Abstract P3-263

Testing of a Homology Model of the Human Estrogen Receptor Ligand Binding Domain by Site-Directed Mutagenesis.

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The binding of ligand to the estrogen receptor (ER) ligand-binding domain (LBD) is thought to trigger a functionally critical conformational change. Currently available three-dimensional structures for the ER LBD have allowed a comparison of the effects different types of ligands, and have suggested that the position of helix 12 has a major effect on the function of the protein. However, because none of the ER LBD structures were solved in the absence of ligand, the structural changes that occur upon ligand binding remain poorly understood. We therefore generated a theoretical model for the ER LBD by homology modeling using the ligand-free retinoic-X-receptor LBD as a template. Comparison of the theoretical model to experimental, ligand-bound, ER LBD structures revealed a similar overall structure, with the alpha-carbons of the three residues (Glu-353, Arg-394, and His-524) that form hydrogen bonds to estradiol located in similar relative positions in the model and the estradiol-bound structure. Most of the deviations were in flexible inter-helix loop regions, and more interestingly, in the region beginning with tyrosine-526. The observed deviation at Tyr-526 was necessary, because the model polypeptide backbone near Tyr-526 was co-localized with the estradiol from the superimposed structure. The model predicted that in the absence of ligand, the side-chain of Lys-531 was buried in the protein, probably forming a salt-bridge with Glu-353 that might stabilize the ligand-free conformation. The predicted effect of ligand binding, therefore, was a disruption of the salt-bridge, and a movement of the backbone near Tyr-526. To test this prediction, Lys-531 was mutated to glutamine (to prevent formation of the Lys-Glu salt-bridge) and to proline (to decrease backbone flexibility). Analysis of estradiol binding to the mutants revealed essentially wild-type binding affinity. However, while the Lys-531-Gln mutant exhibited essentially wild-type cooperativity (Hill coefficient of 1.45 compared to 1.53 for wild-type), the proline mutant exhibited significantly reduced cooperativity (Hill coefficient of 1.19), suggesting that the introduction of the proline reduced the magnitude of the conformational change occurring upon ligand binding. These results suggest that the conformational changes induced by ligand binding involve more than the helix 12 position alteration, and help to explain how the binding of the ligand alters the overall structure of the LBD.

Methods

Molecular Modeling – The estrogen receptor-□ LBD sequence was modeled using the Swiss Model homology modeling server (http://expasy.hcuge.ch/swissmod/SWISS-MODEL.html, see ref. 1-3). The ligand-free RXR-□ LBD structure (PDB database file 1lbd.pdb, ref. 4) was used as a template for the model. The model was compared to the estradiol-bound ER LBD structure 1G50 (ref. 5). The model was assessed using the computer program SwissPDBViewer (version 3.7) running on Macintosh computers; the measurements and graphics presented were generated using this program.

Mutant protein production – The wild-type ER LBD coding sequence was mutated using the Stratagene QuikChange method. The presence of mutations in putative mutant plasmids was verified by DNA sequencing. The mutant plasmids were transformed into TOPP2 cells and the mutant protein expressed as a maltose-binding protein fusion. The wild-type and mutant LBD peptides were isolated as described in ref. 6. During purification, the Lys-531 mutants were somewhat less stable than the wild-type protein, but otherwise behaved normally.

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                 310       320         330         340       350      360
             ++            -         - -    ¥   - ¥-       + Ø -          -+-
human ER-□   SKKNSLALSLTADQMVSALLDAEPPILY SE YDPT    RPFSEASMMGLLTNLADRELVHMINW
                         :.  :  :  .:.::  .    : :       : :    .  . ..::. :  .. :
human RXR-□        SSANEDMPVERILEAELAVEPKTETYVEANMGLNPSSPNDPVTNICQAADKQLFTLVEW
                         --   -+  - -   - + - ¥ -           -         -+  Ø   -
                           230       240       250       260       270       280

                           370       380       390       400       410       420
                ++    Ø -     -     -     -          +  -   +  Ø       -+   +  -
human ER-□      AKRVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFAPNLLLDRNQGKCVEG
                :::.: : .: : ::: ::   : :.:..     ::.      .: :  .  . ::   ... :
human RXR-□     AKRIPHFSELPLDDQVILLRAGWNELLIASFSHRSIAVKDGILLATGLHVHRNSAHSA G
                 ++    Ø -    --      +     -      Ø   +    +-             +
                     290       300       310       320       330       340

                           430       440       450       460       470       480
                  - Ø-         +Ø+       --Ø     +         ¥ Ø      +  --+-    +  -+  -
human ER-□      MVEIFDMLLAT SSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHIHRVLDKITDTLI
                .  ::: .:      :. : :..    :   ::. :.: :                : :      .   . .:.  .:
human RXR-□     VGAIFDRVLTELVSKMRDMQMDKTELGCLRAIVLFNPD          SKGLSNPAEVEALREKVYASL
                    Ø-+    -     + +-    -+ -      +      Ø   -             +        -  -    +-+ ¥
                        350       360       370                  380       390       400

                490       500       510       520       530       540       550
                    +           +           +      +   -   ¥   + +       ¥-    -    -   +
human ER-□      HLMAKAGLTLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLHA
                 .   :          :   :  : ::: :    .:  .    :  .:::.   :       .:.     :.:::.:  .
human RXR-□     EAYCKHKYPE   QPGRFAKLLLRLPALRSIGLKCLEHLFFFKLIGDTPIDTFLMEMLEAPHQMT
                -  ¥  +  +¥  -       +Ø  +     +       +       +    -   ØØØ+     -      -  Ø    -     -
                          410         420       430       440       450       460

¥ = tyrosine     Ø = phenylalanine         = tryptophan
- = glutamate or aspartate + = arginine or lysine
: = identical residue      . = similar residue
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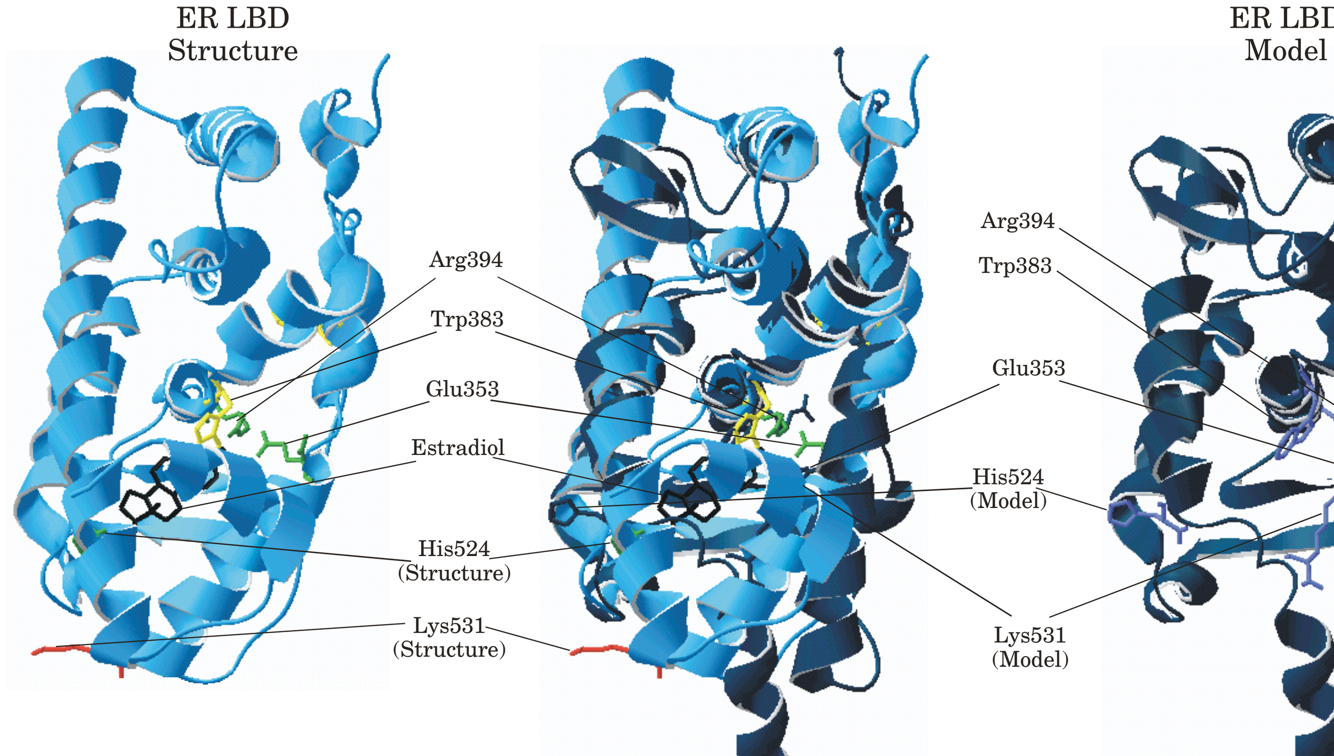
Figure 1. Sequence alignment used for the homology modeling. Because the sequence identity for the estrogen receptor □ LBD and the RXR □ LBD is only ~25%, some of the alignment is based more on structural feature conservation than on sequence.



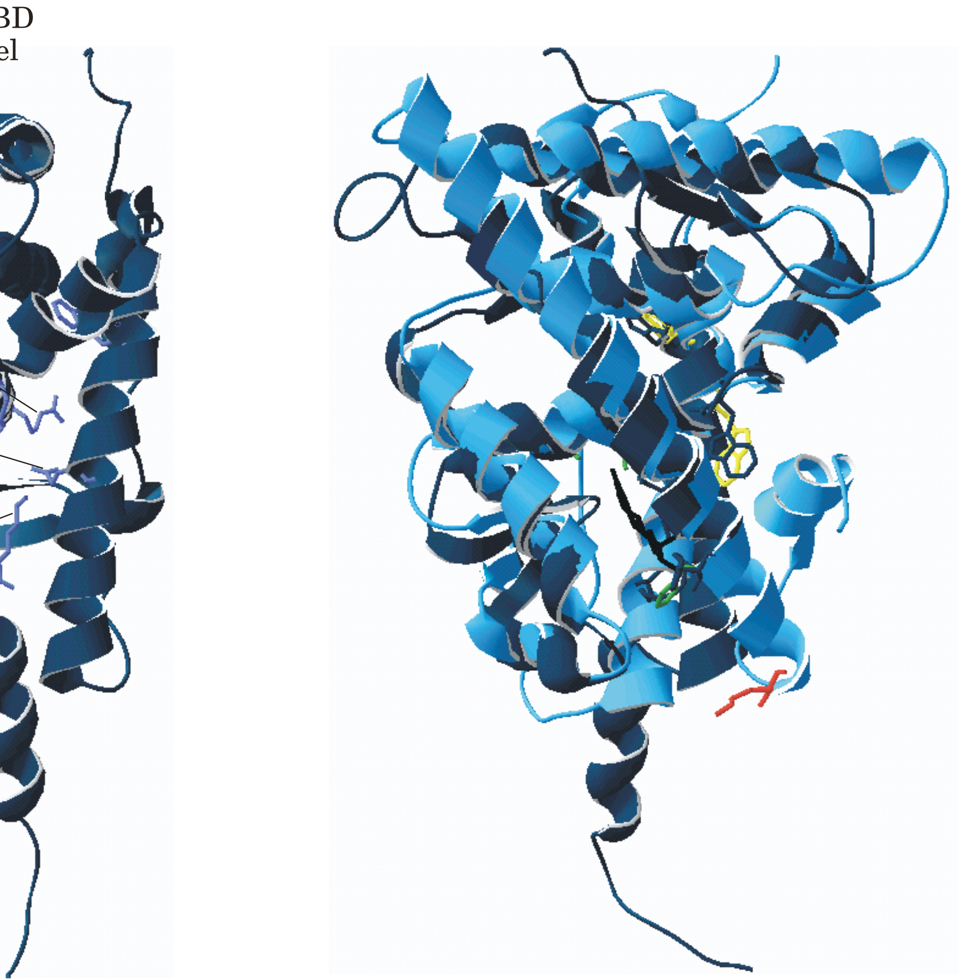


Figure 2. Comparison of the model with the ER LBD structure. The overall fold of the model matches that of the structure. As expected, the modeling software has trouble with loop regions. Most of the secondary structural elements are in similar locations in the model and in the actual structure, with an □-carbon RMS deviation of ~1.5 Å.

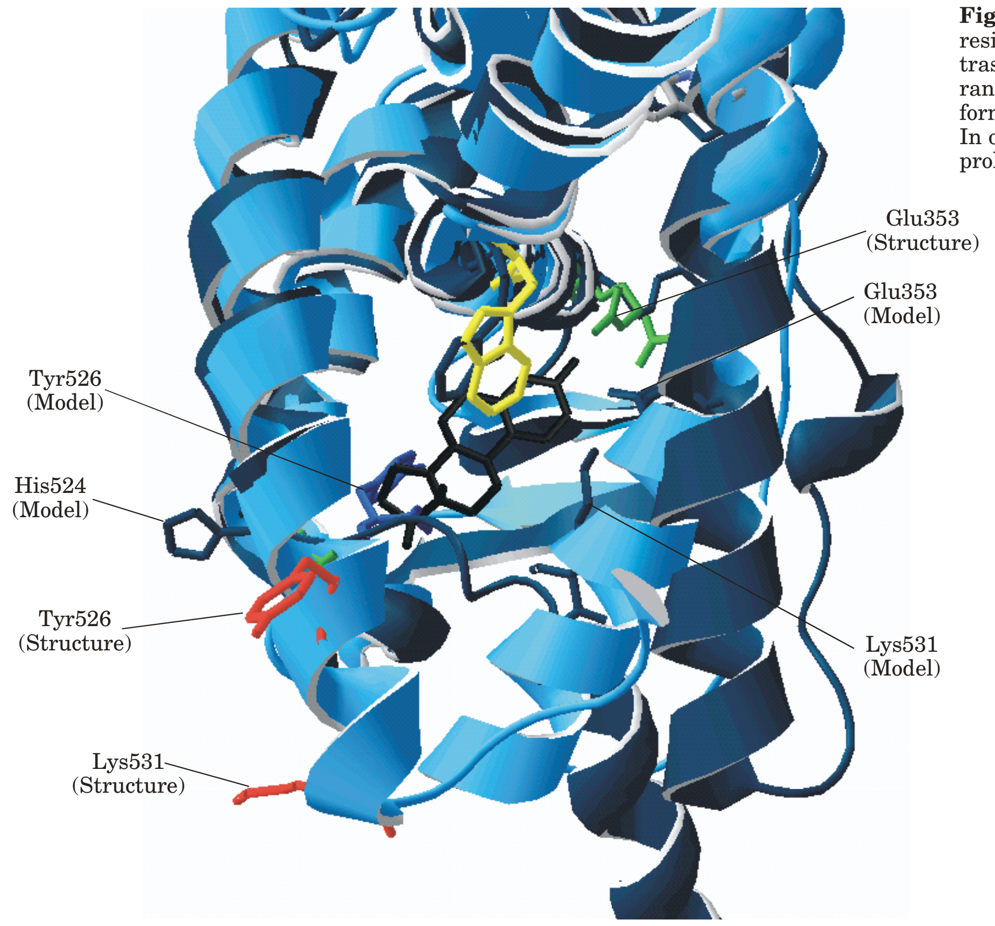


Figure 3. Ligand-binding pocket comparison. The a-carbons of the residues that directly contact the ligand are in similar positions. In contrast, the a-carbon of Lys-531 is shifted by ~14.7 Å. This predicted rearrangement is necessary to allow the ligand to bind. In the model, Lys-531 forms a salt-bridge with Glu-353.
In order to test this prediction, Lys-531 was mutated to glutamine and to proline.

Table I.
Comparative Interatomic Distances for the Structure and the Model

	Interatomic Distances (Å) Structure	Model	Difference
Internal salt-bridges			
Glu323 – Lys449 (□O – □N)	2.9	3.4	0.5
Glu385 – Arg515 (□O – NH)	4.2	4.6	0.3
Glu444 – Arg503 (□O – NH)	2.7	2.7	0.0
Residues H-bonding to estradiol			
Glu353 – Arg394 (C□ – C□)	13.6	13.7	0.2
Glu353 – His524 (C□ – C□)	19.0	18.3	-0.7
Arg394 – His524 (C□ – C□)	21.9	22.4	0.5
Other selected residues			
Glu323 – Trp360 (□O – indole N)	4.0	2.7	-1.3
Lys449 – Trp360 (□N – indole N)	4.5	5.0	0.5
Trp393 – Phe 445 (C□- C□)	4.0	4.2	0.2
Trp360 – Trp383 (C□-C□)	11.1	11.3	0.2
Trp360 – Trp393 (C□-C□)	13.7	12.3	-1.4
Trp383 – Trp393 (C□-C□)	16.1	16.3	0.2
Glu353 – Lys531 (C□ – C□)	23.5	10	-13.5
Glu353 – Lys531 (□O - □N)	24.7	3.6	-21.1

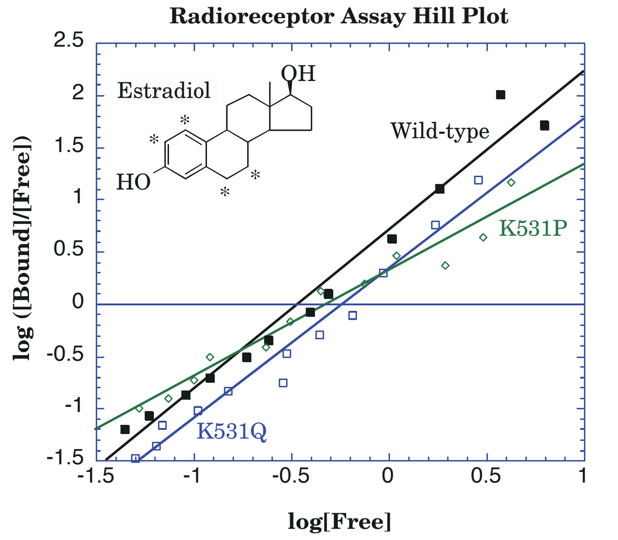


Figure 4. Estradiol binding assay. The tritiated estradiol binding by the wild-type and mutant ER LBD peptides was measured using a standard dextran-coated charcoal radioreceptor assay. The results indicate similar binding affinities for the different peptides, but suggest that the K531P mutant has reduced cooperativity.

Table II.
Estradiol Binding Parameters

Peptide	Hill coefficient (n)	$F_{0.5}$ (nM)
Wild-type	1.51 ± 0.14	0.31 ± 0.08
K531Q	1.45 ± 0.08	0.36 ± 0.22
K531P	1.19 ± 0.07	0.50 ± 0.21

Table III.
Spectral Ligand Binding Parameters

	Coumestrol		Tamoxifen	
Peptide	Hill coefficient (n)	$F_{0.5}$ (nM)	Hill coefficient (n)	$F_{0.5}$ (nM)
Wild-type	1.78 ± 0.08	0.17 ± 0.04	1.18 ± 0.06	0.36 ± 0.03
K531P	0.91 ± 0.05	0.28 ± 0.05	0.98 ± 0.01	0.31 ± 0.12

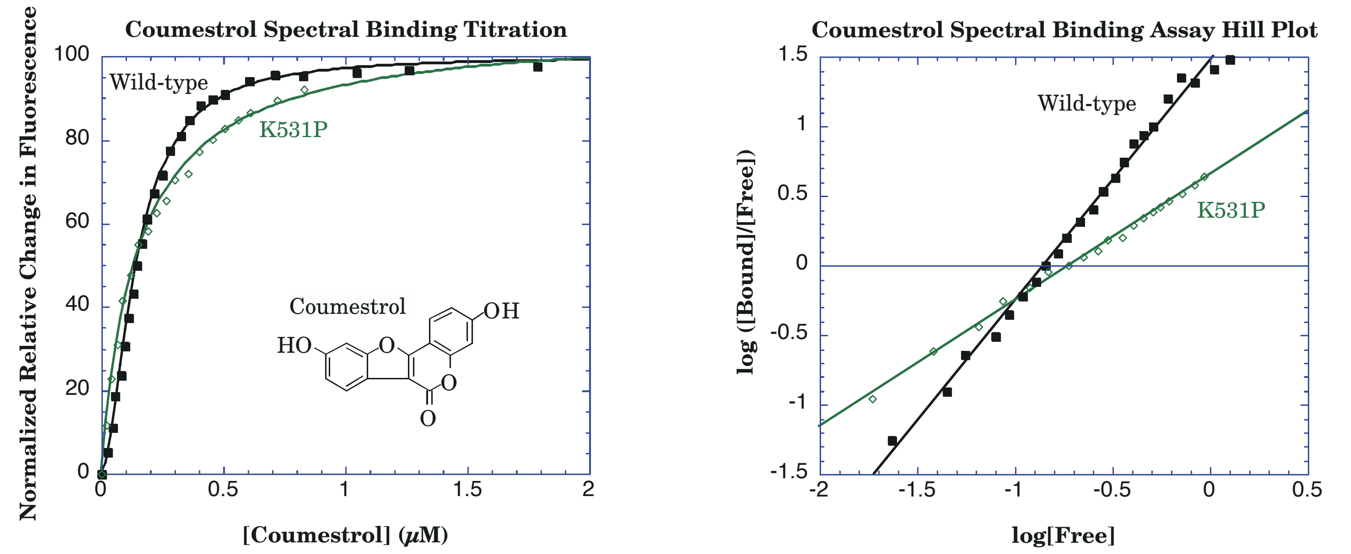


Figure 5. Coumestrol binding receptor assay. The coumestrol binding of the wild-type and K531P LBD peptides was measured by following the reduction in tryptophan fluorescence induced by coumestrol binding. The results indicate similar binding affinities for the different peptides, and again suggest that the K531P mutant has reduced cooperativity.

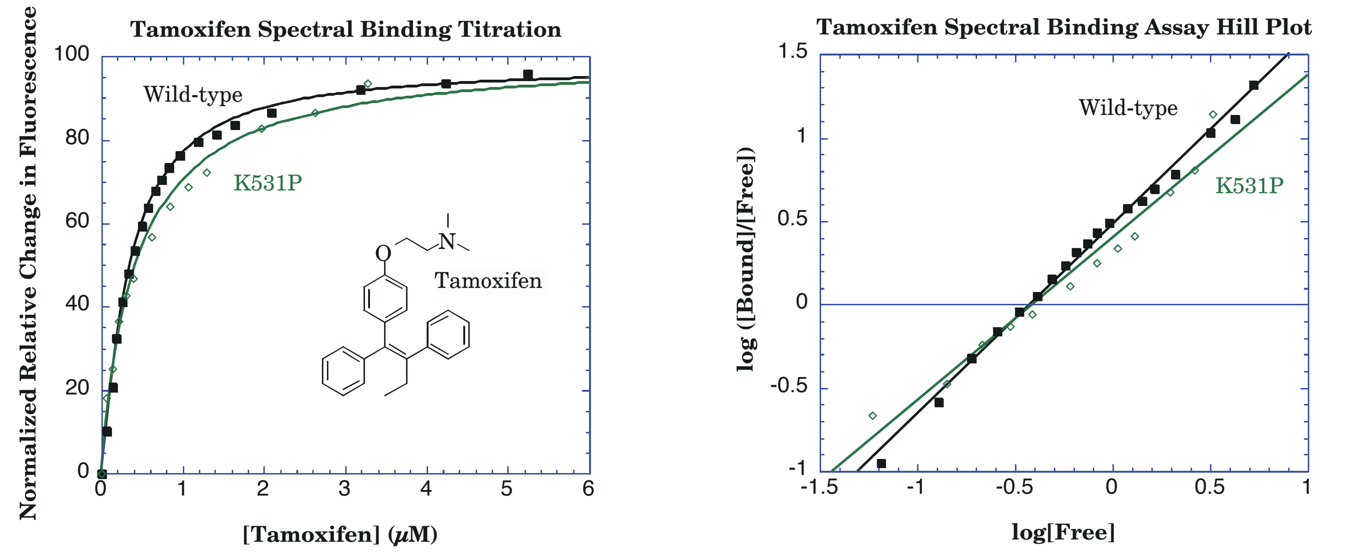


Figure 6. Tamoxifen binding receptor assay. The tamoxifen binding of the wild-type and K531P LBD peptides was measured by following the reduction in tryptophan fluorescence induced by tamoxifen binding. The results indicate similar binding affinities for the different peptides, and again suggest that the K531P mutant has reduced cooperativity.

Conclusions

1. Molecular modeling of the estrogen receptor suggests that ligand-binding alters the protein conformation near Lys-531, and predicts that Lys-531 may form a salt-bridge with Glu-353 in the absence of ligand.
2. Mutations of Lys-531 slightly destabilize the protein, but do not appreciably alter the binding affinity of the ER LBD for its ligands.
3. Mutation of Lys-531 to glutamine has limited effect, suggesting that the predict salt-bridge either does not form or is not important for maintaining the ligand-free conformation.
4. Mutation of Lys-531 to proline reduces or abolishes the normally observed cooperativity of ligand binding.
5. These results suggest that the protein region near Lys-531 is important for the conformational changes that occur upon binding of ligand to the wild-type protein.

References

1. Peitsch, M. C. (1995) *Bio/Technology* **13**, 658-660
2. Peitsch, M. C. (1996) *Biochem. Soc. Trans.* **24**, 274-279
3. Guex, N. and Peitsch, M. C. (1997) *Electrophoresis* **18**, 2714-2723
4. Renaud, J.P., *et al.* (1995) *Nature* **378**, 681-689
5. Eiler, S. *et al.* (2001) *Prot. Express. Purif.* **22**, 165-173
6. Brandt, M.E. and Vickery, L.E. (1997) *J. Biol. Chem.* **272,** 4843-4849
7. Hill, A.V. (1913) *Biochem. J.* **7**, 471

Acknowledgments

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