) or nine (GR+9 in fish) additional amino acid residues were described by several groups (Brandon *et al.* 1991, Bloem *et al.* 1995, Csikos *et al.* 1995, Ducouret *et al.* 1995, Tokuda 1998, Wickert *et al.* 1998). Surprisingly, despite the extension of nine amino acids between the two zinc fingers, the fish GR+9 showed transcriptional activity in a luciferase reporter gene assay (Ducouret *et al.* 1995). Due to a computer-based reconstruction of the phylogenetic tree, these authors suggested that the GR+9 splice variant appears to be the most ancient receptor of the GR group. However it remains obscure how the receptor accomplishes the

binding to the glucocorticoid response element (GRE) which was used in the reporter gene assay (Ducouret *et al.* 1995).

The interfinger region of the GR DBD seems to be flexible and can cope with insertions of up to 27 amino acid residues according to Zandi *et al.* (1993). A receptor fragment consisting of its N-terminal domain and a partial duplication of the second zinc finger in the DBD showed a limited activity of 10–20% when compared with the wild-type receptor in a transactivation test (Zandi *et al.* 1993).

Moreover, an androgen receptor (AR) with 23 additional amino acid residues (AR+23) which were inserted at the splice site junction of the zinc finger coding exons 2 and 3 has been found in a family with partial

androgen insensitivity (Brüggenwirth *et al.* 1997). In this particular case, the acceptor splice site was used alternatively and 69 base pairs of the intronic sequence, upstream from exon 3, were inserted. The additional residues between the two zinc fingers of the AR+23 lead to a non-functioning receptor protein *in vivo* and *in vitro* (Brüggenwirth *et al.* 1997). A gel retardation assay showed that the AR+23 splice variant was unable to contact the corresponding DNA sequence (androgen response element) *in vitro*.

To verify the structural changes in the interfinger region of the glucocorticoid, mineralocorticoid, and androgen receptor splice variants we used computer-based modeling by homology. In this study 24 DBD receptor models were generated using the QUANTA Protein Design tool (Molecular Simulations, Inc. 1997) and/or the MODELLER program (Šali & Blundell 1993) and were compared with the GR wild-type (wt) zinc finger model. Following our structure prediction the hinge region of the zinc finger coding exons of the GR appears to be flexible due to the insertion of several amino acids (up to nine were tested). However, a deletion of four residues upstream or downstream from the exon junction shortened the DNA-recognition helix by about one and a half helical turns. Furthermore, all AR+23 models created by MODELLER (five models per sequence) showed an additional α -helix. Two predicted models according to the sequence AR+23 (GDB M27425) have intimate contact with the DNA molecule, the inserted amino acid residues of one model had only a slight DNA contact, and in two cases the inserted amino acid residues were distorted towards the outside of the protein. The other AR+23 models obtained from sequences GDB M35846 and GDB120556 (see below) resulted in no appreciable difference to the models above. Moreover, the comparison between our prediction of receptor function correlated well with the available transactivation tests.

Materials and Methods

Sequence alignment and secondary structure prediction

Sequence–sequence alignments were computed using standard dynamic programming-based alignment methods. A database search for homologous nucleic acid and amino acid sequences was carried out with BLAST (Altschul *et al.* 1997) over SWISS-PROT (Bairoch & Apweiler 1997). The following sequences were aligned: (i) AVEGWRAR QNTDGGHNY, the inserted nine amino acids (GR+9) with the adjacent four residues (adjacent residues, AVEG and QHNY were involved, since these are conserved amino acid residues of this receptor family); (ii) the inserted 69 bp nucleotide sequence from AR+23 using data from the Genome Databank (GDB): GDB M27425 (Lubhahn *et al.* 1989), GDB M35846 (Tilley *et al.* 1989, Marcelli *et al.* 1990) and GDB 120556 (Brüggenwirth

et al. 1997) respectively; (iii) the corresponding translated amino acid sequence consisting of 23 residues, forward as well as reverse respectively. Secondary structure prediction has been carried out with CoDe, a method combining the results of well-known approaches such as PHD, Predator, and DSC (Rost & Sander 1993, Frishman & Argos 1996, King & Sternberg 1996) to a consensus secondary structure prediction. CoDe is integrated into the ToPLign system (Mevissen *et al.* 1994, Selbig *et al.* 1999).

Molecular modeling

For homology-based modeling the crystallographic analysis of the GR DBD published by Luisi *et al.* (1991) (protein data bank (PDB) entry: 1 glu; PDB (Bernstein *et al.* 1977)) and the GR NMR-structure published by Bauman *et al.* (1993) (PDB entry: 2 gda (24 structures) or 1 gdc (minimized average structure)) were used. The GR DBD sequence was edited using the QUANTA Protein Design tool. The CHARMM force field-based energy minimization was initially performed with the steepest descent algorithm and finalized using the conjugate gradient method. In general, energy minimization calculations as part of homology modeling are performed with parts of the molecule held fixed. This is necessary because alterations of the structure induced by minimization are dragged towards lowering the energy. Structures of low potential energy are useless if they come with poor geometry. For instance, in the case of substituting amino acids, only the energy of the modeled protein side chain should be minimized. The situation is more complicated in cases where several amino acids are deleted or inserted. For this type of alterations there are no coordinates of backbone atoms which can be copied from the template. Therefore, larger regions of the protein have to be set free for minimization. Because we were interested, mainly, in whether secondary structures are preserved or destroyed, we tested several variable residue ranges extending in both the N- as well as the C-terminal direction from the insertion or deletion site (plus/minus 1, 4 and 20 amino acids). The following protein models were subjected to the simulations: human (h) GRwt, marmoset (mt) GR+1, *Onchorhynchus mykiss* (om) GR+9, hGR-G, hGR-Q, hGR-AVEG, hGR-QHNY, MRwt, MR+4, as receptor monomers.

For modeling the AR+23 structure a different protocol had to be applied. Currently, there is no modeling tool that could model segments reliably whose length exceeds 10–12 residues (A Fiser & A Šali, personal communication). A database search of the 23 residue fragment with BLAST against the PDB structures provided the homeo-domain of the thyroid transcription factor 1 (PDB entry: 1 ft) as a possible template. Thus, together with the GR DBD as a template for the basic AR receptor structure, a hybrid model could be derived with the MODELLER program. According to the AR cDNA sequence (Tilley

Table 1 Multiple alignment of different steroid receptors which are alternatively spliced in their DBD. Sequence alignments of the steroid receptor splice variant DBD models. Secondary structures according to the QUANTA assignment are shown in the following colored letters: green=beta bulge, yellow=H-bonded β-strand, violet=α-helix, blue=3er-turn, red=4-turn, black=irregular structure

Receptor	Sequence		
	zinc finger 1	exon junction	zinc finger 2
	P-Box		D-box
hGRwt, hMRwt (1)	LTCGSCKVFFKRAVEG		QHNYLCAGRNDCIIDKIR
hMR+4, rMR+4 (2,3)	LTCGSCKVFFKRAVEG		KCSWQHNYLCAGRNDCIIDKIR
xlMR+4 (4)	LTCGSCKVFFKRAVEG		KCSRQHNYLCAGRNDCIIDKIR
GR+1 (5)	LTCGSCKVFFKRAVEG		RQHNYLCAGRNDCIIDKIR
om, poGR+9 (6,7)	LTCGSCKVFFKRAVEG		WRARQNTDGQHNYLCAGRNDCIIDKIR
hGR exon 3 del G (8)	LTCGSCKVFFKRAVE		QHNYLCAGRNDCIIDKIR
hGR exon 4 del Q (8)	LTCGSCKVFFKRAVEG		HNLYCAGRNDCIIDKIR
hGR exon 3 del AVEG (8)	LTCGSCKVFFKRAVEG		QHNYLCAGRNDCIIDKIR
hGR exon 4 del QHNY (8)	LTCGSCKVFFKRAVEG		LCAGRNDCIIDKIR
hARwt	LTCGSCKVFFKRAAEG		KQKYLCA SRNDCTIDKIR
hAR+23 (GDB M27425) (9)	LTCGSCKVFFKRAAEGKYPKKETLETHYQVYQLLLYFSQKQKYLCA SRNDCTIDKIR		
hAR+23 (GDB M35846) (9)	LTCGSCKVFFKRAAEGKYPKKETLETHYQVYQLLLYFVFSQKQKYLCA SRNDCTIDKIR		
hAR+23 (GDB 120556) (9)	LTCGSCKVFFKRAAEGKYPKKETLETHYQVYQLLLYFVFSQKQKYLCA SRNDCTIDKIR		

h, human; r, rat; xl, *Xenopus laevis* (Anura, amphibia); mt, marmoset (Callitrichidae, new world monkey); om, *Onchorhynchus mykiss* (Teleostei, fish); po, *Paralichthys olivaceus* (Actinopterygii, fish); wt, wild type; del, deletion. (1) Luisi *et al.* (1991); (2) Bloem *et al.* (1995); (3) Wickert *et al.* (1998); (4) Csikos *et al.* (1995); (5) Brandon *et al.* (1991); (6) Ducouret *et al.* (1995); (7) Tokuda (1998); (8) Zandi *et al.* (1993); (9) Brüggewirth *et al.* (1997).

et al. 1989) the following amino acids were substituted: V468 to A; Q471, H472, N473 to K, Q, and K respectively; G478 to S and I483 to T. Sequence specification of human AR intron 2/exon 3 junction varied. Hence, 15 hAR+23 hybrid models were generated according to the sequences from GDB M27425, M35846 and 120556 respectively.

Results and Discussion

Utilizing the zinc finger homology of the steroid hormone receptors we generated 24 computer-based protein models with the QUANTA Protein Design tool and MODELLER by using the data of the crystallographic analysis of the GR DBD (Luisi *et al.* 1991). The colored alignment in Table 1 indicates the changes in the secondary structure of the different steroid receptor DBD models.

Human, rat and *Xenopus laevis* MR+4 (hMR+4, rMR+4, xlMR+4)

The published hMR+4 DBD model with the inserted amino acids KCSW contains a small loop which was distorted to outside the protein (Wickert *et al.* 2000). In our study a new model was created with a transposition of W to R. The superposition of both energy-minimized MR+4 models shows no appreciable difference in the loop shape and orientation. Interestingly, the arginine (R)

residue of the xlMR+4 model oriented towards the tryptophan (W) residue of the hMR+4 model in a perpendicular manner. Apparently, in this case it appears to be of no importance whether the inserted amino acid sequence is KCSW (human and rat) or KCSR (*Xenopus laevis*).

Marmoset GR+1 and *Onchorhynchus mykiss* GR+9 (mtGR+1, omGR+9)

The insertion of an arginine in the marmoset GR (GR+1) occurs at a position which coincides with single amino acid insertions in the human AR (K405) and the V-erb-A and C-erb-A protooncogenes (N68 and N82 respectively) (Brandon *et al.* 1991). According to our model, this additional residue allows for a new hydrogen bond between the oxygen of the glycine (G470) and the hydrogen of the histidine (H472) of the primate GR+1. Therefore, a new 3-turn at the exon junction was formed (Table 1, blue) between the arginine (inserted residue) and the glutamine (Q471). The modified secondary structure in the GR+1 model would support the hypothesis of Brandon and colleagues (1991), who suspected a change in the affinity of receptor–DNA interaction.

Surprisingly, another GR splice variant was described by Ducouret and co-workers (1995). Here, nine additional amino acid residues (WRARQNTDG) were inserted in the interfinger region of the GR DBD of the rainbow trout *Onchorhynchus mykiss*. Transfection studies showed that the GR+9 binds mammalian GRE and is able to activate a reporter gene. As with the MR+4 model

Figure 2 Superposition of the generated GRwt, GR-QHNY and AR+23 DBD models. Crystal structure data for computer-based modeling by homology were obtained from GR (Luisi *et al.* 1991) (PDB 1 glu). Here, only chain A monomers of GRwt (blue), GR-QHNY (red) and AR+23 (yellow) were superimposed. The red arrow shows the shortening of the DNA-recognition helix in the GR-QHNY model. The additional helical structure formed by the inserted amino acid residues of the AR+23 model is marked by a yellow arrow. The ribbons indicate α -helices which are regular regions of the protein fragments. The threads are non-regular regions. DNA is shown as a double-helix ladder with rungs in metallic green.

(Wickert *et al.* 2000), the GR+9 model emulated a loop structure distorted to outside the protein.

Notably, in all generated corticoid receptor DBD models with an insertion of one, four or nine amino acid residues, the glutamic acid (E469) was excluded from forming the DNA-recognition helix (Table 1).

Human GR-G, GR-Q, GR-AVEG and GR-QHNY

In 1993 Zandi and colleagues noted that no functional GR with a deletion in the interfinger region could be obtained. In their opinion a shortening of the interfinger region would force the zinc finger into a different, unsuitable angle. This would disrupt the hydrophobic core which is formed in the wild-type receptor between some residues of the DNA-recognition helix and the following helix in

the second zinc finger (C493–G504). Apparently, the hydrophobic core seems to play an important role in the mutual position of the two zinc fingers (Zandi *et al.* 1993). To verify this hypothesis, we generated GR DBD models with several deletion mutations at the exon junction between exons 3 and 4. One or four amino acid residues were deleted from the donor splice site as well as from the acceptor splice site, GR-G, GR-Q, GR-AVEG and GR-QHNY respectively. Superposition of the GR-G and GR-Q resulted in nearly congruent models and both showed a slight deviation when compared with the wild-type receptor model.

The deletion of four amino acid residues in the DBD results in larger conformational changes in the interfinger region. As the wild-type receptor model showed a helical conformation in the interfinger region from the amino

Table 2 Secondary structure prediction of the 23 amino acids inserted into a human androgen receptor splice variant. Comparison of the results of selected secondary structure prediction methods used by the consensus prediction program CoDe with the secondary structure of the template for the 23 inserted amino acids

23 Inserted amino acids	K	Y	P	K	K	E	T	L	E	T	H	Y	Q	V	Y	Q	L	L	Y	L	F	S	Q
Sequence fragment 1ftt	—	—	—	K	R	R	V	L	F	S	Q	A	Q	V	Y	E	L	E	R	R	F	K	Q
DSSP assignment	—	—	—	C	C	C	C	C	C	C	H	H	H	H	H	H	H	H	H	H	H	H	H
Prediction program																							
PHD	H	H	H	C	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	C	C
Predator	C	C	C	C	C	C	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	C
DSC	C	C	C	C	C	C	C	C	C	C	C	H	H	H	H	H	H	H	C	C	C	C	C
CoDe consensus	C	C	C	C	C	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	C

Amino acid sequences in one-letter code; 1ftt, thyroid transcription factor 1; DSSP, database of secondary structure in proteins (Kabsch & Sander 1983); C, coil; H, α -helix. The boxes indicate identical residues.

acid residues G458 to E469, the α -helical structure in the deletion mutant models GR-AVEG and GR-QHNY were damaged (Table 1, Fig. 2). Both models showed a shortening in the interfinger helix of about six residues that corresponds to one and a half helical turns. The amino acids FKRA (464–467) of the GR-QHNY model build an apparent 5-turn structure, which is really composed of two overlapping 3-turn structures (Fig. 2, red arrow). Our structure prediction of the deletion splice variants supports the hypothesis of Zandi and co-workers (1993) which states that the mutual distance of the two zinc fingers is changed. One consequence might be that these receptors are unable to bind the hormone response element in the promotor region of the target gene. It is known from gene-targeting studies with disrupted glucocorticoid receptors in mouse that a failing GR leads to death within the first hours after birth (Berger *et al.* 1996). This might be the reason why no GR with a deletion in the interfinger region was discovered.

Human ARwt and AR+23

The BLAST-based comparison of the 23 amino acid residues with sequences of known structures from the PDB resulted in a potential relatedness between this fragment and the homeobox domain of the thyroid transcription factor 1. Interestingly, a corresponding search in several sequence databases did not produce this hit. This may result from the fact that the alignment values depend on the size of the database against which a target sequence is compared. The potential relatedness is supported by the secondary structure of the 23 amino acid fragment predicted with CoDe (Selbig *et al.* 1999) (Table 2). The structural fragment from the thyroid transcription factor 1 (PDB: 1 ft) together with the GR DNA-binding domain (PDB entry: 1 glu) allowed us to generate a hybrid model for AR+23. For each of the three observed human AR+23 sequences we derived five models. In all of these models the inserted 23 amino acid residues form a large

loop with an adjacent additional α -helix varying in length by 1–2 residues. Several of these models feature an intimate contact with the DNA molecule, touching it beside the regular cis-active DNA sequence, which is the HRE (Fig. 2, yellow arrow). The loop structure of another AR+23 model (GDB M27425) has only slight DNA contact and in two cases the loop of the inserted amino acid residues was distorted to the protein outside. Figure 2 illustrates an AR+23 DBD model that provided the best structural explanation for the loss of receptor function. In all fifteen models the side chains of the inserted amino acid residues require so much space that a normal contact with the HRE seems to be impossible. The unusual mutated AR+23 caused a partial androgen insensitivity, which can be explained either by a damaged DNA contact through the inserted loop or by a failure in dimerization with the second hormone–receptor complex. The probable loss of the ability of DNA binding is supported by results from a gel-retardation assay obtained from Brüggewirth *et al.* (1997).

Prediction by homology-based molecular modeling versus results obtained from *in vitro* assays

Furthermore, we compared our prediction of receptor function with the available *in vitro* transactivation tests on the basis of the steroid receptor models. As mentioned above, both methods correlated highly (Table 3). All steroid receptor splice variant models allow for structural explanations which confirm the experimental findings. Through revised and further developed methods for sequence–structure alignments and the homology-based modeling the reliability of the predicted structures improved (Kolinski *et al.* 1999, Šali & Kuriyan 1999, Selbig *et al.* 1999). Even sequences with a relatively low identity could be used for modeling by homology (Tramontano 1998) and this method could serve as a tool for gene annotation strategies (Francoijs *et al.* 2000).

Table 3 Prediction of receptor function from *in vitro* experiments of different steroid receptor splice variants in comparison with the predicted receptor function based on modeling by homology

Receptor models	ins/del amino acids in the interfinger region	Receptor function in <i>in vitro</i> experiments	Structure changes in DBD of the receptors	Prediction of the receptor function through modeling by homology
r+hMR+4 (1,2)	ins: KCSW	n.d.	The additional residues form a loop that is distorted to outside the protein	Function comparable to the wild-type receptor and/or different
xMR+4 (3)	ins: KCSR	?	The additional residues form a loop that is distorted to outside the protein	Function comparable to the wild-type receptor and/or different
hGRwt (4)	—	—	Crystal structure used for homology modeling	—
mtGR+1 (5)	ins: R	Yes (change in binding affinity is suspected)	A new hydrogen bond allows the formation of a new 3-turn (aa 470-472)	Function comparable to the wild-type receptor
om+poGR+9 (6,7)	ins: WRARQNTDG	yes	The loop is distorted to outside the protein, QNTD (aa 471-474) have a slight tendency to form a 5-turn	Function comparable to the wild-type receptor
hGR-G (8)	del: G	n.d.	Slight deviation from the wild-type receptor	Function comparable to the wild-type receptor
hGR-Q (8)	del: Q	n.d.	GR-G and GR-Q model were almost congruent	Function comparable to the wild-type receptor
hGR-AVEG (8)	del: AVEG	n.d.	α -Helical structure from aa 458-469 is reduced to aa 458-463	Functions poorly or not at all
hGR-QHNY (8)	del: QHNY	n.d.	α -Helical structure from aa 458-469 is reduced to aa 458-463, FKRA (aa 464-467) build two overlapping 3-turns	Functions poorly or not at all
hAR (9)	ins: KYPKKELETH(Y) YQVYQLLY(F)L(V)FSQ	no (<i>in vivo</i> : no)	All predicted hybrid-structure models formed a wide loop including an additional α -helix which was diverse in its orientation	No function
hAR (10)	aa substitution in the interfinger region and the second zinc finger	no (<i>in vivo</i> : no)	V (581) to F affected protein-DNA interaction, L (616) to P destabilized the protein structure or caused dimerization	Molecular modeling was done by the authors (10); results verified the <i>in vitro</i> experiments

po, *Paralichthys olivaceus*; ins, insertion; del, deletion; n.d., not done; aa, amino acid (amino acids in one-letter code).
(1) Bloem *et al.* (1995); (2) Wickert *et al.* (1998); (3) Csikos *et al.* (1995); (4) Luisi *et al.* (1991); (5) Brandon *et al.* (1995); (6) Ducouret *et al.* (1991); (7) Tokuda (1998); (8) Zandi *et al.* (1993); (9) Brüggewirth *et al.* (1997); (10) Lobaccaro *et al.* (1996).

Actually, we were not able to generate NMR-structures or crystal structures from every splice variant. Therefore, modeling by homology seems to offer the only possibility of describing and explaining alterations in molecular structures. Future experiments where homology models are generated in advance of functional examinations will show whether functional predictions from homology models are possible.

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Received 18 February 2002

Accepted 4 March 2002