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Three-dimensional model for the hormone binding domains of steroid receptors

(nuclear receptors/steroid receptors/protein folding/protein structure prediction)

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ABSTRACT **We have used a motif-based structural search method to identify structural homologs of the hormone binding domains of the nuclear receptors from among a set of known protein structures and have found the closest similarity with members of the subtilisin-like serine proteases. These proteins consist of an open twisted sheet of parallel β-strands flanked on both sides by α-helices. The alignment with the protease scaffold was refined by using multiple sequence prealignment of different sets of nuclear receptors, and alternative model structures were screened by considering their consistency with the results of biochemical experiments defining the ligand binding pocket. In the most favored model, nearly all of the residues thought to be involved in ligand binding map to a pocket of appropriate dimensions where the subtilisin-like proteases have their active site. The three-dimensional model that we propose for the hormone binding domains of the nuclear receptors provides a framework for the design of experiments to further investigate nuclear receptor structure and function.**

Among the most worthy targets for protein structure prediction efforts are the hormone binding domains (HBDs) of the nuclear receptors which constitute a superfamily of gene-regulatory proteins that respond to the steroid estrogen and other hormones (1–3). While the structure of the DNA binding domain of these receptors has been determined by two-dimensional NMR (4–6) and by x-ray diffraction (as a complex with DNA) (7), the structural determination of the HBDs is still under investigation.

Although the detailed structure is experimentally unknown, the HBDs have been the subject of extensive biochemical research, providing information about the residues which are involved in the binding pocket and which presumably must be close to each other in space. In this paper, we show how protein structure prediction algorithms developed by some of us (8–10), along with some biochemical information, can be used to construct a model structure for the HBDs of these receptors. The structural model we have produced is an α/β structure of the type seen in subtilisin-like serine proteases, one that is very different from another recently proposed model (12, 13). The residues identified by affinity-labeling experiments define a cavity in our structural model, appropriate for ligand binding, that corresponds to the active site of the subtilisin-like proteases. This pocket is adjacent to regions of the protein implicated in hormone-regulated activation of transcription, TAF-2. Regions involved in receptor dimerization are found at the surface of our model. The structural model we present provides a framework for considering the location and potential interaction of sites responsible for other known functions of the HBDs of the nuclear receptors and suggests further experiments to evaluate the accuracy of our model.

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METHODS

Motif-Based Structural Search. The sequences of the HBDs of the human estrogen (hER), mouse thyroid hormone (mTRα1), human progesterone (hPR), rat glucocorticoid (rGR), and human retinoic acid (hRAR1) receptors were initially screened against a set of 78 proteins by using an optimized Hamiltonian based on local interactions (R.A.G., Z.A.L.-S., and P.G.W., unpublished work). The proteins selected from the Brookhaven Protein Databank are high-resolution structures varying in length from 150 to 500 residues. There was no preselection of structural motifs, and 40 mixed $\alpha\beta$ protein structures were included, as well as members of other protein folding classes, including the antiparallel β-sheet structures advanced as another model for the nuclear receptors (12, 13). When the highest scoring motifs were found to be of the α/β structural class, additional structures of the α/β type were appended.

The local-interaction Hamiltonian has been demonstrated to successfully identify the correct fold of several proteins of known structure (9). This energy function includes terms for the energy contribution for the various residues to be in an environment defined as a combination of secondary structure and surface accessibility (14), plus a contribution for each pair of residues that are within a certain distance of each other (15–17). In addition, for the alignment to a scaffold protein, a gap penalty is included, which takes the form of a chemical potential. The various parameters in the Hamiltonian were optimized by using spin-glass theory and statistical considerations (refs. 8 and 9; R.A.G., Z.A.L.-S, and P.G.W., unpublished work).

The minimization of this energy function was carried out by a mean-field iterative approach (R.A.G., Z.A.L.-S, and P.G.W., unpublished work), based on a technique originally developed by Finkelstein and Reva (18). The initial alignment was determined with a modified dynamic programming algorithm, where the contact terms were calculated by using the residues of the target interacting with the residues of the scaffold protein (19). The identities of the scaffold residues were then updated with the identity of the corresponding target protein, and the alignment was recalculated. This procedure was iterated until convergence. In some cases, random energy values were added to the Hamiltonian for the initial alignment as a means to generate alternative, distinct local minima.

Abbreviations: GR, glucocorticoid receptor; HBD, hormone binding domain; PR, progesterone receptor; RAR, retinoic acid receptor; TR, thyroid receptor; species designations for receptors are given by prefixes: h, human; m, mouse; r, rat.

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With the exception of hRAR1, the scaffold with the lowest energy for each of the receptors was in the class of the subtilisin-like proteases. For the more distantly related hRAR1 receptor, another α/β protein, glyceraldehyde-3-phosphate dehydrogenase, had a comparable but slightly higher score.

Although the motif-based structural search identified the subtilisin-like serine proteases as the most likely folding topology for the nuclear receptor HBDs, the exact alignments are fluxional. This suggests that the structure is only an imperfect analog. There are additional uncertainties because the gaps in several places are rather long. The alignment thus depends upon knowledge of the statistically less certain gap penalties. Also, the precise boundaries of the HBDs are not known.

To restrict our focus to a few structures corresponding to the different alignments, we used two strategies. One of these is based on multiple sequence alignment, and the other is a restriction of our search to configurations conforming to the results of the least ambiguous biochemical experiments.

Multiple Sequence Alignment. Multiple sequence alignment helps in two ways: (*i*) it yields a structure which will be in common to many members of the nuclear receptor superfamily, so that biochemical data from different receptors can be mapped onto a single model for evaluation, and (*ii*) when reliable, it allows one to average out local minima that are caused by fluctuations in residue identity between the representative proteins. Multiple sequence alignment provides a sort of signal averaging leading to a more stable threading of the unknown sequence onto the scaffold during iteration.

Four multiple sequence alignments were set up with the CLUSTAL V alignment algorithm (20). In three of these, multiple alignments were created for three receptor families, the ER family, the GR family (GR, PR, androgen receptor, and mineralocorticoid receptor), and the TR and RAR family. The sequence similarity within these families is relatively high, so the multiple sequence alignment algorithm is robust. In the fourth multiple sequence alignment, 12 receptors including some from each of the above three families were all multiply aligned. The resulting alignment was adjusted by hand to preserve some of the features of the alignment of Laudet *et al.* (21). As the evolutionary distance between these families leads to a less robust multiple sequence alignment, the latter alignment was used only for reconciling biochemical data from different receptors, and not for multiple alignment with the scaffold protein.

The threading of the sequences to the scaffold was done by using the iterative dynamic programming algorithm on each of the sequences, enforcing the condition that all residues aligned to each other by the multiple sequence alignment must reside at the same position in the scaffold. The energies of each of the sequences were evaluated as in the individual alignments and averaged together. The signal averaging resulting from threading these multiply prealigned sequences considerably reduced the variety of distinct alignments produced. The subtilisin-like serine proteases were still the highest scoring candidates for the three sets, with the energy gap distinguishing these from other known structures considerably augmented over single alignments.

Sorting Structures with Biochemical Data. While the signal averaging derived from the multiple sequence alignment helps, the randomization process still generates many local minima of the free energy corresponding to different alignments of the HBDs to the protease scaffolds. Many features are preserved in these local minima, but there can be both local adjustments and serious realignments in these structures with energies only slightly higher than the deepest ones found. Some of these realignments assign secondary structure elements to entirely different strings of residues. This may be partly because we are dealing with a single domain of a multidomain protein. The approximately repetitive nature of the α/β motif in the subtilisin fold allows phase shifts with little energy cost. We therefore used biochemical data relating to the binding site, described in detail in a later section, to screen these structures.

Among the high-scoring alignments, there are some which are consistent with virtually none of these biochemical experiments, many of which are consistent with a few of the experiments, and a few closely related alignments which at our low resolution fit nearly all of the data. We discuss one of these structures in detail here. We hope in future publications to address the other possible alignments and their confrontation with experiment. Although these experiments seem unambiguous, any error in their interpretation could lead to a predilection for one of the other alignments.

Reevaluation of the Proposed Structure by the Hamiltonian. As a further step in completing the structures, we developed several scaffolds based on the low-energy alignment that satisfied the biochemical data. In these scaffolds, unused residues were excised, and unassigned receptor residues (loops) were constructed by using the loop search subroutines (22) provided in SYBYL 6.0 (Tripos Associates, St. Louis). The coordinates are available from the authors through anonymous file-transfer protocol. No attempt was made to refine the position of loop residues or residues flanking deletions, so steric conflicts and long C_α–C_α bond distances (up to 7.5 Å) exist in the structure.

These scaffolds correspond to "recombinant" proteases and can be considered as candidates in the energy minimization search. With loops built into a good alignment, one expects the free energy to become more favorable, because it will no longer be necessary to introduce gaps, eliminating the gap penalty. Pair contacts and profile energy terms may also be changed. Indeed, all components of the free energy are favorably improved by the gap completion. Even better values were obtained by realigning the GR family to this created scaffold. This caused only minor local shifts in the alignment.

In Fig. 1 we show a histogram of the free energies obtained in our survey for the multiple sequence-aligned GR family.

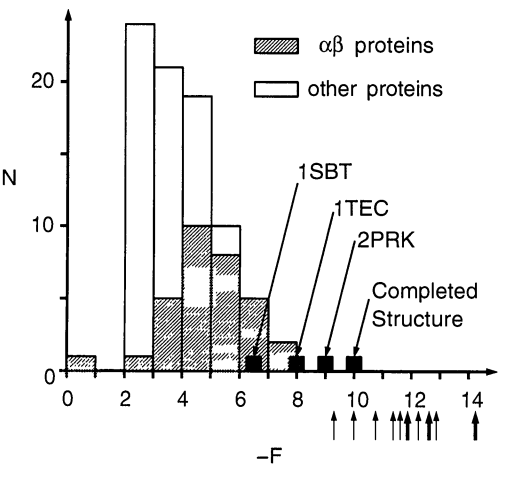

FIG. 1. Histogram of minimum free energies for the GR HBD family as aligned with 83 protein structures. As shown, the structures with the best free energies are the mixed $\alpha\beta$ proteins, with the subtilisin-like proteases [subtilisin (1SBT), thermitase (1TEC), and proteinase K (2PRK)] the best of these. Also shown in the figure is the free energy of the completed structure created by excising deletions and building-in insertions in the alignment of the GR family with thermitase. The minimum-free-energy alignments of each of the subtilisin-like proteases against themselves and each other are indicated by thick arrows and regular arrows, respectively. The arrow at a free energy of 6.12 corresponds to the alignment of the distant homolog human furin with thermitase.

Since these are all local minima of the free energy, there is a cosmologically large number of random structures with much higher energies, centered about a free energy of zero, that are not shown in the graph. Clearly, of these local minima, the α/β proteins in general provide the best results, and the subtilisin-like serine proteases the best of these. There is a clear gap in the energy spectrum, with the completed structure having even lower free energy. For normalization, we also include the free energy associated with the recognition of the different subtilisin-like proteases against each other and themselves.

RESULTS

A completed low-resolution structure of the backbone of the GR family is shown in Fig. 2. The alignment of sequences from the GR family with the serine protease thermitase is shown in Fig. 3. The α-helices and β-strands defining the core structure are given consecutive number and letter designations, respectively, except for the two strands corresponding to a distinct β-turn, which are designated as X and Y. Portions of other receptor sequences, as aligned to the GR class with the multiple sequence alignment, are also included in Fig. 3.

The overall fold is a doubly wound parallel β-sheet consisting of a core of hydrophobic parallel β-strands displayed in a twisted open sheet, packed between helices. The two inner helices are essentially hydrophobic, whereas the three outer helices are amphipathic in the serine protease, and these features are well preserved in the receptor as aligned to it. The one exception is helix 4, which in thermitase is buried and nonpolar, whereas in our model, it is amphipathic, truncated, and partially exposed. There are 191 conserved core residues in the subtilisin-like serine proteases, located primarily in the sheets and helices (23). As shown in Fig. 3, gaps in the alignment of the receptor families occur primarily at turn positions in the proposed structure. Thus, the structural assignment is consistent with the evolutionary sequence conservation of the receptors.

The Steroid Binding Site. A set of affinity-labeling and mutation experiments give important constraints on the location of the ligand binding site. As noted previously, these results were used to screen the alternative alignments of the HBD sequences. The location of the steroid A ring has been probed through photoaffinity labeling with synthetic steroids having their reactive functions in the A ring. Triamcinolone acetonide labels Met^{622} and Cys^{754} in rGR (24), while R5020 labels Met^{759} and Met^{909} in hPR (25). Dexamethasone mesylate, an affinity label with a reactive function on carbon-21, has been shown to label Cys^{656} in rGR (26). Dexamethasone also binds better to a $Cys^{656} \rightarrow$ Gly rGR mutant with increased affinity (27). Thus, this site defines a region in the receptor that binds the D ring of glucocorticoids. Mutational analysis of the binding of 11β-substituted progestins showed that the change $Cys^{575} \rightarrow$ Gly in chicken PR permitted the binding of the bulky antiprogestin RU486 (28). This residue, corresponding to Gly^{722} in hPR, should then influence a region that accommodates an 11β steroid substituent. Finally, the nonsteroidal affinity label tamoxifen aziridine alkylates Cys^{530} in hER (29), and when this residue is mutated to alanine, alkylates Cys^{381} (30). If we assume that the affinity label is bound in the same orientation by wild-type and mutant receptor, the two cysteine thiols must be within 11 Å of each other.

In our model, the A-ring-labeled residues, shown in blue in Figs. 2 and 3, are found clustered together in the interior of the protein, in the middle of α-helices 1 and 4 and on a strap covering both helices. The D-ring contact site, shown in red, is positioned on an external loop before α-helix 2, and the residue affecting 11β substituents, shown in green, is at an internal site on a loop after sheet A. The residues corresponding to hER Cys^{530} and Cys^{381}, colored yellow in Figs. 2 and 3, have a C_α separation of 12.5 Å. The binding pocket, of a size appropriate to bind a steroidal ligand and lined with largely hydrophobic residues, is accessible from the front of the protein. Its mouth is between α-helices 1 and 2 and β-strands A and D. This is a β–α loop crossover region where the active site of most α/β enzymes is found (31), also corresponding to the catalytic site of the subtilisin-like proteases (32).

We have also investigated a model of comparable energy based on a rather different alignment, where ligand access is from the rear of the structure. Although this model agrees with most of the results of biochemical experiments, it does not position the hER cysteines in a way that can accommodate both tamoxifen aziridine labeling experiments.

Interactions with Other Biomolecules. Mutations in mER corresponding to sites Arg^{503}, Leu^{507}, and Ile^{514} in hER affect receptor dimerization (33). The three sites that affect receptor dimerization, shown in purple in Figs. 2 and 3, are located in coil regions flanking β-strand G, near the surface of our model, where they might influence intermolecular interactions.

The ligand-dependent transcriptional activation function TAF-2 has been mapped to the C-terminal portion of the mER HBD by a limited mutational study (34). This region, corresponding to residues 535–548 in hER, indicated in Fig. 2 in darker gray, constitutes most of α-helix 4 and the turn to helix 5. The initial part of this sequence is close to the ligand

FIG. 2. Completed predicted structure of the GR HBD family, based on an alignment with thermitase, in crossed stereo. Residues identified through biochemical experiments are colored as described in the text.

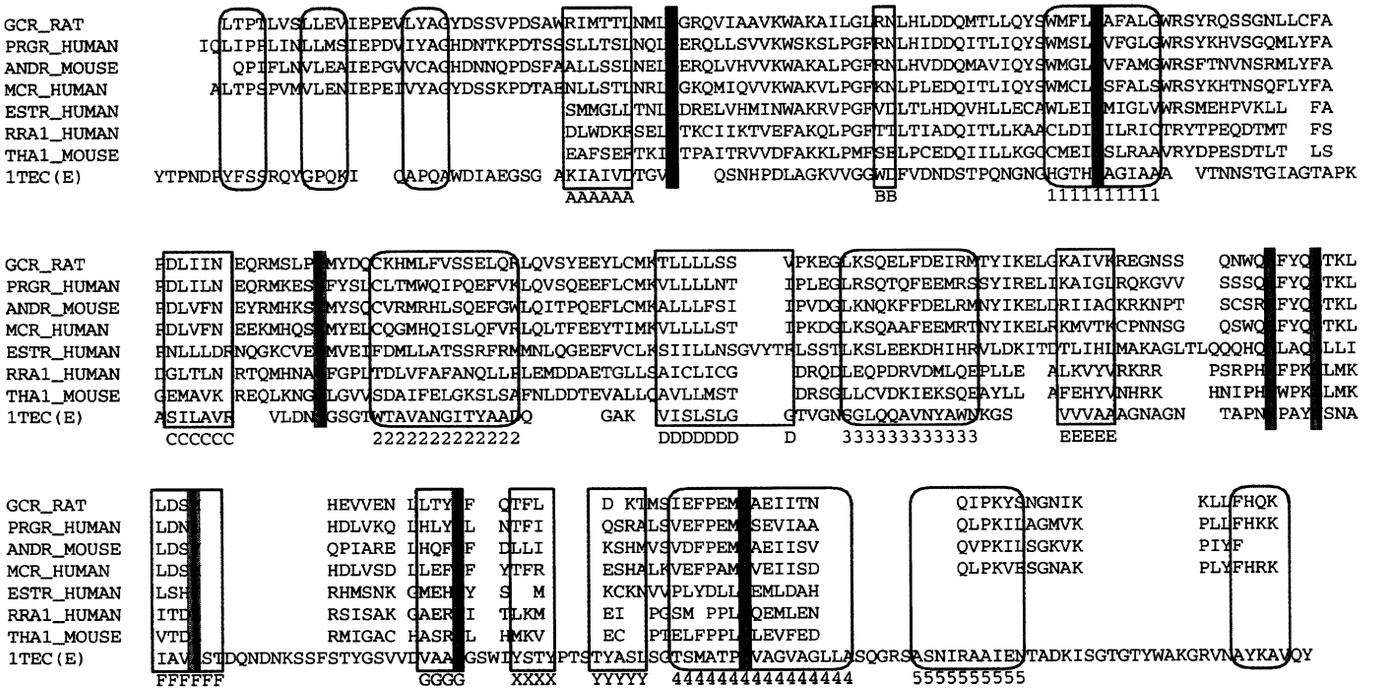

FIG. 3. Alignment of the GR family with thermitase. Also shown is the multiple alignment of portions of other members of the nuclear receptor superfamily with the GR family. The receptor sequences are given their Swiss-Prot Database designations and are listed in the order rGR, hPR, mouse androgen receptor, human mineralocorticoid receptor, hER, hRAR1, and mTRα1. Thermitase is identified by its Brookhaven Protein Databank designation 1TEC(E). Residues identified through biochemical experiments are colored with the same scheme used in Fig. 2, as described in the text. α-Helices and β-sheets are enclosed in rounded and squared boxes, respectively. Core secondary structural elements are notated in the last line, with the strands of the central β sheet designated A–G, the surrounding α-helices numbered 1–5, and an exposed β-bend designated X and Y.

binding site, and the latter part is exposed at the rear of our model.

There are many other mutations, natural and synthetic, affecting hormone binding, dimerization, and transcriptional activation. Their evaluation in terms of receptor structure must be undertaken with care, since mutational changes resulting in a loss of binding may indicate improper or incomplete folding. Some of the receptors may interact with heat shock proteins, suggesting that a portion of the molecule may be incompletely folded *in vivo* (35). This should be borne in mind in interpreting biochemical evidence.

Proteolytic Activity. Although the subtilisin-like serine proteases were identified by our method without any preconception of searching for this functional class, there are a number of reports linking steroid receptors with serine proteolytic activity (36, 37). Molinari *et al.* (11) ascribe chymotrypsin-like proteolytic activity to the ER that is activated by estradiol and is blocked by the mammalian serine protease inhibitor aprotinin and the inactivator diisopropyl fluorophosphate. While these observations are curious, they do not speak directly to the relationship between the ligand binding site in our model for nuclear receptors and the usual location of the catalytic site in proteins of the subtilisin-like folding class.

Other Proposed Models. Recently, based on a homology method termed hydrophobic cluster analysis, Lemesle-Varloot *et al.* (12) and Ojasoo *et al.* (13) have proposed that the nuclear receptors are related to serum serine protease inhibitors (e.g., α_1-antitrypsin, ovalbumin). Those investigators have presented an extensive discussion of the validity of their model, but the location of the ligand binding site is uncertain. The antiparallel β-sheet folding pattern of these serum proteins is of a different topology than our model, and although members of this folding class were among the set of proteins examined in our search, they did not give high scores.

CONCLUSION

The model we propose for the HBD is consistent with most of the results of affinity labeling and the most definitive mutation studies. It is attractive in its positioning of residues involved in ligand binding in a constellation that provides an appropriate size environment for a ligand. When interpreting the model, it should be borne in mind that the model is unlikely to be correct on an atomistic level, since it was based on a prediction scheme intended to produce low-resolution structures. The use of experimental information in pinning down the alignment also means that the prediction relies upon those experimental results. Despite possible shortcomings of the model, it should be a helpful starting point for designing more-specific experiments involving affinity labeling, site-specific mutagenesis, and protein engineering to further test the model.

Finally, we note that protein structure prediction is still in an embryonic stage of development. Making predictions in "blind tests" is an essential step in learning whether important interactions in protein folding are understood and whether search strategies for minimal-energy structures are well conceived. Thus, the determination of the structure of the binding domains of the nuclear receptors by x-ray diffraction or NMR and the comparison of the structure with our predictions are eagerly awaited.

Note Added in Proof. Our further studies on the receptors have revealed a systematic difference from the training set used to determine the energy parameters. The variance of random structure energies for the receptor is unusually large, explaining the fluxional alignments. The receptor is especially rich in small hydrophobic residues whose pair interactions dominantly contribute to the variance. The core of the subtilisin protease structures provides a particularly efficient mode of accommodating these residues. Recent spectroscopic experiments on the thyroid hormone receptor (38) have come to our attention which have been interpreted as giving

secondary structure content: 28% helix, 28% β-sheet, 11% turns, and 33% unordered. This is in harmony with our model for the hormone binding domains and the general assignment to the mixed α/β class.

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1. Parker, M. G. (1991) *Nuclear Hormone Receptors* (Academic, London).
2. Evans, R. (1988) *Science* **240,** 889–895.
3. Beato, M. (1988) *Cell* **56,** 335–344.
4. Härd, T., Kellenbach, E., Boelens, R., Maler, B., Dahlman, K., Freedman, L. P., Carlstedt-Duke, J., Yamamoto, K. R., Gustafsson, J.-Å. & Kaptein, R. (1990) *Science* **248,** 157–160.
5. Schwabe, J. W. R., Neuhaus, D. & Rhodes, D. (1990) *Nature (London)* **348,** 458–461.
6. Lee, M. S., Kliewer, S. A., Provencal, J., Wright, P. E. & Evans, R. M. (1993) *Science* **269,** 1117–1121.
7. Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R. & Sigler, P. B. (1991) *Science* **352,** 497–505.
8. Goldstein, R. A., Luthey-Schulten, Z. A. & Wolynes, P. G. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4918–4922.
9. Goldstein, R. A., Luthey-Schulten, Z. A. & Wolynes, P. G. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9029–9033.
10. Goldstein, R. A., Luthey-Schulten, Z. A. & Wolynes, P. G. (1993) in *Proceedings of the 26th Annual Hawaii International Conference on System Sciences*, eds. Mudge, T. N., Milutinovic, V. & Hunter, L. (IEEE Comp. Soc. Press, New York), Vol. 1, pp. 699–707.
11. Molinari, A. M., Abbondanza, C., Armetta, I., Medici, N., Minucci, S., Moncharmont, B., Nigro, V. & Puca, G. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4463–4467.
12. Lemesle-Varloot, L., Ojasoo, T., Mornon, J.-P. & Raynaud, J.-P. (1992) *J. Steroid Biochem. Mol. Biol.* **41,** 369–388.
13. Ojasoo, T., Doré, J.-C., Mornon, J.-P. & Raynaud, J.-P. (1992) in *Molecular Structure and Biological Activity of Steroids*, eds. Bohl, M. & Duax, W. L. (CRC, Boca Raton, FL), pp. 157–207.
14. Luthy, R., Bowie, J. U. & Eisenberg, D. (1992) *Nature (London)* **356,** 83–85.
15. Miyazawa, S. & Jernigan, R. L. (1985) *Macromolecules* **18,** 534–552.
16. Sippl, M. J. (1990) *J. Mol. Biol.* **213,** 859–883.
17. Sippl, M. J. & Weitckus, S. (1992) *Proteins* **13,** 258–271.
18. Finkelstein, A. & Reva, B. (1991) *Nature (London)* **351,** 497–499.
19. Godzik, A. & Skolnick, J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 12098–12102.
20. Higgins, D. G. & Sharp, P. M. (1988) *Gene* **73,** 237–244.
21. Laudet, V., Hänni, C., Coll, J., Catzeflis, F. & Stéhelin, D. (1992) *EMBO J.* **11,** 1003–1013.
22. Shenkin, P. S., Yarmush, D. L., Fine, R. M., Wang, H. & Levinthal, C. (1987) *Biopolymers* **26,** 2053–2087.
23. Siezen, R. J., de Vos, W. M., Leunissen, J. A. M. & Dijkstra, B. W. (1991) *Protein Eng.* **4,** 719–737.
24. Carlstedt-Duke, J., Strömstedt, P.-E., Persson, B., Cederlund, E., Gustafsson, J.-Å. & Jörnvall, H. (1988) *J. Biol. Chem.* **263,** 6842–6846.
25. Strömstedt, P.-E., Berkenstam, A., Jörnvall, H., Gustafsson, J.-Å. & Carlstedt-Duke, J. (1990) *J. Biol. Chem.* **265,** 12973–12977.
26. Simons, S. S., Pumphrey, J. G., Rudikoff, S. & Eisen, H. (1987) *J. Biol. Chem.* **262,** 9676–9680.
27. Chakraborti, P. K., Garabedian, M. J., Yamamoto, K. R. & Simons, J. (1991) *J. Biol. Chem.* **266,** 22075–22078.
28. Garcia, T., Benhamou, B., Gofflo, D., Vergezac, A., Philibert, D., Cambon, P. & Gronemeyer, H. (1992) *Mol. Endocrinol.* **6,** 2071–2078.
29. Harlow, K. W., Smith, D. N., Katzenellenbogen, J. A., Greene, G. L. & Katzenellenbogen, B. S. (1989) *J. Biol. Chem.* **264,** 17476–17485.
30. Reese, J. C., Wooge, C. H. & Katzenellenbogen, B. S. (1992) *Mol. Endocrinol.* **6,** 2160–2166.
31. Brändén, C. (1980) *Q. Rev. Biophys.* **13,** 317–338.
32. Brändén, C. & Tooze, J. (1991) *Introduction to Protein Structure* (Garland, New York).
33. Fawell, S. E., Lees, J. A., White, R. & Parker, M. G. (1990) *Cell* **60,** 953–962.
34. Danielian, P. S., White, R., Lees, J. A. & Parker, M. G. (1992) *EMBO J.* **11,** 1025–1033.
35. Pratt, W. B. (1990) *Mol. Cell. Endocrinol.* **74,** C69–C76.
36. Ganesan, S., Bashirelahi, N., Young, J. D. & Cohen, S. P. (1987) *Life Science* **41,** 2767–2776.
37. Baker, M. E. & Kimlinger, W. R. (1982) *Biochem. Biophys. Res. Commun.* **108,** 1067–1073.
38. Toney, J. H., Wu, L., Summerfield, A., Sanyal, G., Forman, B., Zhu, J. & Samuels, H. (1993) *Biochemistry* **32,** 2–6.