Role of Substrate Conformational Features in the Stereospecificity of Aromatase†

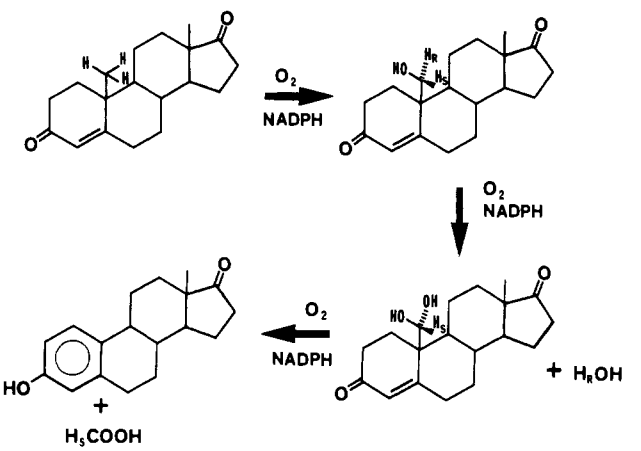
Denise D. Beusen, Barry L. Kalman, and Douglas F. Covey*
Department of Pharmacology, Washington University Medical School, St. Louis, Missouri 63110
Received May 6, 1985

ABSTRACT: Hydroxylation of 19-hydroxyandrost-4-ene-3,17-dione (19OHA) by aromatase occurs at the 19-pro-*R* hydrogen, suggesting that the C_{19} group has a preferred conformation in the enzyme active site. X-ray crystallographic studies have led to a postulate that the steroid plays a role in determining this conformation. In an effort to quantitate the steroid's role, we estimated conformational constraints about the C_{10}–C_{19} bond of 19OHA using molecular mechanics calculations. Rotational barriers ≤6 kcal/mol and energy differences between conformers ≤1 kcal/mol were found. We perturbed these conformational constraints by preparing an altered substrate, 19-hydroxyandrosta-4,6-diene-3,17-dione (19OHAD). The stereospecificity of aromatization for 19OHA and 19OHAD was found to be the same. Thus, theoretical and experimental approaches both indicate that conformational constraints intrinsic to 19OHA cannot be a major determinant in the stereospecificity of its oxidation by aromatase.

During the conversion of androstenedione to estrone by the microsomal P-450 aromatase, 19-hydroxyandrostenedione is generated in the first of three NADPH-requiring monooxygenations (Longchampt et al., 1960; Meyer, 1955; Wilcox & Engel, 1965). Two subsequent monooxygenations by this enzyme transform 19OHA[1] into the final product and result in release of the angular C_{19} group as formic acid (Starka & Breuer, 1970; Axelrod et al., 1965; Skinner & Akhtar, 1969). As depicted in Scheme I, this formate retains the 19-pro-*S* hydrogen of 19OHA. The 19-pro-*R* hydrogen of 19OHA is released to water, presumably by oxygen insertion into the 19-pro-*R* carbon–hydrogen bond during the second of the three oxygenations (Arigoni et al., 1975; Osawa et al., 1975). The first two oxygenations of aromatase are believed to occur in the same active site (Kelly et al., 1977).

Some investigators have proposed that aromatase delivers oxygen to C_{19} from the out-of-ring position, trans to the C_5–C_{10} bond (Osawa, 1973; Duax & Osawa, 1980). If one assumes that aromatase delivers oxygen from the same site each time, rotation about the C_{10}–C_{19} bond of 19OHA must occur in order to present a new carbon–hydrogen bond to the enzyme for the second oxygenation. This rotation could give rise to the two conformers shown in Scheme II. The first conformer corresponds to that observed for crystalline 19OHA (Duax & Osawa, 1980) and has the correct 19-hydrogen poised for oxygenation trans to the C_5–C_{10} bond. The second conformer of Scheme II has the pro-*S* C_{19}–H bond in the out-of-ring position, and oxygenation of this conformer would result in release of the 19-pro-*S* hydrogen to water. Because of possible 1,3-diaxial interactions between the 19-hydroxyl and the 6β- and 8β-axial hydrogens, this latter conformer may be higher in energy than conformer 1. A large enough energy difference could preclude 19OHA from assuming conformer 2 in the enzyme active site, and the stereospecificity of 19OHA aromatization could then be a consequence of the sole presence of conformer 1 in the active site. On the basis of crystallographic studies, some investigators have in fact invoked a role for conformational features of the steroid in the stereospecificity of aromatization (Duax et al., 1976).

Scheme I



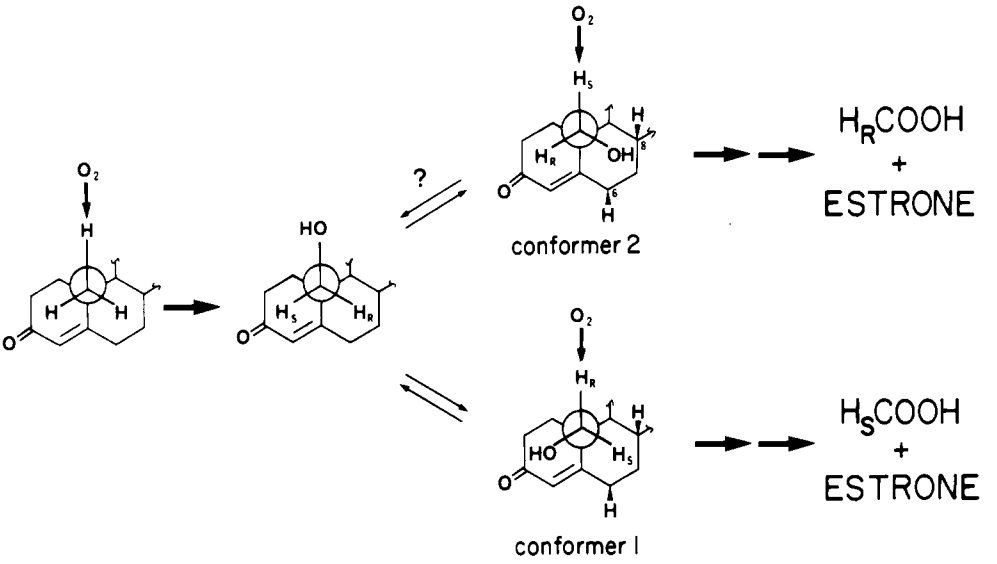
Clearly, the interaction of aromatase with its substrate must result in immobilization of the C_{10}–C_{19} bond during the second oxygenation in order to yield the enzyme's stereospecificity. The qualitative argument outlined previously moved us to seek a quantitative assessment of the steroid's contribution to this process. Accordingly, we performed molecular mechanics calculations on 19OHA to obtain estimates of (1) the steroid's rotational energy barriers about the C_{10}–C_{19} bond and (2) the relative population of the steroid's three minimum-energy conformers. Our concerns about the limitations of these calculations prompted us to experimentally evaluate the steroid's contribution by determining the stereospecificity of 19-hydroxyandrosta-4,6-diene-3,17-dione aromatization. This substrate lacks a 6β-axial hydrogen, and the 1,3-diaxial in-

† This work was supported by National Institutes of Health Grants RR-00954 and CA-23582. D.F.C. is the recipient of Research Career Development Award CA-00829 from the National Institutes of Health. D.D.B. is supported by National Institutes of Health Training Grants 5T32GM07067 and 5T32HL07275.

[1] Abbreviations: 19OHA, 19-hydroxyandrost-4-ene-3,17-dione; 19OHAD, 19-hydroxyandrosta-4,6-diene-3,17-dione; BSTFA, bis(trimethylsilyl)trifluoroacetamide; FID, flame ionization detection; GC, gas chromatography; MS, mass spectrometry; *S*-19OHA, (19*S*)-19-hydroxyandrost-4-ene-3,17-dione-*19-d*; *R*-19OHA, (19*R*)-19-hydroxyandrost-4-ene-3,17-dione-*19-d*; d_2-19OHA, 19-hydroxyandrost-4-ene-3,17-dione-*19,19-d*$_2$; *S*-19OHAD, (19*S*)-19-hydroxyandrosta-4,6-diene-3,17-dione-*19-d*; *R*-19OHAD, (19*R*)-19-hydroxyandrosta-4,6-diene-3,17-dione-*19-d*; d_2-19OHAD, 19-hydroxyandrosta-4,6-diene-3,17-dione-*19,19-d*$_2$; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-0662$01.50/0

Scheme II



teractions described previously must be less than those for 19OHA. Both approaches led us to conclude that these 1,3-diaxial interactions are insufficient in themselves to dictate a particular 19OHA conformer in the active site of aromatase.

Experimental Procedures

Materials. NADPH and 9-anthraldehyde were purchased from Sigma. Human placental microsomes were prepared and assayed for protein and aromatase activity as described previously (Covey et al., 1981). Baker extraction system and columns were purchased from J. T. Baker Chemical Co., Inc. BSTFA and GC supplies were obtained from Supelco, Inc. The 19OHA was purchased from G. D. Searle, Inc. $NaBD_4$, $NaBH_4$, MnO_2, and chloranil were purchased from the Alpha Division of Ventron Corp. Silica gel activity grade III was purchased from Woelm. The 9-anthryldiazomethane was prepared by the method of Barker et al. (1980) and had physical properties identical with those reported previously. It was stored as a 10 mM solution in ether at −80 °C.

Preparation of Substrates. The 19-oxoandrostenedione and (19*S*)-19-hydroxyandrost-4-ene-3,17-dione-*19-d* were prepared from 19OHA by the method of Akhtar et al. (1982). By use of similar methods, (19*R*)-19-hydroxyandrost-4-ene-3,17-dione-*19-d* was prepared by oxidizing *S*-19OHA to 19-oxoandrostenedione-*19-d* and reducing this aldehyde with $NaBH_4$. The 19-hydroxyandrost-4-ene-3,17-dione-*19,19-d*$_2$ was prepared by reduction of 19-oxoandrostenedione-*19-d* with $NaBD_4$. The physical properties of each deuterated 19OHA were identical with those of authentic 19OHA.

19-Hydroxyandrosta-4,6-diene-3,17-dione, (19*R*)-19-hydroxyandrosta-4,6-diene-3,17-dione-*19-d*; (19*S*)-19-hydroxyandrosta-4,6-diene-3,17-dione-*19-d*, and 19-hydroxyandrosta-4,6-diene-3,17-dione-*19,19-d*$_2$ were prepared from their corresponding 19-hydroxyandrostenediones in the following fashion. Typically, 19OHA (373 mg, 1.2 mmol) was refluxed for 3 h with chloranil (1.5 g, 6.1 mmol) and 2-methyl-2-propanol (19 mL). An equal volume of 2-propanol was added, and the reaction mixture was filtered through Celite. Solvent was removed under vacuum, and the residue was purified on a silica gel column (2 × 45 cm, eluant 1:1 benzene/ethyl acetate). The crude product was recrystallized from methanol/water: yield 169 mg (46%); mp 202.5–204.5 °C.

NMR Methods. Quantitative analysis of the C_{19} deuterium position by ^1H NMR, on the basis of a reduction in the integrated area of the C_{19} proton absorbances upon incorporation of deuterium, was performed in $CDCl_3/D_2O$ on a Brucker WH360. The 19-proton resonances centered at δ 3.9 were integrated for 19OHA, and those centered at δ 3.8 were integrated for 19OHAD.

Incubation of Substrates with Placental Microsomes and Isolation of Products. Incubations were done in a shaking water bath (37 °C) in air with 50-mL Erlenmeyer flasks. Each contained 100 mM KCl, 10 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 0.2 mM substrate (in 0.18 mL of ethanol), 7.5 mM NADPH, and 27 mg of microsomal protein in a total volume of 9 mL. For each experiment, five parallel incubations were prepared containing the following substrates: (1) nondeuterated steroid; (2) 19*R*-deuterated steroid; (3) a 1:1 mix of 19*R*- and 19*S*-deuterated steroids; (4) 19*S*-deuterated steroid; (5) 19-dideuterated steroid. The fifth incubation was necessitated by our discovery of significant amounts of endogenous formate in the microsomes. With these 19-dideuterated substrates, any formate produced by aromatase would contain deuterium regardless of the enzyme's stereospecificity. Thus, an upper limit could be obtained for formate production due solely to aromatase despite the presence of large amounts of endogenous formate.

After 60 min of incubation, the contents of each flask were centrifuged for 1 h at 4 °C, 100000*g*. Supernatants were applied to C_{18} Baker extraction columns preconditioned with methanol and incubation buffer. The aqueous effluent and a 2-mL buffer rinse of the column were combined and saved. Steroids eluted from the column with 90:10 methanol/water were collected, dried under N_2, and taken on to GC analysis.

The steroid-free incubation supernatants were adjusted to pH 9 with 0.4 N NaOH, transferred to 50-mL round-bottom flasks, and lyophilized overnight. The resulting powder was taken up in 1 mL of 1 N *p*-toluenesulfonic acid and lyophilized and the distillate collected in a U-tube submerged in liquid N_2. The U-tube contents were transferred to a 4-mL vial and adjusted to pH 9 with 0.4 N NaOH. These vials were lyophilized overnight, and the remaining residue, containing formic acid, was derivatized as described below.

GC Methods. All FID–GC analyses were performed on a Varian 3700 (N_2 40 mL/min, injector 280 °C, detector 280 °C). Steroids were analyzed as their trimethylsilyl derivatives prepared as follows: 0.3 mL each of BSTFA and pyridine was added to ~2 μmol of steroid, and the mixture was shaken overnight at room temperature. The reaction mixture was

dried under a stream of N_2 and taken up in 0.2 mL of methylene chloride. FID–GC analysis of derivatized steroids was done in a 6 ft × 2 mm i.d. glass column containing 1% SP 2401 on 80/100-mesh Gaschrom Q (initial temperature 190 °C; final temperature 250 °C; rate 6 °C/min). Retention times were as follows: from incubations of 19OHA, estradiol, 2.5 min; estrone, 4.1 min; 19-hydroxytestosterone, 6.7 min; 19OHA, 9.7 min; from incubations of 19OHAD, 1,3,5-(10),6-estratetraene-3,17β-diol, 2.9 min; 3-hydroxy-1,3,5-(10),6-estratetraen-17-one, 4.4 min; 17β,19-dihydroxyandrosta-4,6-dien-3-one, 6.5 min; 19OHAD, 9.2 min. The identity of each peak was established by GC–MS. Percent conversion of starting steroid to estrogens was calculated from peak areas. In all cases, 17β-hydroxysteroid dehydrogenase products were observed in addition to aromatase products.

Formic acid isolated from incubations was derivatized as follows: the final lyophilization residue was taken up in 1 N HCl (0.6 mL), and 9-anthryldiazomethane (0.3 mL) was added. Vials were capped, wrapped in aluminum foil, and shaken overnight at room temperature. After addition of 0.4 N NaOH (0.2 mL), each vial was vortexed, followed by the addition of 0.05 mL of 1:1 acetic acid/water and more vortexing. The aqueous layer was extracted 2 times with fresh ether; combined ether extracts were dried over Na_2SO_4, dried under N_2, and taken up in 0.15 mL of methylene chloride. Derivatized formate was analyzed by GC on a 6 ft × 4 mm i.d. glass column containing 3% OV-1 on 100/120 Supelcoport (215 °C). The retention time of 9-anthracenemethanol formate was 6.8 min.

Mass Spectrometric Analyses. All analyses were performed on a Finnigan 3200 GC–MS with either a 2 ft × 4 mm i.d. glass column containing 1% SP2401 on 80/100 Gaschrom Q at 235 °C (for derivatized steroids) or a 2 ft × 4 mm i.d. glass column containing 3% OV-17 on 100/120 Supelcoport at 215 °C (for 9-anthracenemethanol formate). Instrument conditions were as follows: He 30 mL/min; separator 240 °C; injector 220 °C; ionizing energy 70 eV. Selected ion monitoring was used to determine relative intensities of ions in the molecular ion region, and percent incorporation of deuterium was calculated from these intensities.

Calculations. Since aromatase is stereospecific in hydroxylating the C_{19}–H_R bond (Osawa, 1975), one can predict[2] the deuterium present in formate generated from deuterated 19OHA by aromatase. For the mixed-substrate incubation, this value is the average of the theoretical values for *R*-19OHA or *S*-19OHA alone. These theoretical values would be observed experimentally in the absence of endogenous microsomal formate.

The corrected percent deuterium in formate was calculated[3] from the experimentally observed percent deuterium in formate and the percent conversion of substrate to estrogen. It enabled comparison between like incubations in different experiments by correcting for (1) dilution by endogenous formate and (2) differences in percent conversion between incubations. It does assume that dilution by endogenous formate is the same for all incubations in a given experiment.

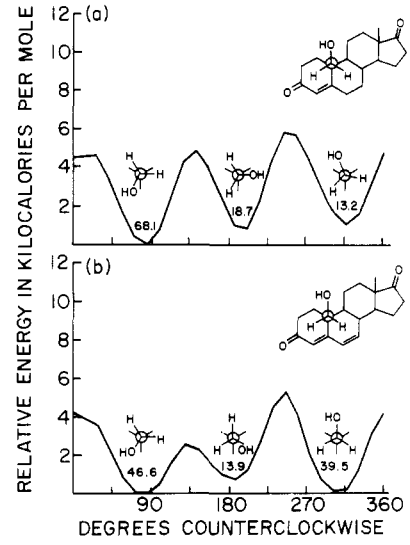


FIGURE 1: Relative strain energy of aromatase intermediates as a function of rotation about the C_{10}–C_{19} bond. Numbers shown in the energy troughs represent the percentage of molecules found in the corresponding conformer.

Conformational Analyses. By use of an MMS-X molecular graphics system (Molnar et al., 1976), an initial structure for 19OHA was generated from coordinates obtained by an X-ray crystallographic determination (Duax & Osawa, 1980). An initial structure for 19OHAD was constructed by fusing the A, B, and C rings of the crystal structure for canrenone (Weeks et al., 1976), a $\Delta^{4,6}$-3-one steroid, with the D ring of the 19OHA structure and subsequently modifying with standard fragments.

The dihedral driver option of MM2 (Allinger & Yuh, 1980), a strain energy minimization program, was used to calculate rotational profiles for 19OHA and 19OHAD. For both, the C_1–C_{10}–C_{19}–O_{19} torsional angle was varied from 0 to 360° in 15° increments. The relative strain energy (kcal/mol) at each increment was plotted vs. degrees of rotation counterclockwise to generate a rotational profile.

RESULTS

Modeling Studies. Rotational profiles for 19OHA and 19OHAD about the C_{10}–C_{19} bond are shown in Figure 1. For 19OHA (Figure 1A), these calculations revealed that barriers to rotation are moderately low, ranging from 4.6 to 5.8 kcal/mol. These barriers are not large enough to preclude the steroid from attaining any of the three minimum-energy conformers, inasmuch as a barrier of 16–20 kcal/mol is required to freeze out rotational motion (Hanack, 1965). The difference in energy between the three minimum-energy conformers is small (≤1 kcal/mol). By use of the steric partition function (Pitzer, 1940), the relative population of each minimum-energy conformer was calculated for 19OHA and is given in each energy minimum. These calculations reveal that at 37 °C all of the three possible minimum-energy conformers of 19OHA are significantly populated (68, 19, and 13%).

The results of similar calculations done on 19OHAD are shown in Figure 1B and reveal that the extra double bond of 19OHAD significantly reduced one rotational barrier (by 2.3 kcal/mol). This barrier lies between the conformer having the 19-pro-*R* hydrogen out of ring and that having it over ring A. The other two rotational barriers appear to have been only

[2] The theoretical percent deuterium in formate was calculated as

(theoretical % D in formate) =
(% D incorpn in substrate) × (fraction of D in 19*S* position)

For d_2-19OHA, all of the monodeuterated species is assumed to be *S*-19OHA, yielding a value of 77.3 + 21.7 = 99% theoretical deuterium in formate.

[3] The corrected percent deuterium in formate was calculated from

(cor % D in formate) =
(raw % D in formate) × (% conversion, d_2-19OHA incubation)/(% conversion in incubation) × (theor % D in formate from d_2-19OHA)/(raw % D in formate from d_2-19OHA incubation)

Table I: Deuterium Content and Position in Aromatase Substrates

substrate	deuterium incorporation (%)[a]	relative amounts of deuterium at[b] 19R	19S
R-19OHA	86.8 ± 0.4 (d_1)[c]	0.82	0.18
S-19OHA	94.2 ± 0.6 (d_1)	0.11	0.89
d_2-19OHA	77.3 ± 0.03 (d_2) 21.7 ± 0.01 (d_1)	ND[d]	ND
R-19OHAD	83.0 ± 0.2 (d_1)	0.80	0.20
S-19OHAD	96.6 ± 0.3 (d_1)	0.07	0.93
d_2-19OHAD	78.6 ± 0.2 (d_2) 20.7 ± 0.1 (d_1)	ND	ND

[a] Determined by GC–MS, selected ion monitoring. [b] Determined by 360-MHz ^{1}H NMR. [c] Represents standard error of the mass spectral determination. [d] Not determined.

slightly reduced. The lowest energy conformer is the same for both 19OHA and 19OHAD. An analysis of the relative population of each conformer of 19OHAD reveals that its C_6–C_7 double bond has shifted conformer populations to make the conformer having the 19-hydroxyl over neither ring more favorable (going from 13% in 19OHA to 40% in 19OHAD). This shift is at the expense of the other two conformers, both of which become less populated than their counterparts in 19OHA.

Deuterium Incorporation into Substrates. The amount and position of deuterium in substrates as determined by GC–MS and 360-MHz NMR, respectively, are given in Table I. As reported previously (Osawa et al., 1975), reduction of 19-oxoandrostenedione by $NaBD_4$ proceeds with a high degree of stereoselectivity to yield *S*-19OHA with 89% of its deuterium in the 19*S* position. Subsequent oxidation and $NaBH_4$ reduction of this steroid yields *R*-19OHA, which is deuterated predominantly (82%) in the 19*R* position. Only a small amount (~7%) of the total deuterium in *S*-19OHA is lost during its transformation into *R*-19OHA. Dideuterated 19OHA was generated by two successive cycles of oxidation and $NaBD_4$ reduction of 19OHA. By GC–MS, d_2-19OHA was found to contain predominantly two deuterium atoms (77%), while an additional 22% contained a single deuterium atom. On the basis of the stereoselectivity of the reduction and the retention of deuterium during the oxidation, one can calculate that virtually all of this monodeuterated species must have its deuterium in the 19*S* position.

The deuterated 19OHADs were prepared from 19OHA in a procedure identical with that outlined above with an additional final chloranil oxidation to generate the Δ^6 double bond. Since this final step would be unlikely to affect the stereochemistry at carbon 19, deuterium incorporation and distribution are, as expected, similar to those of their Δ^4-3-one analogues (Table I).

Determination of the Stereospecificity of 19OHA and 19OHAD Aromatization. The theoretical, uncorrected, and corrected percent deuterium in formate as well as percent conversion to estrogens are presented in Table II for studies with 19OHA. Our experimental results are consistent with previous studies demonstrating retention of the 19-pro-*S* hydrogen in formate produced during aromatization of 19OHA. Only small amounts of deuterium were found in formate isolated from incubations containing 19*R*-deuterated substrate (13.7, 9.2, and 7.5%). Conversely, sizable amounts of deuterium were observed in formate from 19*S*-deuterated steroid (67.8, 67.4, and 62.1%). As anticipated, incubations having a 1:1 mix of *R*-19OHA and *S*-19OHA yielded formate with deuterium incorporation midway between (34.3 and 49.3%) that isolated from incubations containing *R*-19OHA or *S*-19OHA alone. This result establishes the ability of our method to detect the case in which the enzyme is nondiscriminate with respect to the 19-pro-*R* and 19-pro-*S* hydrogens.

For each experiment, a comparison of the corrected values for percent deuterium in formate with the theoretical values reveals that the corrected values tend to be lower than expected (e.g., for *S*-19OHA, corrected values of 62.1–67.8% vs. theoretical 83.8%, and for *R*-19OHA, corrected values of 7.4–13.7% vs. theoretical 15.6%). The reason for this finding is unclear. Although a lack of absolute stereospecificity in aromatase can explain this anomaly for *S*-19OHA, it cannot provide an explanation for the *R*-19OHA results since the corrected percent deuterium in formate would be greater than the theoretical in this event. Alternatively, the dilution factor could have varied between incubations or something anomalous may have occurred in the d_2-19OHA incubation against which all others were normalized. Such an anomaly might take the form of a conversion rate different from that of the other substrates. However, our results do not bear this out in that the percent conversion for d_2-19OHA is comparable to that of other substrates in each experiment. Furthermore, the only kinetic isotope effect observed for this enzyme is in the first hydroxylation, which generates 19OHA (Caspi et al., 1983). Our assignment of a 19*S* configuration to the monodeuterated species present in d_2-19OHA is also unlikely to provide an explanation, since any aportionment of this species between 19*R* and 19*S* would only lead to a further reduction in the corrected percent deuterium values.

Despite the disparity between the theoretical and the corrected experimental values, the results do establish the ability of our method to reproduce previous findings on the stereopreference of aromatase (Arigoni et al., 1975; Osawa et al., 1975). Knowing this, we could move on to evaluate the stereospecificity of aromatization for 19OHAD, a compound previously shown to undergo aromatization (Braselton et al., 1974). The results from experiments using the appropriately deuterated forms of 19OHAD are shown in Table III. Generally, conversion of substrate to estrogens tended to be lower for 19OHAD than for 19OHA. This study revealed that the Δ^6 double bond of 19OHAD did not lessen the enzyme's ability to discriminate between the 19*S* and 19*R* positions. Very little deuterium was retained in formate arising from *R*-19OHAD (0, 8.5, and 5.7%), while a much greater amount was found in formate arising from *S*-19OHAD (78.7, 86.8, and 91.7%). As expected, incubations containing a mixture of substrates (*R*-19OHAD plus *S*-19OHAD) yielded formate with a deuterium incorporation midway (47.7, 42.4, and 54.0)

Table II: Percent Deuterium in Formate Isolated from Incubation of Placental Microsomes with Deuterated 19-Hydroxyandrostenediones

	incubation substrate			
	R-19OHA	1:1 *R*-19OHA/*S*-19OHA	*S*-19OHA	d_2-19OHA
theoretical	15.6	49.7	83.8	99.0
expt I	13.7[a] (5.4 ± 0.02,[b] 21.1[c])	not done	67.7 (34.8 ± 0.2, 27.4)	99.0 (43.8 ± 0.1, 23.6)
expt II	9.2 (3.4 ± 0.2, 37.8)	34.3 (15.9 ± 0.3, 47.4)	67.4 (32.1 ± 0.8, 48.7)	99.0 (34.1 ± 0.5, 35.2)
expt III	7.4 (2.2 ± 0.04, 12.3)	49.3 (12.2 ± 0.1, 10.3)	62.1 (17.3 ± 0.1, 11.6)	99.0 (23.3 ± 0.04, 9.8)

[a] Represents percent deuterium in formate corrected for variation in percent conversion and dilution by endogenous formate. [b] Represents uncorrected percent deuterium in formate. [c] Represents percent conversion of substrate to estrogens.

Table III: Percent Deuterium in Formate Isolated from Incubation of Placental Microsomes with Deuterated 19-Hydroxyandrosta-4,6-diene-3,17-diones

	incubation substrate			
	R-19OHAD	1:1 *R*-19OHAD/*S*-19OHAD	*S*-19OHAD	d_2-19OHAD
theoretical	16.6	53.2	89.8	99.3
expt I	0.0[a] (0.0 ± 0.1,[b] 20.1[c])	47.7 (16.9 ± 0.1, 18.6)	78.7 (27.6 ± 0.1, 18.4)	99.3 (29.9 ± 0.02, 15.8)
expt II	8.5 (2.0 ± 0.04, 6.2)	42.4 (12.5 ± 0.1, 7.8)	86.8 (24.9 ± 0.1, 7.6)	99.3 (19.5 ± 0.02, 5.2)
expt III	5.7 (2.1 ± 0.1, 8.3)	54.0 (27.7 ± 0.1, 11.6)	91.7 (59.2 ± 0.1, 14.6)	99.3 (40.4 ± 0.1, 9.2)

[a] Represents percent deuterium in formate corrected for variation in percent conversion and dilution by endogenous formate. [b] Represents uncorrected percent deuterium in formate. [c] Represents percent conversion of substrate to estrogens.

between that of incubations having *R*-19OHAD or *S*-19OHAD alone.

Discussion

The C_{19} rotational profile of 19OHA as determined by MM2 calculation suggested that conformational constraints intrinsic to the steroid were insufficient to dictate a particular conformation in the enzyme active site. However, MM2 is known to underestimate torsional constraints in congested molecules (Ōsawa et al., 1979), so we felt that calculations alone could not adequately answer the question. As discussed below, the computations suggested that the reduced rotational barrier of 19OHAD could lead to a decrease in aromatase stereospecificity that would have been observable by our method. Alternative probes of the steroid's contribution to aromatase stereospecificity might have been 19-hydroxyandrosta-1,4-diene-3,17-dione (lacking a 2β-hydrogen) or 19-hydroxyandrosta-4,9(11)-diene-3,17-dione (lacking a 9β-hydrogen). Androsta-1,4-diene-3,17-dione is known to be a time-dependent inactivator of aromatase (Covey & Hood, 1982), and therefore, 19-hydroxyandrosta-1,4-diene-3,17-dione did not hold much potential as a compound to evaluate by our experimental method. Computer modeling revealed that a 9(11) double bond did reduce rotational barriers at C_{19}, but this effect was not as large as that observed for 19OHAD; therefore, 19-hydroxyandrosta-4,9(11)-diene-3,17-dione was not evaluated.

The role of substrate internal rotation in the stereospecificity of aromatase can be considered from either a kinetic or a thermodynamic perspective. Viewed in a kinetic sense, the enzyme cannot exhibit any stereospecificity if the C_{19} group is rotating as fast as the enzyme can catalyze oxygenation. Thus, if $k_{cat} \simeq k_{rot}$, the enzyme would hydroxylate the 19-pro-*R* and 19-pro-*S* positions with equal probability. In order for the enzyme to be 99% stereospecific, the enzyme's rate of catalysis must be 100 times greater than the rate of rotation at C_{19}, or $k_{cat} \simeq 100\ k_{rot}$. Using this assumption, the Arrhenius equation ($k_{rot} = 10^{11} \exp[-\Delta E/(RT)]$; Harris, 1958), and reported values of k_{cat} for other (nonaromatase) androstenedione P-450 hydroxylations (0.3–14.7 min^{-1} $P\text{-}450^{-1}$; Waxman et al., 1983), one can calculate that an effective rotational barrier of 20.8–23.2 kcal/mol between enzyme-bound conformers would be required for 99% stereospecificity. Our computer modeling shows the intrinsic rotational barriers in 19OHA to be approximately 5 kcal/mol. If the barrier to rotation in the enzyme active site is a combination of steroid-derived and enzyme-derived forces, the enzyme would be required to contribute 15.8–18.2 kcal/mol to the rotational barrier. A large part of this energy could come from hydrogen bonds between the enzyme and the 19-hydroxyl of 19OHA (Fersht, 1977). If the effective rotational barrier were to drop from 20.8–23.2 to 17.9–20.3 kcal/mol, a loss of stereospecificity would be predicted since k_{cat} would equal k_{rot}. This is a reduction of 2.9 kcal/mol in the appropriate barrier. In 19OHAD, our MM2 calculations revealed that one of the steroid's C_{19} rotational barriers was reduced by 2.3 kcal/mol. If this barrier corresponds to that exploited by the enzyme in immobilizing the C_{19} group, the stereospecificity of aromatization for 19OHAD should have been only ~70% that of 19OHA (calculated from principles outlined above). Our experimental method would have been capable of detecting such a reduction. Since we observed no difference in the stereospecificity of aromatization of 19OHA and 19OHAD, we are forced to conclude that if rotational barriers in 19OHA are important to the observed reaction stereospecificity, then one of the other two rotational barriers of 19OHA would be critical.

From a thermodynamic perspective, aromatase's stereospecificity could be the result of relative conformer populations. If oxygen is delivered trans to the C_5–C_{10} bond, the conformer having the 19-hydroxyl in the out-of-ring position would be catalytically nonproductive. Of the remaining two conformers, one must be negligibly (<1%) populated in the enzyme active site to explain the stereospecificity of aromatase. This condition would be met if the difference in energy between bound conformers was >2.8 kcal/mol. For 19OHA and 19OHAD, our calculations indicate the largest energy difference between conformers in the free steroid to be ~1 kcal/mol, implying that enzyme–steroid interactions must stabilize one conformer by at least 1.8 kcal/mol. Consequently, even if structural modifications could be made that would result in equal solution populations of the two catalytically productive conformers, the enzyme would still discriminate between the two conformers by 1.8 kcal/mol and thus remain at least stereoselective. Only if the energy difference between enzyme-bound conformers were to decrease to <0.5 kcal/mol would a loss of stereospecificity be predicted, since both conformers would be equally populated.

The aromatization of 19OHAD was of interest to us for reasons other than its use as a probe of substrate conformational effects in the enzyme's stereospecificity. Our studies of 10β-propynyl inhibitors of aromatase demonstrated the ability of (1*S*)-10-(1-hydroxy-2-propynyl)estr-4-ene-3,17-dione to act as a time-dependent inhibitor of aromatase while (1*R*)-10-(1-hydroxy-2-propynyl)estr-4-ene-3,17-dione was only a competitive inhibitor (Covey et al., 1981). After modification of these inhibitors with a Δ^6 double bond (analogous to the conversion of 19OHA to 19OHAD), *both* diastereomers were found to act as time-dependent inhibitors of aromatase (Covey et al., 1982). One possible explanation for this result was that structural modification of the inhibitor had impaired the enzyme's ability to discriminate between diastereomeric positions at C_{19}. The results presented here argue against this explanation.

In summary, the analysis of the molecular mechanics calculations together with the experimental finding that 19OHA and 19OHAD are aromatized with similar specificities have provided the data for the first attempt to critically evaluate the role of conformational constraints intrinsic to 19OHA in its stereospecific oxidation by aromatase. We believe that these

results contribute significantly to defining the magnitude of conformational effects previously suggested by crystallographic studies of 19OHA.

ACKNOWLEDGMENTS

We thank Dr. Garland Marshall for providing access to the MMS-X modeling system and Dr. Linda Kurz for performing the quantitative ^{1}H NMR studies.

Registry No. *R*-19OHA, 99630-90-7; *S*-19OHA, 82227-91-6; d_2-19OHA, 71995-64-7; *R*-19OHAD, 99606-27-6; *S*-19OHAD, 99606-28-7; d_2-19OHAD, 99606-29-8; 19OHA, 510-64-5; 19OHAD, 14507-55-2; aromatase, 9039-48-9.

REFERENCES

Akhtar, M., Calder, M. R., Corina, D. L., & Wright, J. N. (1982) *Biochem. J. 201*, 569–580.

Allinger, N. L., & Yuh, Y. H. (1980) *QCPE 395*.

Arigoni, D., Battaglia, R., Akhtar, M., & Smith, T. (1975) *J. Chem. Soc., Chem. Commun.*, 185–186.

Axelrod, L. R., Matthijssen, C., Rao, P. N., & Goldzieher, J. W. (1965) *Acta Endocrinol. (Copenhagen) 48*, 383–391.

Barker, S. A., Monti, J. A., Christian, S. T., Benington, F., & Morin, R. D. (1980) *Anal. Biochem. 107*, 116–123.

Braselton, W. E., Jr., Orr, J. C., & Engel, L. L. (1974) *Steroids 24*, 411–433.

Caspi, E., Arunachalam, T., & Nelson, P. A. (1983) *J. Am. Chem. Soc. 105*, 6987–6989.

Covey, D. F., & Hood, W. F. (1982) *Cancer Res. (Suppl.) 42*, 3327S–3333S.

Covey, D. F., Hood, W. F., & Parikh, V. D. (1981) *J. Biol. Chem. 256*, 1076–1079.

Covey, D. F., Beusen, D. D., & Hood, W. F. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 882 (Abstr. 3582).

Duax, W. L., & Osawa, Y. (1980) *J. Steroid Biochem. 13*, 383–386.

Duax, W. L., Weeks, C. M., & Rohrer, D. C. (1976) *Recent Prog. Horm. Res. 32*, 81–109.

Fersht, A. (1977) *Enzyme Structure and Mechanism*, pp 230–231, W. H. Freeman, San Francisco.

Hanack, M. (1965) *Conformation Theory*, p 40, Academic Press, New York.

Harris, M. (1958) *Prog. Stereochem. 2*, 157–195.

Kelly, W. G., Judd, D., & Stolee, A. (1977) *Biochemistry 16*, 140–145.

Longchampt, J. E., Gual, C., Ehrenstein, M., & Dorfman, R. I. (1960) *Endocrinology (Philadelphia) 66*, 416–419.

Meyer, A. S. (1955) *Biochim. Biophys. Acta 17*, 441–442.

Molnar, C. E., Barry, C. D., & Rosenberger, F. U. (1976) *MMS-X Molecular Modeling System*, Computer Systems Laboratory Technical Memorandum 229, Washington University, St. Louis, MO.

Ōsawa, E., Shirahama, H., & Matsumoto, T. (1979) *J. Am. Chem. Soc. 101*, 4824–4832.

Osawa, Y. (1973) *Int. Congr. Ser.–Excerpta Med. No. 273*, 814–819.

Osawa, Y., Shibata, K., Rohrer, D., Weeks, C., & Duax, W. L. (1975) *J. Am. Chem. Soc. 97*, 4400–4402.

Pitzer, K. S. (1940) *J. Chem. Phys. 8*, 711–720.

Skinner, S. J. M., & Akhtar, M. (1969) *Biochem. J. 114*, 75–81.

Starka, L., & Breuer, H. (1970) *Endocrinol. Exp. 4*, 201–206.

Waxman, D. J., Ko, A., & Walsh, C. (1983) *J. Biol. Chem. 258*, 11937–11947.

Weeks, C. M., Hazel, J. P., & Duax, W. L. (1976) *Cryst. Struct. Commun. 5*, 271–274.

Wilcox, R. B., & Engel, L. L. (1965) *Steroids, Suppl. 1*, 49–57.