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Molecular Modelling of the Human Estrogen Receptor and Ligand Interactions Based on Site-directed Mutagenesis and Amino Acid Sequence Homology

David F. V. Lewis,[1]* Malcolm G. Parker[2] and Roger J. B. King[3]

[1]*Molecular Toxicology Group and* [2]*Cell Regulation Group, School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH, U.K. and* [3]*Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, U.K.*

A molecular model of the human estrogen receptor is reported based on a new alignment with the α_1-antitrypsin sequence, a homologous protein of known crystal structure. The putative ligand binding site is situated roughly equidistant between the DNA binding and dimerization regions. This binding site contains a number of amino acid residues shown by site-directed mutagenesis to be associated with the binding of agonists and antagonists. This putative ligand binding pocket is well-defined within a loop of peptide, containing complementary amino acids for binding interactions with agonists and antagonists. A leucine-rich region, common to most steroid-binding proteins, is in an optimum position for dimerization leading to DNA interaction. It is likely that ligand binding influences dimerization and DNA interaction by a conformational change in the receptor via the transcriptional activation residues. This model suggests that ligand binding may affect the hydrogen bonding pattern such that transpeptide signalling is initiated. The model accommodates steroidal estrogens and antiestrogens as well as the non-steroidal partial antagonist, hydroxytamoxifen.

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INTRODUCTION

The human estrogen receptor (hER) is a member of the steroid receptor superfamily [1, 2] which includes the androgen, glucocorticoid, progesterone [3], retinoic acid, peroxisome proliferator [4] and thyroid hormone [5] receptors [6–10] all of which share common structural motifs, such as a DNA binding region consisting of two "zinc fingers" [11, 12], a dimerization region (leucine zipper) and a ligand binding domain [13, 14].

The crystal structure of the glucocorticoid receptor DNA binding domain has been reported recently [15], together with a solution structure [16] and NMR studies have been carried out on the human estrogen receptor DNA binding region [17]. In addition to structural information from X-ray crystallography and NMR data for DNA binding and dimerization regions [18], it has been possible to construct molecular models for portions of some steroid superfamily receptors based on amino acid sequence homology with proteins of known crystal structure [19–22]. In particular, it appears that human α_1-antitrypsin [23] possesses both structural similarity and sequence homology with many members of the steroid hormone-binding receptor superfamily [20, 22] and also with other steroid-binding proteins [20], such as human corticosteroid binding globulin [24].

Site-directed mutagenesis experiments on the mouse estrogen receptor have identified specific residues involved in ligand binding, dimerization and transcriptional activation [25–28]. Previous molecular modelling studies have indicated that potentially useful information about ligand binding sites and relative binding energies can be obtained from computational approaches [19, 29] applied to steroid-hormone receptor ligand interactions. Any such modelling should take account of the different effects of agonists and antagonists. Agonists such as estradiol promote ER dimerization, DNA binding and transcriptional activation

*Correspondence to D. F. V. Lewis.
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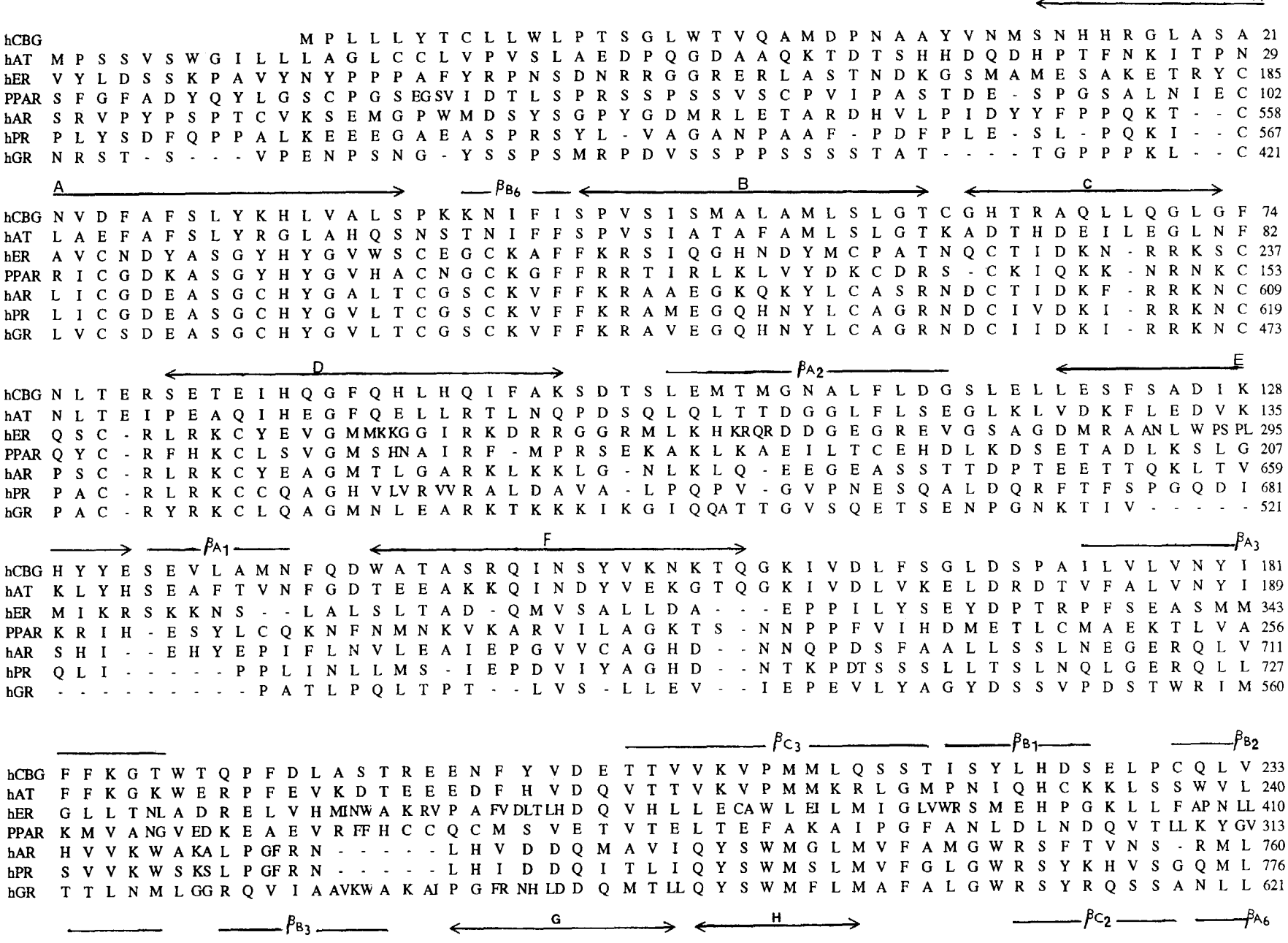
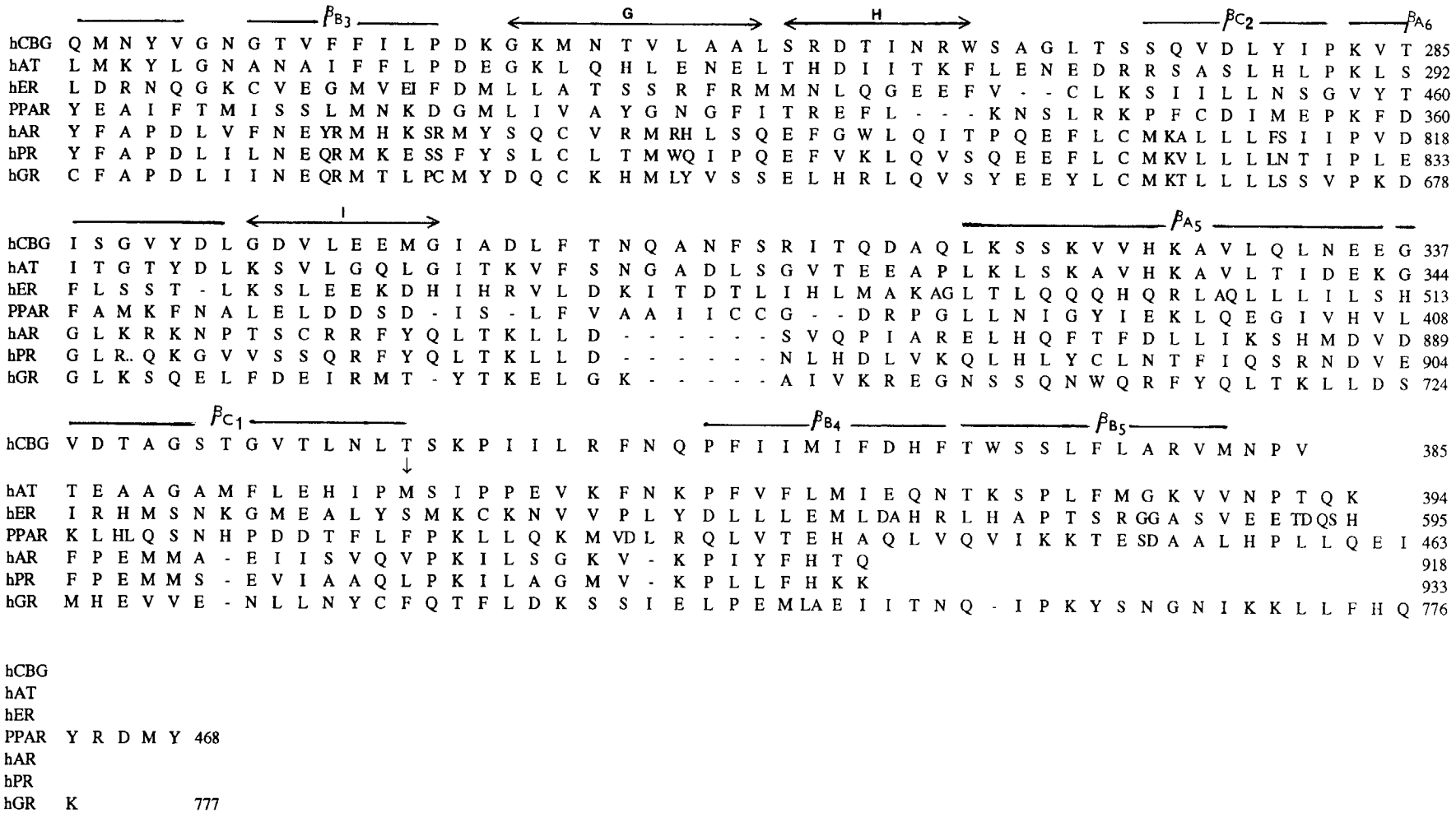
Table 1. Alignment of the amino acid sequences of steroid hormone receptors and human α_1-antitrypsin





hCBG, human corticosteroid binding globulin; hAT, human α_1-antitrypsin; hER, human estrogen receptor; PPAR, peroxisome proliferator-activated receptor; hAR, human progesterone receptor; hGR, human glucocorticoid receptor.

↓, elastase cleavage site.

—, 23 residues omitted to facilitate alignment.

by interaction with other transcription factors through the transactivation domains (TAF). Antagonists of the triphenylethylene class, such as tamoxifen, will achieve all of the above except the TAF2 interaction [30]. The steroidal antagonists substituted via position 7, such as ICI 164384, have a more controversial action. Some studies suggest that DNA binding of ER does not occur, whereas others indicate that it does [31]. Whatever the true situation might be, there is a clear difference in biological profile of compounds in the tamoxifen and ICI 164384 series. Hence, modelling of the interaction of representative compounds of agonist and two types of antagonist with ER would be informative.

This work represents the application of molecular modelling to examining and estimating human estrogen receptor ligand interactions based on sequence homology, site-directed mutagenesis and crystallographic information.

METHODS

Based on previous sequence alignments [20, 21, 23, 24, 32] we have produced a new alignment between a number of steroid-binding proteins and the human α_1-antitrypsin sequence (Table 1). Although the percentage identity between the α_1-antitrypsin and ER sequences was quite low (11%) consideration of conservative changes improved the overall homology to 25%, and both of these criteria were employed in the alignment process. Using this alignment we have constructed a model for the hER by amino acid replacement from the human α_1-antitrypsin crystal structure [33] to that of the human estrogen receptor [7] followed by energy minimization, utilizing the Sybyl molecular modelling software package (Tripos Associates, St Louis). Geometry optimization was carried out using the Tripos force field [34] from within the Sybyl program, running on an Evans and Sutherland ESV30 graphics workstation. Ligand binding interaction energies were estimated from the differences between calculated minimum energies of the ligand–receptor complex and its individual components. The ligands chosen for these binding studies were estradiol (agonist), hydroxytamoxifen (partial agonist) and ICI 164384 (pure antagonist).

RESULTS

The overall proposed molecular structure of the ligand-bound human estrogen receptor is shown in Fig. 1, where the positions of the zinc finger DNA binding motifs, dimerization and putative ligand binding sites have been indicated. The latter is represented by a well-defined pocket resulting from a loop in the polypeptide chain. A tyrosine residue position (459) lies at the mouth of this pocket and is the most likely side-chain for interaction with the A-ring of estradiol, due to the possible hydrogen bonding and π–π stacking interactions between the two phenolic substructures. This residue corresponds to phenylalanine in the peroxisome proliferator receptor (ppar) in our alignment, and this particular phenylalanine appears to form part of the ligand binding site in a previously reported model of the ppar [19].

The putative ligand binding site in hER contains a number of hydrophobic residues and a glutamate residue (419) which is able to accept a hydrogen bond from the D-ring hydroxyl group of estradiol as shown in Fig. 2. The pocket tapers as it extends further into the protein towards the DNA binding domain where aspartate-545 forms an ion pair with arginine-210, which is itself part of the signal peptide Lys-206 Ala-207 Phe-208 Phe-209 Arg-210, a highly conserved sequence that interacts with the DNA response element.

Trans-4-hydroxytamoxifen is able to fit this pocket with its phenolic group occupying the same position as that of the A-ring of estradiol. Glutamate-419 is unable to form a hydrogen bond with this antagonist, however, as there is no available hydrogen bond donor species corresponding to the same position as the D-ring hydroxyl of estradiol. However, the long side-chain of tamoxifen extends into an essentially hydrophobic channel at the end of the putative binding site, where the protonated nitrogen can ion-pair with aspartate-545, preventing this residue from interacting with arginine-210. The binding orientation of *trans*-4-hydroxytamoxifen is shown in Fig. 3. Interestingly, the *cis*-isomer of 4-hydroxytamoxifen, which acts as a partial agonist, is able to fit this putative binding site, but the side-chain cannot form an ion-pair with aspartate-545. Instead, serine-554 will accept a hydrogen bond from the protonated tertiary amino group on *cis*-hydroxytamoxifen. This ligand interaction is displayed in Fig. 4. A second antagonist, ICI 164384, is also capable of binding in the same pocket, even though this estradiol analogue possesses an extremely long side-chain. In this case, the ligand accepts a hydrogen bond from serine-522 to the amide carbonyl oxygen on the side-chain, which adopts a slightly angled conformation when bound, as shown in Fig. 5.

The antagonists give rise to greater interaction energies with the hER model than do the agonists, as is presented in Table 2, where it can be seen that *trans*-4-hydroxytamoxifen and ICI 164384 display approximately two times the binding energy of the agonists estradiol and *cis*-4-hydroxytamoxifen.

The DNA binding domain, spanning residues 185 to 250, comprises two helix-turn-helix motifs with eight conserved cysteine residues (185, 188, 202, 205, 221, 227, 237 and 240) which lies at the bottom of two "zinc fingers". At the end of the first set of four cysteines lies the signal peptide sequence (Lys-206 to Arg-210) required for binding to the hER response element on DNA, which consists of one half of a palindromic

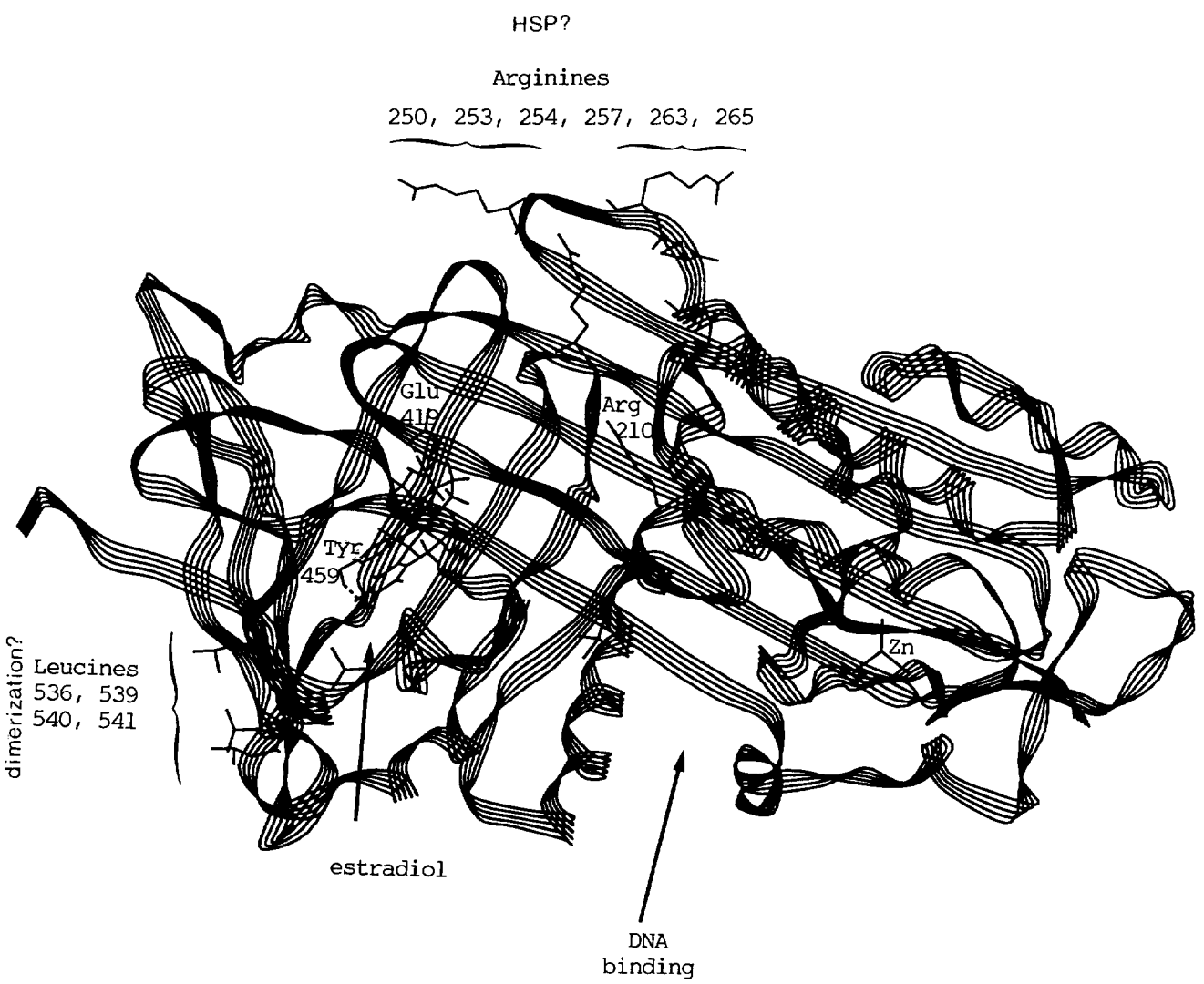


Fig. 1. Structure of the hER model showing potential ligand binding, DNA binding, heat shock protein interaction and dimerization.

nucleotide sequence requiring dimerization of the receptor on DNA for transcriptional activation to occur. The signal recognition penta-peptide residues lie on the surface of the receptor protein and, presumably, these become exposed prior to DNA interaction as a result of the conformational changes which accompany ligand binding.

The dimerization region containing a number of leucines and isoleucines which form a "leucine zipper" motif of surface residues (Ile-451, Ile-452, Leu-453 and Leu-454) lies in relatively close proximity to the putative ligand binding site, at a distance of about 12 Å. There is also a stretch of leucine residues (Leu-507, Leu-508, Leu-509 and Leu-511) which, in some instances, form hydrophobic contacts with all of the bound ligands. Site-directed mutagenesis experiments indicate that this region is sensitive to ligand binding [28]. In fact, the serine residue (position 522) which is able to hydrogen bond with the side-chain amide group of ICI 164384, lies within this region probed by mutagenesis experiments and known to modify ligand binding characteristics. Furthermore, aspartate-545, which ion pairs with the tamoxifen protonated amino group, forms part of the transcriptional activation region [26]. As this latter residue also forms an ion-pair with arginine-210 in the estradiol-bound receptor, the combination of site-directed mutagenesis and homology modelling suggests that a key stage in the mechanism of activation of the hER is the electrostatic interaction between the ligand binding and DNA binding site, which may be modulated by the conformational change that accompanies binding to the putative ligand binding site.

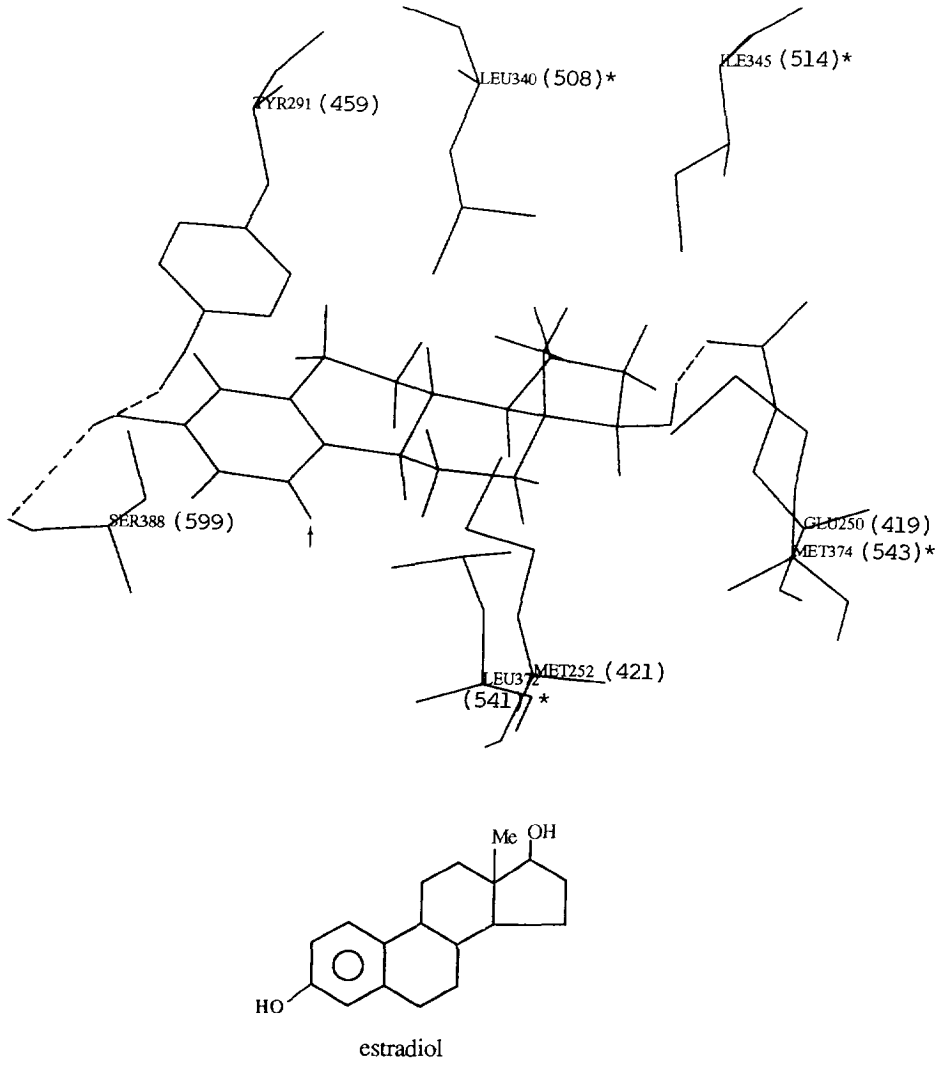


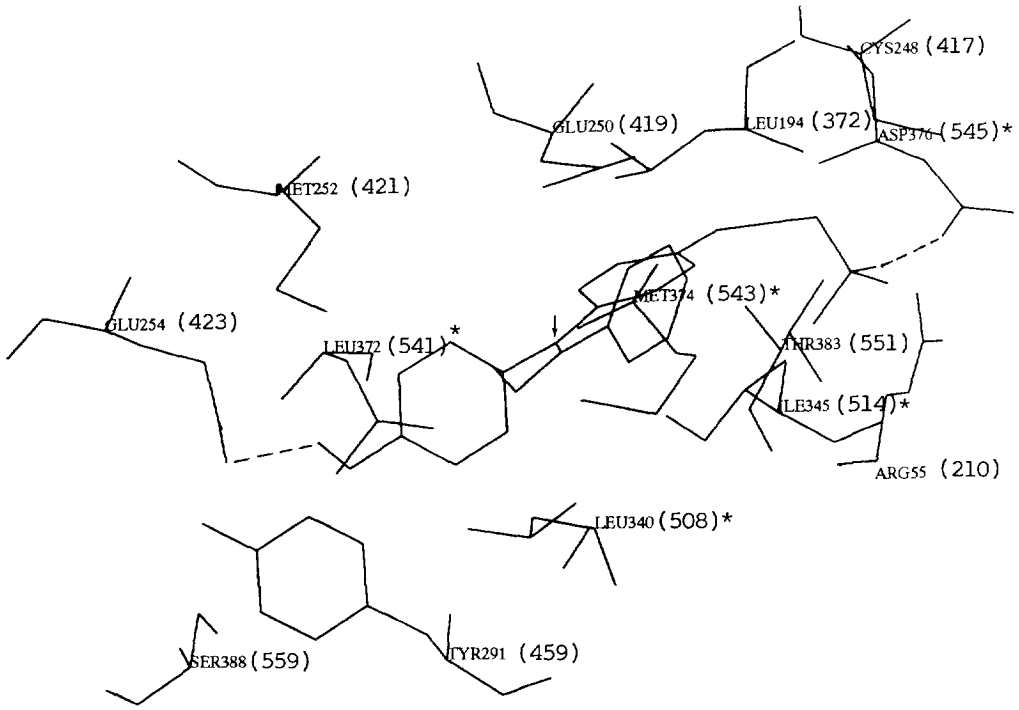
Fig. 2. Putative ligand binding site in the hER model with bound estradiol. The ligand is arrowed in the figure and also shown as a conventional 2D structure beneath. *Denotes residues shown by site-directed mutagenesis to be involved with ligand binding. Residues are numbered according to their alignment with α_1-antitrypsin. Actual sequence positions are placed in parentheses.

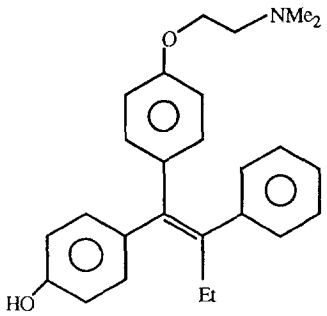
DISCUSSION

Presumably, opening of the DNA binding region would result from disruption of the electrostatic interactions between aspartate-545 and arginine-210, thus exposing the latter for interaction with the DNA response element. The putative ligand binding site lies roughly equidistant between the DNA binding and leucine zipper regions, being approximately 12 Å from each. The close proximity of the ligand binding site to both of these regions of mechanistic importance, suggests that there are likely to be cooperative conformational changes in this section of the receptor following ligand binding. There is sufficient energy associated with binding to drive the required conformational change to produce activation of the receptor, and the orientation of the leucine zipper region is such that dimerization of the receptor will facilitate interaction between the zinc finger motifs and the palindromic DNA response element sequence.

The calculated ligand binding interaction energies for the agonist, estradiol, the agonist, *cis*-hydroxytamoxifen, the partial antagonist, *trans*-hydroxytamoxifen, and the pure agonist, ICI 164384, are presented in Table 2, where it can be seen that the antagonists show higher interaction energies than the agonists. The reason for this is probably that the former produce more favourable conformational changes in the receptor than do the latter and these are reflected in the overall internal energy changes, although hydrogen bonding may also play a part.

The ligand binding energies shown in Table 2 are, undoubtedly, rough approximations for the binding free energies, as the effect of desolvation of both receptor and ligand on the overall free energy change, together with the entropy changes resulting from



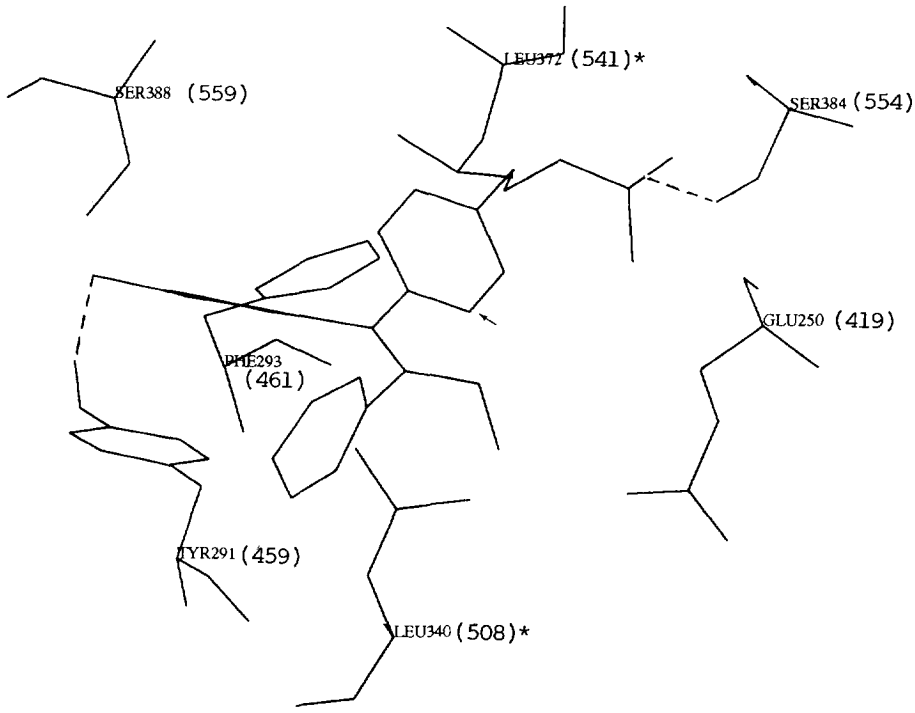


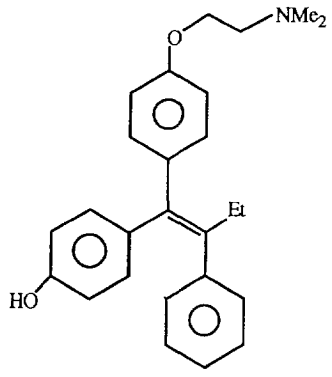
4-hydroxytamoxifen (trans)

Fig. 3. Proposed binding interaction between *trans*-tamoxifen with the putative ligand binding site of hER. The ligand is arrowed in the figure and also shown as a conventional 2D structure beneath. *Denotes residues shown by site-directed mutagenesis to be involved with ligand binding. Residues are numbered according to their alignment with α_1-antitrypsin. Actual sequence positions are placed in parentheses.

loss of translational and rotational degrees of freedom accompanying binding, have not been estimated. However, to some extent, these effects will effectively cancel each other out, although there are also the conformational energy changes in the receptor to consider.

The binding energies and affinity constants calculated here differ markedly from experimentally determined values (Table 2). Hydroxytamoxifen and estradiol have approximately equivalent K_Ds (10^{-10} M) whereas ICI 164384 has a weaker interaction ($K_D = 10^{-9}$ M). The possible reasons for the discrepancies between the calculated and experimental values lie in the differences in hydrogen bonding and ionic interactions, and in the different contributions to the entropy change for the binding process. For example, estradiol and ICI 164384 are capable of forming three hydrogen bonds with the receptor, whereas the two hydroxytamoxifen isomers only form two. Additionally, *trans*-hydroxytamoxifen can form an electrostatic interaction with the binding site, although the other ligands do not. Set against this are the entropy changes in the binding process, and the desolvation entropy change is roughly proportional to the molecular size of the ligand; more precisely, its surface area. Finally, there is the loss in





4-hydroxytamoxifen (cis)

Fig. 4. Proposed binding interaction of *cis*-tamoxifen with the putative ligand binding site of hER. The ligand is arrowed in the figure and also shown as a conventional 2D structure beneath.*Denotes residues shown by site-directed mutagenesis to be involved with ligand binding. Residues are numbered according to their alignment with α_1-antitrypsin. Actual sequence positions are placed in parentheses.

translational and rotational freedom which occurs on binding and this effect, also related to the size of the ligand, will tend to oppose the contribution to the free energy change brought about by desolvation. Precise calculations of these additional energy terms are not easy or straightforward but it is possible to make estimates. In general, inclusion of such contributions from non-bonded interactions will increase the binding free energies and, consequently, also the binding affinities. However, the calculated interaction energies (Table 2) are, to some extent, dependent on the size of the ligand and its orientation in the pocket. The antagonists possess long side-chains which fit the pocket via hydrogen bonding and hydrophobic interactions, and this contributes to their higher binding energies.

It can be seen, from an inspection of Figs 3 and 4, that *trans*-hydroxytamoxifen occupies the binding site in a different orientation to the *cis* isomer, and this brings about the variation in interaction energies between the two ligands. It is also of interest to note that *cis*-hydroxytamoxifen participates in an aromatic ring interaction with phenylalanine-461.

Furthermore, there appears to be a region of surface residues rich in arginines (see Fig. 1) 40 residues downstream from Arg-210, which could represent a

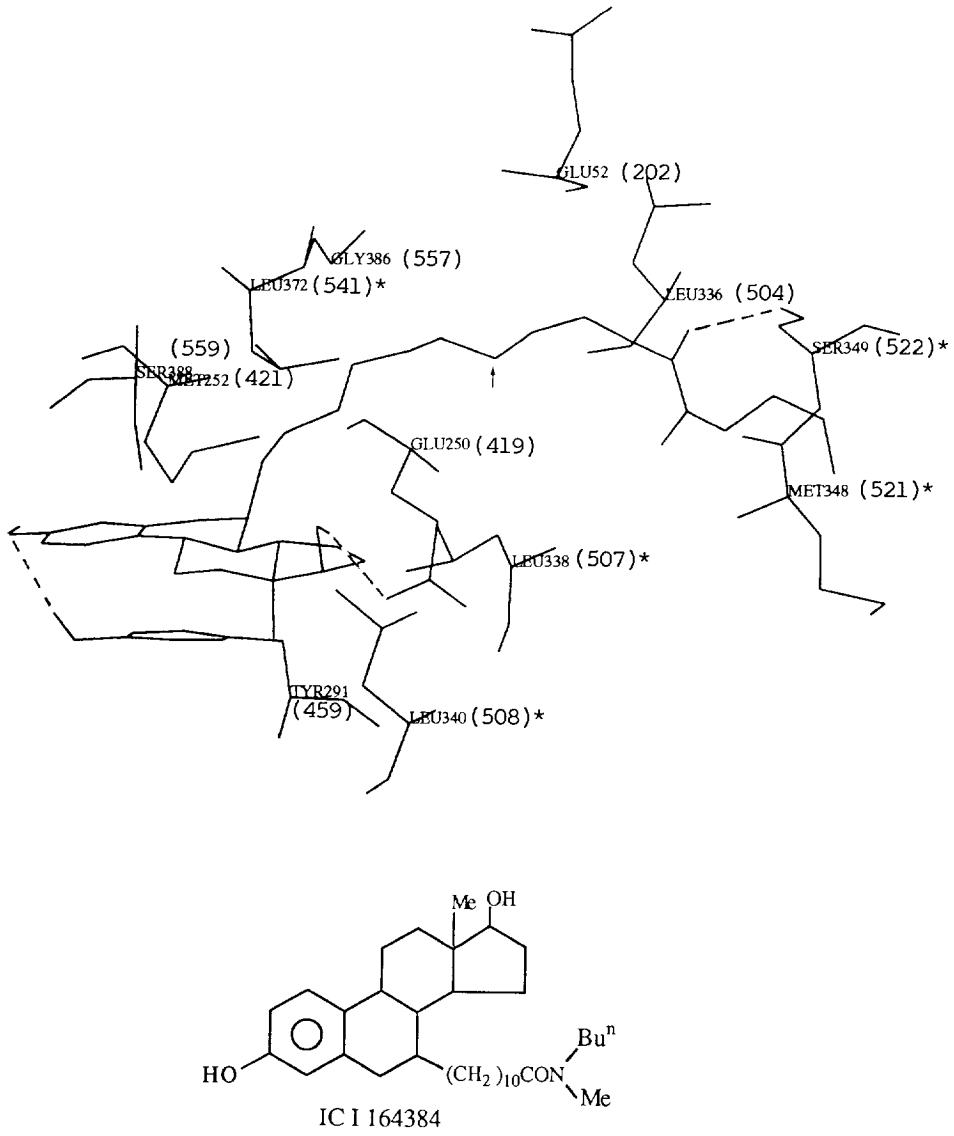


Fig. 5. Proposed mode of binding between ICI 164384 and the putative ligand binding site of hER. The ligand is arrowed in the figure and also shown as a conventional 2D structure beneath. *Denotes residues shown by site-directed mutagenesis to be involved with ligand binding. Residues are numbered according to their alignment with α_1-antitrypsin. Actual sequence positions are placed in parentheses.

site for ionic interactions with the heat shock protein, as this molecular recognition process is thought to be electrostatic in origin. Another possible role for this unusual concentration of basic residues could be to interact with the DNA phosphate moieties, which will be negatively charged, for long-range sensing and orientation of the DNA recognition sequence prior to short-range docking interactions. It is established that DNA binding will not occur when HSP 90 is bound to the receptor [35].

In addition to the putative ligand binding sites mentioned previously, there is a second hydrophobic

Table 2. Calculated ligand binding interaction energies (kcalmol^{-1})

Ligand	Energy of ligand	Energy of complex	Interaction energy	logK	$K(M^{-1})$	$K_D(M)$
Estradiol	6.241	−924.958	−6.000	4.235	1.72×10^{4}	5.82×10^{-5}
Cis-tamoxifen	10.480	−924.707	−9.988	7.050	1.12×10^{7}	8.91×10^{-8}
Trans-tamoxifen	10.709	−930.110	−15.620	11.026	1.06×10^{11}	9.42×10^{-12}
ICI 164384	5.831	−935.268	−15.900	11.223	1.67×10^{11}	5.98×10^{-12}

Energy of ligand free receptor = −925.199 kcalmol^{-1}.
$\Delta G = -1416.7$ logK where: ΔG = Energy complex − Σ Energies ligand, receptor.

pocket nearer to the DNA binding domain which is able to bind antagonists, preferentially. However, there is no obvious tyrosine residue present in this region for binding to the A-ring; instead, serine-432 fulfils this function, whereas glutamate-542 forms an ion-pair with the protonated nitrogen of tamoxifen. The ICI 164384 anti-estrogen compound is also able to fit this second site but, due to its structural differences from tamoxifen, it interacts with serine-433 and serine-554 via hydrogen bonding. The binding of agonists, such as estradiol, to this second site is not favourable due to the fact that this pocket does not contain two hydrogen bond donor/acceptor residues at the optimum distance and orientation for docking with the A-ring and D-ring hydroxyl groups.

In contrast, the binding of estradiol to the first site is energetically favourable and the binding energies are within the range expected for the formation of two hydrogen bonds (0.5–4.0 kcal mol^{-1} per hydrogen bond). In a previously reported molecular modelling and site-directed mutagenesis study involving testosterone binding to its monoclonal antibody, an interaction energy of 3.9 kcal mol^{-1} suggested that a single hydrogen bond had been formed between the steroid D-ring OH and a tyrosine residue of the antibody [36]. From an inspection of the hydrogen bonding network in the estrogen receptor model, it appears that ligand binding completes a "circuit" of hydrogen bonded electron conduits between different portions of the peptide, such that some form of "signal" is transmitted through the protein which triggers the activation process. As the binding interactions of antagonists are different from those of the agonists (see Figs 2–5) this hydrogen bonding transmitted signal is likely to be modulated such that one activates while the other deactivates the receptor. In the case of *cis*-hydroxytamoxifen, as opposed to *trans*-hydroxytamoxifen, for example, hydrogen bonds are formed with tyrosine-459 and serine-554 in the former, but with glutamate-423 and aspartate-545 in the latter.

The model reported here, for the human estrogen receptor can readily explain the loss of activity produced by modifying the steroid to either estrone or 17α-estradiol, because no hydrogen bonding is possible at the D-ring due to changes in the ligand. Furthermore, 16α-estradiol (estriol) would be expected to bind to the receptor but with diminished activity, as the 16α-hydroxyl group will interfere with the 17α-hydrogen bond to glutamate-419. This also agrees with the diminished agonism of estriol [37].

In conclusion, therefore, this study demonstrates that the α_1-antitrypsin structure, as employed by Raynaud and co-workers for modelling steroid hormone receptors, can represent a useful template for the hER which is in agreement with site-directed mutagenesis experiments. Moreover, molecular modelling of the ligand–receptor interaction suggests that ligand binding modulates the hydrogen bonding network which provides interpeptide communication in the hER between binding and DNA activation. When an agonist molecule completes this electron transport "circuit", the resulting effect may disrupt the existing framework of hydrogen bonds to free the DNA binding zinc finger domain. It is hoped that further developments of this model, utilizing molecular dynamics to investigate conformational energy changes following ligand binding, can lead to an increased understanding of hER activation of potential relevance to the design of novel anti-estrogens [38] and facilitate the generation of further models of steroid hormone receptors which may provide a rational explanation of hormonal action at the molecular level [39].

ADDITIONAL NOTE

Since the submission of this manuscript for publication, we have become aware of an additional model for the steroid hormone binding domain of a number of steroid receptors, which utilized an alternative crystal structure template [40]. This appears to represent a general model for a variety of steroid receptor ligand binding domains and does not focus on any single steroid receptor. Although this alternative model merits further investigation, it is not clear whether the structure was energy minimized in the presence (or absence) of ligands, and no binding site models were presented. Consequently, it is difficult to make any comparisons between the two models, particularly with respect to ligands, such as ICI 164384 which can present difficulties due to its molecular shape. In the alignment presented in this work, we have included the sequence for the human corticosteroid binding globin, which also maps quite well with the anti-trypsin sequence. The uteroglobin structure which binds progesterone also shows some degree of sequence similarity with our alignment, and is of known crystal structure. However, a further potential utility of anti-trypsin as a model template for steroid receptors lies in a possible prototype sequence stretch for modelling the DNA binding domain.

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