Progesterone Receptor Ligand Binding Pocket Flexibility: Crystal Structures of the Norethindrone and Mometasone Furoate Complexes

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Although progesterone, the natural ligand of the progesterone receptor (PR), has a hydrogen atom at the 17α position, other potent steroid agonists such as norethindrone and mometasone furoate have larger substituents at this position that are accommodated by the PR ligand binding pocket. Crystallographic analysis of PR ligand binding domain complexes clearly demonstrated that these moieties were accommodated by local shifts of the protein main chain and by adoption of alternative side chain rotamer conformations of ligand-proximal amino acids. These conformational changes imparted a ligand-specific volume to the binding pocket, from 490 Å^3 in the metribolone complex to 520 Å^3 in the norethindrone complex, 565 Å^3 in the progesterone complex, and 730 Å^3 in the mometasone furoate complex. Despite these marked alterations in binding pocket volume, critical interactions essential for establishment of an active AF2 conformation were maintained.

Introduction

The progesterone receptor (PR) has long been an important drug target because of its role in the female reproductive cycle.[1] The natural agonist of PR, progesterone, plays a central role in the establishment and maintenance of pregnancy. Increased progesterone levels in plasma are responsible for the lack of ovulation during pregnancy through negative feedback on the hypothalamus, pituitary, and luteinizing hormone release system. This inhibitory effect of progesterone is the basis of oral contraceptives, which contain synthetic progesterone analogues called progestins.[2]

All progestins currently used in oral contraceptives are derivatives of 19-nortestosterone and differ from testosterone by the elimination of the C19 methyl group.[3] The early progestins, termed estranes, consist of norethindrone (17α-ethynylnortestosterone, Figure 1A)[4] and its derivatives norethindrone acetate, norethynodrel, and ethynodiol diacetate. The activity of norethindrone derivatives depends on in vivo conversion to norethindrone itself.[3] The 17α-ethynyl group of norethindrone resulted in increased oral bioavailability with surprisingly little effect on binding to PR.[5,6] Clearly, steroid receptors can tolerate an increase in ligand size in this region of the binding pocket.

The natural hormone for PR, progesterone, has only a hydrogen atom at 17α, while other ligands such as metribolone have a methyl group at 17α (Figure 1A). However, the PR potency and selectivity of synthetic steroid agonists can be enhanced by even larger chemical moieties at the 17 position of steroid backbones[7] (Figure 1B). Mometasone furoate (MF) is a potent progestin and glucocorticoid[7] with a furoate ester at the 17α position. Thus, PR can tolerate groups at the 17α position with a molecular weight as large as 87 Da (Figure 1).

To determine how steroid receptors structurally accommodate the added bulk at the 17α position, the structures of PR complexed with the potent progestins norethindrone and MF were determined by X-ray crystallography. These structures demonstrated that differences in protein conformation caused by compound binding were confined to protein regions immediately adjacent to the binding pocket.

Results

Potent PR Agonists with Different Substituents at the 17α Position. Norethindrone and MF differ significantly in structure from progesterone and metribolone (Figure 1A), but both compounds are potent PR agonists with K_d values of 0.4 and 0.08 nM, respectively (Figure 1B). Progesterone has 23 non-hydrogen atoms and a molecular volume of 350 Å^3 in the conformation observed in its complex with PR.[8] Compared with progesterone, norethindrone has a larger ethynyl substituent at the 17α position and a smaller hydroxyl group at the 17β position and lacks the C19 methyl group. Overall, norethindrone has 22 non-hydrogen atoms, one less than progesterone, but the molecular volume is larger (370 Å^3) (Table 2). The difference in molecular volume may be due to the ethynyl group, which projects outward from the steroid core, whereas the substituents in progesterone tend to occupy volume that is already partly inaccessible to solvent. By contrast, MF has larger substituents at 17α and 17β, as well as a C19 methyl group and additional substituents at the 9 and 11 positions. MF has a total of 35 heavy atoms and a molecular volume of 485 Å^3 (Table 2). From these differences in ligand size, it was of interest to determine how PR accommodated extra mass while maintaining transcriptional activity.

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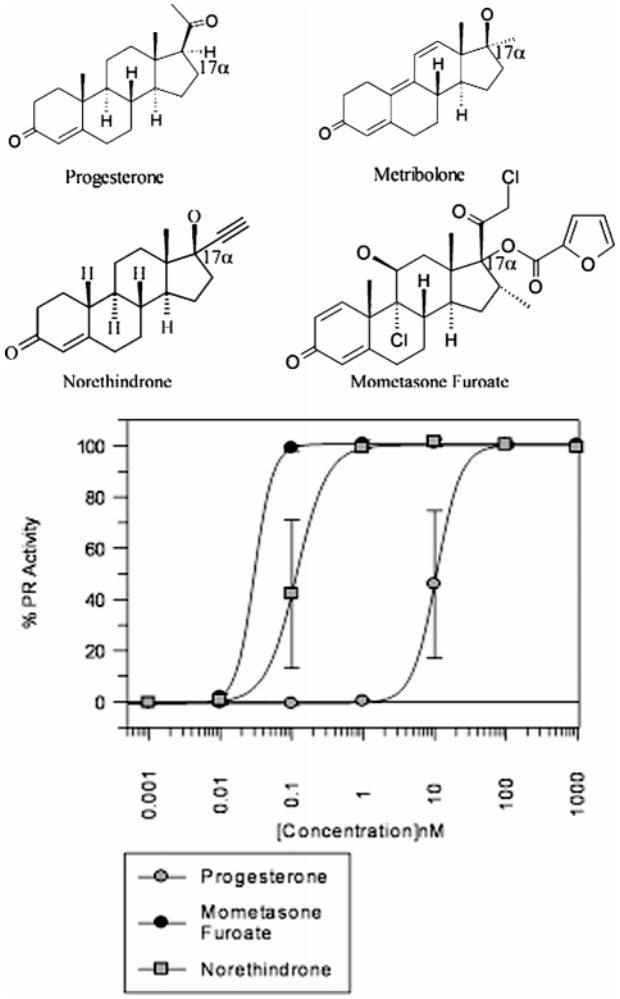

Figure 1. Increased bulk at the 17α position leads to increased potency on PR: (A) structures of progesterone, metribolone, norethindrone, and mometasone furoate showing moiety size at 17α; (B) norethindrone and MF are potent agonists of the progesterone receptor.

Structurally Conserved Regions of the PR/Ligand Complexes. To measure deviations in atomic positions caused by ligand binding, each molecule of the asymmetric unit of the PR/progesterone structure was overlaid with the corresponding molecules from the PR/norethindrone and PR/MF structures (Table 1) using an iterative superimposition algorithm.[9] As shown in Figure 2A, the overall position of residues 682−932 was similar in the three structures. Compared with the standard progesterone complex,[8] the metribolone,[10] norethindrone, and MF complexes gave Cα rms deviations of 0.43−0.52, 0.21−0.24, and 0.31−0.37 Å, respectively. By contrast, comparisons of Cα backbone shifts from the noncrystallographically related complexes for the progesterone, metribolone, norethindrone and MF complexes gave rms deviations of 0.84, 0.40, 0.88, and 0.87 Å, respectively. Thus, global ligand-dependent shifts of these residues are smaller than differences between the two crystallographically independent complexes of each PR/ligand structure. For these complexes, the largest deviations occur in residues that are packed around the MF furoate group. Compared with the PR/progesterone complex, five residues that are packed around the furoate group (Leu714, Phe794, Leu797, Cys798, and Tyr890) had side chain atom rms deviations of 2.02−2.35 Å in the PR/MF structure but only 0.26−0.60 Å in the PR/norethindrone structure. Visual comparisons of these structures (Figure 2B) suggested that helix-6, helix-7, and the connecting loop were shifted outward in the MF structure, with the largest deviations in residues 791−794. Compared with the crystallographically related subunit of the PR/progesterone structure, backbone atoms in residues 791−794 had rms deviations of 0.77−0.80 Å in the MF structure and only 0.25−0.36 Å in the norethindrone structure. Virtually all of these atoms were shifted outward, effectively expanding the pocket available to the 17α substituent. These results suggest that backbone shifts incurred upon binding of the bulkier ligand resulted in local rearrangements that were not propagated throughout the structure.

Table 1. Crystallographic Data and Refinement

	PR/norethindrone	PR/MF
Crystallographic Data		
space group	$P2_1$	$P2_1$
a, Å	57.7	58.1
b, Å	64.1	64.3
c, Å	70.1	70.1
β, deg	95.8	96.9
resolution range	20−1.7	20−1.6
observations (unique)	460 483 (54 571)	402 501 (66 100)
completeness (final shell)	99.3 (97.0)	98.5 (90.0)
I/σ (final shell)	20.1 (3.8)	19.7 (3.0)
R_{merge}, % (final shell)	5.3 (22.9)	5.0 (24.6)
Refinement Statistics		
resolution range	20−1.7	20−1.6
% R_{free}	7	7
R_{cryst} (R_{free})	18.6 (21.5)	17.6 (21.0)
protein atoms	3968	4007
ligand atoms	44	70
solvent molecules	276	297
rmsd bonds, Å	0.03	0.04
rmsd angles, deg	2.0	3.1

Table 2. Pocket Volumes

		ligand volume, Å^3		pocket volume, Å^3	
ligand	heavy atoms	subunit A	subunit B	subunit A	subunit B
metribolone	21	302	299	486	526
norethindrone	22	368	380	527	521
progesterone	23	349	351	566	569
MF	35	485	485	734	730

Conserved Interactions with the Steroid A-Rings. Inspection of the protein/ligand contacts for PR and different steroid agonists identified a number of conserved interactions that most likely represent common contacts required for recognizing and binding progestins (Figure 3). As previously observed in other PR/steroid complexes, a hydrogen bond was observed between O3 of the steroid A-rings of both norethindrone and MF and the side chain of Gln725. This hydrogen bond between a conserved glutamine and the A-ring oxygen has been observed in all crystal structures of 3-keto steroid receptors.[8,10−13] As observed with other 3-keto steroids, the side chain of a conserved arginine, PR Arg766, formed the linchpin of a hydrogen bond network centered on the A-ring O3. This network also included a hydrogen bond between Arg766 and the main chain amide of Phe788, which made van der Waals contact with the steroid A-ring. Most of the remaining PR−ligand interactions were hydrophobic, but some

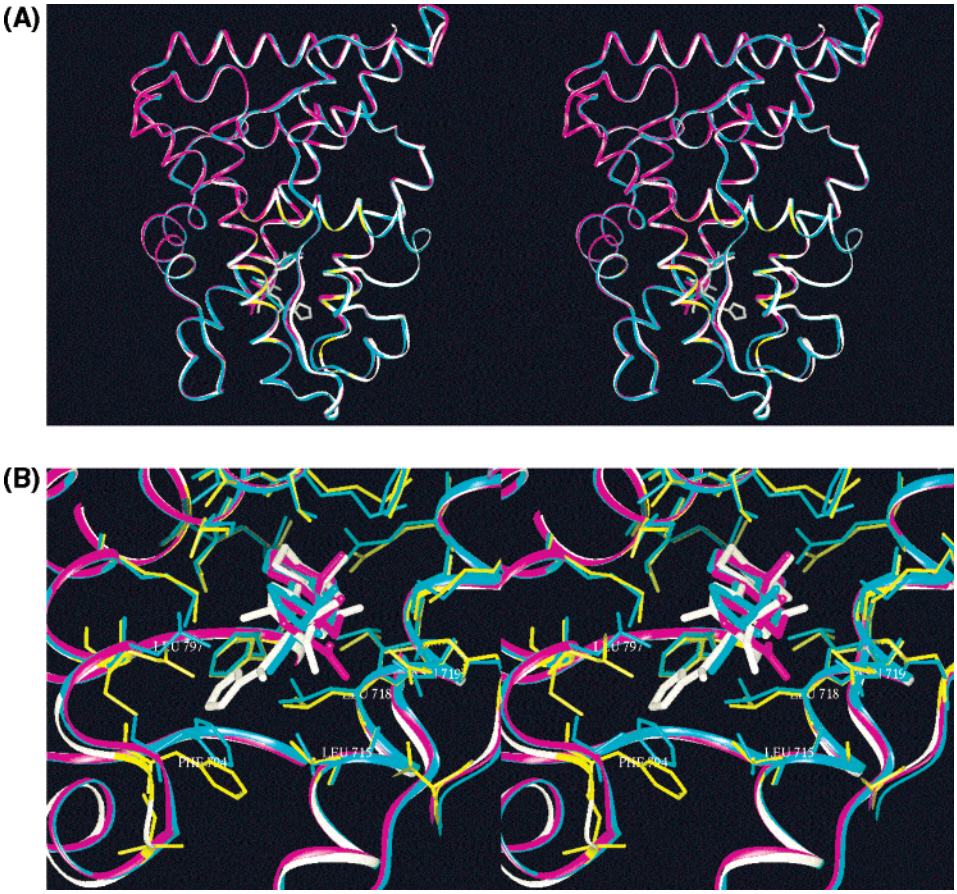

Figure 2. Ribbon representation showing the overlay of PR bound to progesterone (magenta), norethindrone (cyan), and MF (white). (A) The overall changes in the main chain position are small. (B) Interactions with the bulkier furoate moiety of MF cause a slight shift of the main chain from helix-6 to the bottom of helix-7 (residues 784−801). Residues within 5 Å of MF are colored yellow.

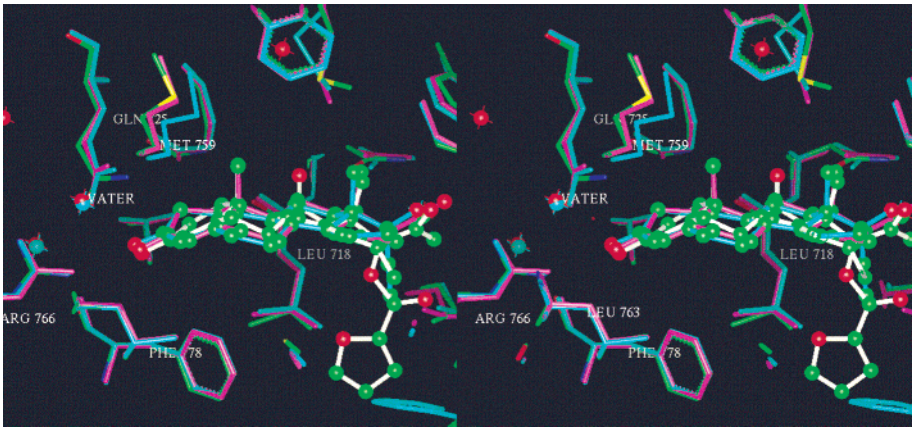

Figure 3. Overlay of PR bound to progesterone (magenta protein and ligand bonds), norethindrone (cyan protein and ligand bonds), and MF (protein is colored by atom; ligand has white bonds) shows the conservation of interactions around the steroid A-ring. Ligand atoms and MF protein atoms are colored green, blue, and red for carbon, nitrogen, and oxygen, respectively.

polar interactions involving the D-ring may be responsible for molecular recognition and increased affinity.

Ligand-Specific Interactions with the Steroid Core. Although norethindrone and progesterone are structurally similar, the unique groups at C17 of the D-ring produced a shift in the position of norethindrone within the pocket such that this ligand made contacts not observed in the PR/progesterone complex. Compared to progesterone, norethindrone has a larger group at 17α (an ethynyl) and a smaller substituent at 17β (a hydroxyl replaces a formyl). These two changes produced a slight rotation of norethindrone within the pocket that displaced the D-ring further from helix-3 and provided room for a water molecule to mediate an additional hydrogen bond between the C17 hydroxyl and the side chain of Asn719 (Figure 4A). This water-mediated hydrogen bond was observed in both noncrystallographically related molecules.

Unlike other steroids observed in nuclear receptor crystal structures, the mometasone core has a hydroxyl group at C12, which made an additional hydrogen bond with Asn719. The large 17α substituent of mometasone

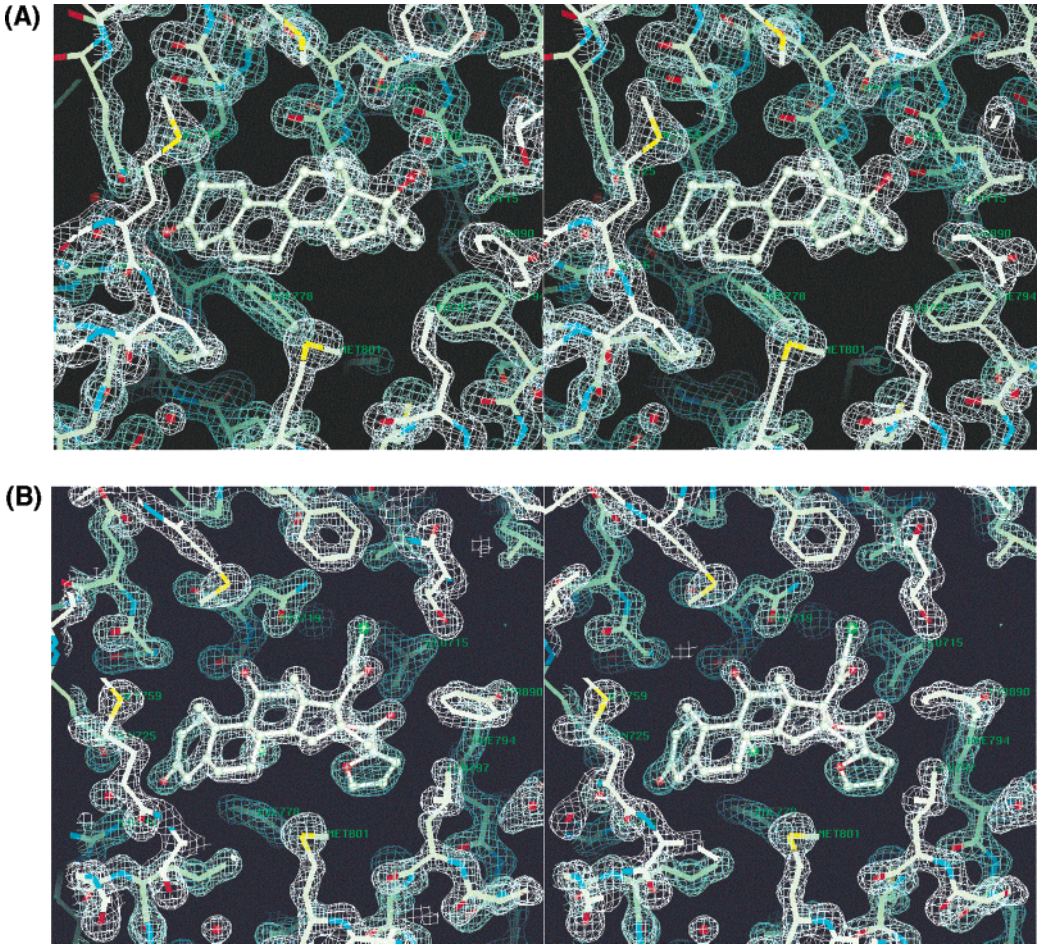

Figure 4. Additional interactions between PR and the two ligands increase potency. Electron density maps shown are $2F_o - F_c$ maps contoured at 2σ. (A) Norethindrone has a water-mediated hydrogen bond to Asn719. Norethindrone is drawn as a ball-and-stick figure, while PR is a stick figure. Ligand and protein atoms are colored white, blue, and red for carbon, nitrogen, and oxygen, respectively. Distances are in angstroms. (B) MF causes some side chain rearrangements in the 17α back pocket. Ligand and protein atoms are colored white, blue, red, yellow, and green for carbon, nitrogen, oxygen, sulfur, and chlorine, respectively.

is inserted into the same pocket as the 17α-ethynyl group of norethindrone. Possibly because of this 17α substituent, the D-ring of mometasone furoate is rotated away from helix-3, similar to norethindrone. While this rotation opened space for a water molecule bridging the 17β-hydroxyl to Asn719 in the norethindrone complex, the same volume is occupied by the 17β-chloroacetyl group in the mometasone furoate structure. As observed with the acetyl group in progesterone, the 17β-chloroacetyl oxygen formed no hydrogen bonds with the protein. Although there were polar residues in the vicinity of this oxygen (Asn719, Tyr890, and Thr894), none were positioned to form a strong hydrogen bond. Also, although there are several polar groups in the 17α substituent, none formed hydrogen bonds with the protein.

Accommodation of 17α Position Groups within a "Back" Pocket. PR accommodated norethindrone through a slight rotation and translation of the steroid core, with the binding pocket amino acid side chains retaining conformations similar to those observed in the progesterone complex. The 17α-ethynyl group of norethindrone was projected into a 17α "back" pocket in PR. Although progesterone has no substituent at the 17α position, an overlay of the PR/progesterone complex onto the PR/norethindrone complex showed that the 17α back pocket existed in the progesterone complex but was unoccupied. The PR residues lining the 17α back pocket adopted similar conformations in both structures, with deviations of 0.7 Å or less in the backbone. The largest deviations occurred in Phe794 and Leu797, which swing 0.9 and 1.3 Å away from the ligand in the norethindrone complex. The C19 methyl group in progesterone projects into a bubble-like pocket in PR. Norethindrone has no methyl group at this position, allowing Met759 to shift about 0.9 Å closer to the ligand in both molecules in the asymmetric unit and allowing Met909 to shift 1.5 Å closer in one of the two subunits. These shifts significantly reduced the volume of the pocket (Table 2), more than compensating for the very slight expansion in the 17α back pocket. The volumes of the pockets in the norethindrone complex were 527 and 520 Å^3 for the two subunits. Applying the same pocket volume measurement technique to the PR/progesterone complex yielded 566 and 569 Å^3 for the two subunits (Table 2).

The MF furoate group was projected into the same 17α back pocket as the ethynyl group of norethindrone. However, it occupied more volume and induced larger conformational changes upon the protein. Residues lining the 17α back pocket in the PR/MF complex experienced main chain deviations of up to 1.1 Å from the corresponding subunit in the progesterone complex (Figure 2B). The side chains of Phe794 and Leu797 rotated, moving their tips 2.6 and 2.9 Å relative to their

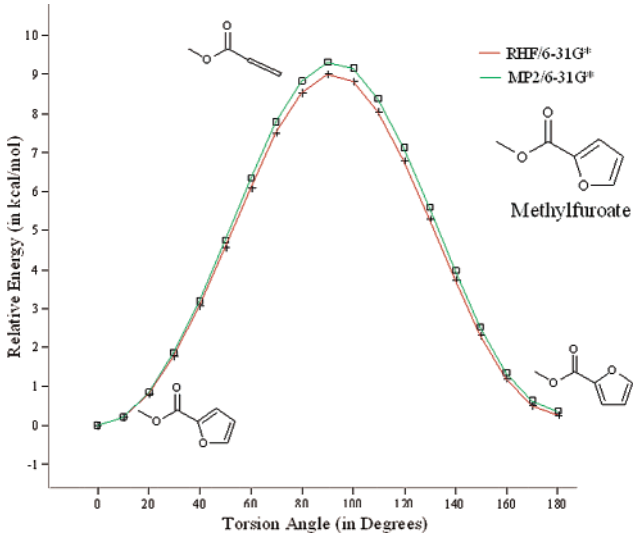

Figure 5. Ab initio generated torsional profile of methyl furoate illustrating that the furan ring of the furoate ester can adopt either the "cis" or "trans" orientation.

positions in the progesterone complex, and a number of other side chains also underwent movements of more than 1.5 Å (Figures 2B and 4B). Like progesterone, MF does have a C19 methyl group, and the protein accommodated it in a "bubble-like" pocket similar to that in the progesterone complex. These conformational changes resulted in increased pocket volumes of 734 and 730 Å^3 for the two subunits of the MF complex (Table 2). Although we were unable to ascertain the absolute orientation of the furan ring, there were no discernible polar interactions between the protein and the furoate ester in either orientation. Quantum mechanical analysis of the torsion properties of the bond between the furan ring and ketone function indicated that either orientation of the furan is equally likely in the gas phase (Figure 5).

Conclusions

The progestins in oral contraceptives, apart from their primary function, provide an array of short- and long-term noncontraceptive health benefits that include menstrual cycle control, treatment of dysfunctional uterine bleeding, protection against several gynecologic and nongynecologic cancers, and preservation of bone density.[14] Understanding the interactions between PR and its ligands will aid in the design of more potent and selective drugs.

We have identified a novel water-mediated interaction between the 17β-hydroxyl group of norethindrone and Asn719. This interaction enhanced PR's affinity for norethindrone and only was possible because norethindrone rotated 5° relative to progesterone within the pocket, thereby creating sufficient room for a water molecule to bind with good hydrogen bond geometry to both norethindrone and Asn719. Modeling suggested that if norethindrone were bound in the same orientation as progesterone, then the water would be squeezed out of its optimal position and the hydrogen bonds would be much weaker. The D-ring interaction observed in the PR/norethindrone complex is different from that observed for other steroid receptor complexes involving testosterone derivatives. Relative to progesterone, dihydrotestosterone (DHT), when complexed with the androgen receptor (AR), and metribolone complexed with PR are rotated 8° and 7°, respectively, in the opposite direction from norethindrone, which brings the 17-hydroxyl close enough to AR Asn705 or PR Asn719 to hydrogen-bond directly.[10,11] The rotation of DHT may be enhanced by the presence of threonine 877 in AR, which can also hydrogen-bond directly with DHT, versus cysteine 891 in PR. Given that norethindrone, metribolone, and DHT all have the 17β-hydroxyl group, why does norethindrone rotate in the opposite direction? Modeling suggested that the 17α-ethynyl group of norethindrone would bump Leu719 if it rotated in the same direction as DHT, which is supported by the observation that MF rotated in the same direction as norethindrone. Only the rotation of norethindrone permitted the bridging water molecule to bind in a position where all the hydrogen bonds have good geometry.

The molecular volume of mometasone furoate is 39% larger than either norethindrone or progesterone, with most of the additional bulk comprising the furoate ester moiety at the 17α position of the steroid core. PR managed to fit this additional bulk within the pocket with minimal change in protein conformation. The changes included the following: (1) an outward shift of residues 787 to 800 by 0.8 Å opened the pocket slightly; (2) Leu797 and Phe794 adopted alternative rotamers; (3) the side chain of Tyr890 moved 0.9 Å. These changes expanded the existing 17α back pocket and increased the pocket volume by 165 Å^3, thereby permitting the protein to completely enclose MF. The residues lining the back pocket and surrounding the furoate ester were all hydrophobic. The furoate ester made no specific interactions with the protein. However, the increase in buried surface area substantially increased the potency of the compound and led to this compound's tight binding to the progesterone receptor.[7]

The conformational flexibility of proteins often makes ab initio prediction of ligand binding modes difficult, and the PR structures reported here illustrate this point clearly. Because both norethindrone and MF are 3-keto steroids, the positions of the A-rings remained essentially unchanged because of the conserved interaction of the carbonyl oxygen with Gln725 and a water molecule. However, the D-ring substituents of norethindrone and MF differ significantly from progesterone, necessitating some alteration of either the binding orientations of these compounds or the protein conformation in this region. Despite the additional group at the 17α position, norethindrone binding to PR essentially maintained the backbone and side chain conformations of the progesterone complex. Norethindrone was accommodated in the binding pocket simply through a rotation of the ligand and inclusion of a water molecule. However, MF, which differs significantly from progesterone in both the 17α and 17β positions, induced alterations of both the protein main chain and side chain conformations to accommodate the ligand.

Given these and other crystal structures, knowing how PR will respond upon binding of different ligands is difficult to predict. Small molecule docking coupled with dynamics calculations could potentially address this problem. However, the sheer number of conformations that the ligand and the protein may adopt and the

questionable accuracy of such calculations limit one's ability to select the appropriate ligand pose and protein conformation. Thus, the results of such calculations may lead to inappropriate conclusions about the ligand-binding mode. Upon inspection of the PR/ligand complexes reported in this study, the global protein conformation remained relatively unchanged while ligand-proximal amino acid side chains adopted different conformations in order to accommodate the bulky substituents. However, extending these observations to more diverse steroid or nonsteroid ligands with fewer conserved interactions is questionable given the apparent conformation changes. In the usual case, PR ligand binding could involve a more complex and coordinated movement of residue side chains and backbone movements, unpredictable by traditional means.

Experimental Section

Transcriptional Activation Measurements. Activation measurements were performed as described previously.[7]

Protein Expression and Purification. The ligand binding domain (LBD) of the human progesterone receptor (amino acids 678−933) was cloned into pET24a (Novagen) with a MKKGHHHHHHG tag at the N-terminus of GST and a thrombin protease site between GST and the PR LBD.[12] Transformed BL21*[DE3] *Escherichica coli* were grown in 2XYT media with 10 μM ligand present (norethindrone or mometasone furoate). Cells were lysed in a buffer containing 2 M urea, 300 mM NaCl, 50 mM Tris (pH 8.0), and 10 μM ligand, and the lysate was clarified by centrifugation. The supernatant was loaded onto a nickel NTA affinity column (Qiagen) and eluted with a 25−500 mM imidazole gradient. The peak fractions were pooled and dialyzed in four steps to remove the 2 M urea and to add 10% glycerol and 0.1% β-octylglucoside. The fusion protein was cleaved with thrombin by incubation for 20 h at 4 °C at a mass ratio of 1:250. Glutathione sepharose resin was added batchwise and incubated for 30 min to remove the 6-his GST. The sample was diluted to give a total NaCl concentration of 30 mM and loaded onto a High Performance S column (AP Biotech), and the protein eluted with a 25−250 mM NaCl gradient. Peak fractions were collected and adjusted to 100 mM NaCl and then concentrated to 7 mg/mL for crystallization trials.

Protein Crystallization. The best crystals were obtained when 100 mM $LiSO_4$ was exchanged for the 100 mM NaCl in the protein buffer. Crystals formed in 3 days at room temperature in 12−15% PEG 1000, 100 mM $LiSO_4$, 50 mM HEPES (pH 6.5), and 10% glycerol. Crystals were placed in 25 μL of a 4 °C harvesting buffer (25% PEG 1000, 10% glycerol, 100 mM $LiSO_4$, 50 mM HEPES (pH 6.5)). A slow-exchange cryobuffer (25% PEG 1000, 40% glycerol, 100 mM $LiSO_4$) was added in 3, 4, 5, 6, and 7 μL increments with an hour between additions (also at 4 °C). The crystals remained in the final glycerol concentration (25%) overnight before being frozen in liquid nitrogen.

Data Collection and Processing: Structure Solution and Refinement. X-ray diffraction data were collected at a wavelength of 1 Å at the Advanced Photon Source on the IMCA 17-ID beamline using an ADSC 210 detector and was reduced using HKL2000.[15]

The PR complexes crystallized in the space group $P2_1$, with $a = 58.1$ Å, $b = 64.3$ Å, c=70.1 Å, $\beta = 96.9°$, and two molecules per asymmetric unit. The structures were solved by molecular replacement with AMORE,[16] using a single protein subunit of the PR/progesterone complex as a search model. Protein rebuilding utilized Quanta. Refinement was completed with Refmac5.[17]

Overlays and Root Mean Square Deviations. The iterative superimposition facility in the MVP program[9] was used to overlay each subunit of the PR/norethindrone, PR/MF, and PR/metribolone complexes onto the A-subunit of the PR/progesterone complex. This facility generated an initial superimposition from the sequence alignment and then iteratively refined the superimposition by using only the residues that deviate by less than 1.0 Å from their counterparts in the reference structure. Residues at the ends of the chain may be disordered, and residues 706, 707, and 861 were not modeled in all of the structures, so positions of backbone N, Cα, C, and O atoms of residues 686−705, 708−860, and 862−930 were used. The rms deviations reported here for the backbone, specific segments, and specific sets of residues were calculated within MVP without any further rotation or translation.

Cavity and Ligand Volumes. Cavities were defined with the program MVP[9] by filling the pockets and covering the protein with layers of closely spaced water-sized spheres and then using cluster analysis to identify distinct cavities. The cluster analysis defines the boundary between interior and exterior water-sized spheres by considering the degree to which the protein occludes the solid angle from the position of each sphere. Cavity volumes were then calculated using the program GRASP[18] with a 0.2 Å grid. The sphere placement and cavity volume calculations were all done using explicit hydrogens, with the atomic radius values of Bondi.[19] Although cavity volumes are reported here to three significant figures, the accuracy is much poorer than this. The exact accuracy depends on the grid spacing and is difficult to assess because calculations with very fine grids are not practical. Ligand volumes were calculated directly with GRASP on a 0.2 Å grid using Bondi radius values and should be more accurate than cavity volumes.

Calculation of Furan Ring Orientation. In an attempt to resolve the conformational ambiguity of the 17α-furoate group of MF, methyl furoate was selected as a model system and a systematic ab initio investigation of this molecule was undertaken. Standard torsional driving calculations were carried out in order to determine the torsional profile about the furan−ester bond. All calculations were undertaken at both the Hartee-Fock (RHF) and Moller−Plesset (MP2) levels of theory with the 6-31G* basis set using the Gaussian 98 programs.[20] During the torsional profile calculations, only the torsion about the furan−ester bond remained fixed; all other internal degrees of freedom were allowed to move without constraint.

The torsional driving began at the "0° conformer" (as defined by the proper torsion among the ester oxygen, carbonyl carbon, furan carbon, and furan oxygen) and systematically examined all conformers between 0° and 180° at 10° increments along the profile. The initial geometry at 0° was obtained from CONCORD 4.0.7,[21] while the remaining conformers along the profile were generated from the previous local minimum. After completion of the torsional profile calculation, full geometry optimization and vibrational frequency calculations (at the respective levels) were carried out on the 0° and 180° conformers. These calculations ensured that an accurate appraisal of the energy difference between the two conformations had been determined and true minima had been located.

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