‘Slow-binding’ sixth-ligand inhibitors of cytochrome *P*-450 aromatase

Studies with 19-thiomethyl- and 19-azido-androstenedione

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The progress curves for the inhibition of aromatase by 19-thiomethylandrostenedione and 19-azidoandrostenedione were found to be non-linear where the extent of inhibition increased with time. Further experiments enabled these compounds to be classified as ‘slow-binding’ inhibitors of aromatase. The phenomenon was attributed to the formation of an initial $E \cdot I$ complex that rearranged to another species ($E \cdot I^*$) in which the interaction between the enzyme and inhibitor had been maximized, giving rise to tighter binding. When 19-thiomethylandrostenedione was used as the inhibitor the $t_{0.5}$ (half-time) for the dissociation of $E \cdot I^*$ was calculated to be 12.6 min with K_i and K_i^* values of 2.4 and 1.4 nM respectively. In the case of 19-azidoandrostenedione, the two separate dissociation constants were not determined, and a single K_i value of 5 nM was obtained. The conclusions drawn from kinetic studies were confirmed by absorption spectrometry, when time-dependent formation of complexes between aromatase and either 19-thiomethylandrostenedione or 19-azidoandrostenedione were observed by the formation of ‘Type II’ spectra. The two complexes respectively had maxima at 429 and 418 nm. The spectral data suggested that the two inhibitors interact with the haem iron of aromatase, forming hexa-co-ordinated species for which structural models are presented.

INTRODUCTION

The conversion of androgens into oestrogens (1→4, Scheme 1) is catalysed by cytochrome *P*-450 aromatase ([1] and references cited therein) and occurs through the participation of three sequential oxidation reactions at C-19, each requiring 1 mol of NADPH and 1 mol of O_2 [2–5]. We have extensively studied the mechanism of action of the enzyme, and the salient feature of our

findings [1,3–5], which have received support from the studies of other groups [6–8], are summarized in the sequence of Scheme 1. This subject has recently been reviewed [5].

There is much recent medical interest in aromatase, since inhibitors of the enzyme may be of value as contraceptives and also for treating oestrogen-dependent diseases, particularly breast cancer. This potential application prompted us to initiate a programme of work aimed at designing specific inhibitors of

Scheme 1. Pathway for the aromatase-catalysed conversion of androstenedione (1) into oestrone (4)

The fate of the two *pro*-chiral hydrogen atoms (H_R and H_S) is highlighted by identifying these as H^O and H^A respectively. The scheme shows that, whereas H^O is removed as H^O_2O in the conversion 2→3, H^A is expelled with formate. $\odot_2$, $\otimes_2$ and $\bullet_2$ denote the oxygen molecules used in each of the three steps. The oxygen molecule ($\odot_2$) used in the first step is incorporated into 2 and remains undisturbed during the conversion of 2 into 3. This oxygen ($\odot$) and another from $\bullet_2$ used in the third step are found in formate. The scheme also shows that 1β and 2β hydrogen atoms, denoted as $H^\square$, are eliminated in the final aromatization process as water.

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aromatase. This endeavour has culminated in the discovery that steroids containing heteroatoms at C-19, as exemplified by 19-azidoandrostenedione and 19-thiomethylandrostenedione (subsequently referred to as the azidosteroid and thiomethylsteroid respectively; see structures 5 and 6 in Fig. 7 below) are particularly promising inhibitors of human placental aromatase [9,10]. This paper describes experiments which have shed light on the mode of action of these inhibitors. A part of the work has appeared in a preliminary communication [9].

METHODS

Materials

Chemicals were generally obtained from Sigma Chemical Co., Poole, Dorset, U.K. except for the following: [$1\beta,2\beta$ - $^3\text{H}_2$]-Androst-4-ene-3,17-dione (New England Nuclear, Stevenage, Herts., U.K.); Nonidet P40 (BDH, Poole, Dorset, U.K.); hydroxyapatite, (Bio-Gel HTP; Bio-Rad Laboratories, Watford, Herts., U.K.). 19-Azidoandrost-4-ene-3,17-dione (5) and 19-thiomethylandrost-4-ene-3,17-dione (6) were synthesized as described previously [10].

Preparation of placental microsomes

This was carried out using the original method of Ryan [11] as described previously [10].

Purification of aromatase

Aromatase was partially purified by the method of Kellis & Vickery [12], except that the DE-52 anion-exchange step was omitted, owing to the loss of an unacceptably large amount of enzyme activity at this stage, which was attributed to the dissociation of haem from cytochrome *P*-450. Furthermore, in order to obtain substrate-free aromatase for use in the spectral studies, androstenedione was not present in the buffers used for the final hydroxyapatite chromatographic step. Processing of six placentae gave 10 ml of the final extract containing 20 nmol of aromatase as determined spectrally from a molar absorption coefficient ($\epsilon_{429-393}$) of $126 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ [12]. The estimation of total haem [13] gave a value which showed that 90 % of haem in the aromatase was bound as cytochrome *P*-450. The enzyme [in 10 mM-sodium phosphate buffer (pH 7.4)/0.1 mM-EDTA/0.1 mM-dithiothreitol, 20 % (w/v) glycerol/0.1 % Nonidet P40] was stored at -70°C .

The enzyme was reconstituted and assayed as follows. The incubation mixture contained $5 \mu\text{mol}$ of NADP^+ , $32 \mu\text{mol}$ of glucose 6-phosphate, 10 units of glucose 6-phosphate dehydrogenase, $100 \mu\text{g}$ of phosphatidylcholine, 1 unit of NADPH:cytochrome *P*-450 reductase ($80 \mu\text{l}$) and 2.5 nmol of [$1\beta,2\beta$ - $^3\text{H}_2$]androstenedione (in $50 \mu\text{l}$ of methanol containing $0.5 \mu\text{Ci}$ of ^3H) in 100 mM-sodium phosphate buffer (5 ml), pH 7.4, which was also 5 % (w/v) in glycerol and 0.025 % (v/v) in Nonidet P40. The mixture was equilibrated at 37°C and supplemented with 20 pmol of aromatase ($40 \mu\text{l}$; for composition see above). Aliquots of the incubation mixture were removed at various time intervals and processed for the measurement of $^3\text{H}_2\text{O}$ [1]. The enzyme had an activity of 3.0 nmol of androstenedione aromatized/min per nmol of cytochrome *P*-450 aromatase.

Kinetic studies

For the experiments of Fig. 1 (below), aromatase assays were performed using placental microsomes (microsomal fractions), as described previously [10], except that samples were taken at 0, 2, 4, 6, 8, 10, 15, 20, 25, 30 min. The substrate concentration was $0.5 \mu\text{M}$ and the inhibitor concentration was varied between 0 and 100 nM .

For preincubation experiments (Fig. 2 below), human placental microsomes ($100 \mu\text{g}$ of protein) and inhibitor (100 nM final concn.) were preincubated for 15 min in 4 ml of buffer before the reaction was initiated by adding substrate (to give $0.5 \mu\text{M}$ final concn.) and NADPH-regenerating system in 1 ml of buffer. These experiments were performed in parallel with incubations containing substrate and inhibitor in 4 ml of buffer and initiated by addition of human placental microsomes and NADPH-regenerating system contained in 1 ml of buffer. Each of these two experiments had a control where no inhibitor was present to check that preincubation itself did not alter the enzyme rate. All other details of the incubation, including composition of buffers, NADPH-regenerating system and concentration of microsomes, were as described previously [10].

Spectral studies

Stock solution ($200 \mu\text{l}$), containing 0.4 nmol of aromatase in pH 7.4 buffer (10 mM-sodium phosphate/0.1 mM-EDTA/0.1 mM-dithiothreitol/20 % glycerol/0.1 % Nonidet P40), was diluted with $600 \mu\text{l}$ of pH 7.4 buffer containing 10 mM-sodium phosphate. The solution, initially at 0°C , was divided into two $400 \mu\text{l}$ portions and placed in two 1 cm-pathlength cells and allowed to equilibrate to ambient temperature for 5 min. All ligands were made up in solutions of methanol, and appropriate amounts were added to the experimental cell. The reference cell received methanol only. All measurements were taken at room temperature on a Kontron Instruments U-930 spectrophotometer. Baselines are shown in all spectra, and these have been automatically subtracted from the sample spectra shown.

RESULTS

Enzyme preparation

As in other cytochrome *P*-450 systems, aromatase-catalysed reactions require the participation of another enzyme, NADPH:cytochrome *P*-450 reductase, which is responsible for the transfer in two steps of a hydride equivalent from NADPH to the haem iron of the cytochrome. Carefully prepared placental microsomes contain both the enzymes (aromatase and the reductase) and, in the presence of NADPH and O_2 , catalyse the formation of oestrogen from androstenedione (Scheme 1).

Aromatase was also solubilized using detergents and further purified by hydrophobic and hydroxyapatite chromatography [12]. In the experiments described below, such a purified enzyme was used to study spectroscopically the binding of substrate and inhibitory ligands to the haem component of aromatase. The solubilized preparation was reconstituted to obtain a catalytically functional preparation through dilution of the detergent and supplementation with the reductase. The kinetic parameters of the reconstituted enzyme, in addition to the concentrations of NADPH, the reductase and detergents, are dependent on several other factors. These include the type of vesicles formed when the detergent-solubilized protein is diluted with the assay buffer, the extent of dispersion of the water-insoluble substrate in the aqueous medium and whether phospholipids are included during the reconstitution process (see [14]). Since some of these factors are difficult to control precisely, the kinetic parameters obtained with a reconstituted system are only of value for internal comparison. In our hands the reconstituted system gave K_m values for androstenedione of 130 nM , whereas values of 60 and 130 nM were reported by another group in two different papers ([12] cf. [18]) and a wider variation obtained by other workers (see [15]). The variability is less extensive with the microsomal fraction, and therefore, for convenience and reproducibility, this system was used for the detailed kinetic experiments of Figs. 1 and 2.

Fig. 1. Progress curves for the inhibition of aromatase by thiomethylsteroid (6)

Androstenedione (500 nM) was used as the substrate, with inhibitor concentrations of 0 nM (○), 10 nM (●), 25 nM (□), 50 nM (▲) and 100 nM (△), and the reaction was initiated by the addition of microsomal aromatase (100 µg/5 ml of incubation mixture). The broken line shows an example of the graphical method for estimating the apparent first-order rate constant, k .

Non-linear rate of aromatization

When the aromatization reaction was monitored in the presence of various concentrations of the thiomethylsteroid, a family of non-linear progress curves were obtained in which the extent of inhibition increased with time (Fig. 1). This behaviour is reminiscent of compounds which either irreversibly inactivate an enzyme or act as 'slow-binding' inhibitors [16]. In order to differentiate between these alternatives, the microsomes were preincubated with the inhibitor and, after 15 min, the mixture was supplemented with the substrate and other components of the overall reaction and the time course of product formation assayed (Fig. 2a). Once again, a non-linear progress curve was obtained, except that, in this case, the extent of inhibition decreased with time until a new steady-state rate had been established (curve ●, Fig. 2a). The rate of reaction in the steady-state was the same as that in which the reaction was commenced by the addition of enzyme to a preincubation mixture containing inhibitor and substrate (curve □, Fig. 2a). In Fig. 2(b) analogous behaviour was observed with the azidosteroid (5), though with this inhibitor the extent of non-linearity was less pronounced than with the thiomethylsteroid (6). These experiments clearly indicate that with both the ligands (5 and 6, Fig. 7 below) the

Fig. 2. Progress curves for the inhibition of aromatase by (a) azidosteroid (100 nM) and (b) thiomethylsteroid (100 nM)

The substrate (androstenedione) concentration was 500 nM. Reactions were initiated either by addition of microsomal aromatase (100 µg/5 ml of incubation mixture) and NADPH-regenerating system to inhibitor and substrate (□) or by addition of substrate and NADPH-regenerating system to a preincubation containing microsomal aromatase and inhibitor (●). Control incubations contained no inhibitor (■).

enzyme-inhibitor complexes formed in the preincubation phase were reversible and that the overall kinetic behaviour is characteristic of 'slow-binding' inhibition [16].

Several models have been proposed to account for this type of inhibition [16]. The observation that the initial rates vary with inhibitor concentration (see Fig. 1) and that the inhibitor was present in large excess over the enzyme leads us to believe that the model which best explains our results is one where binding involves the sequence:

In this case the rapidly formed enzyme-inhibitor complex, $E \cdot I$ undergoes slow isomerization to another complex $E \cdot I^*$ where the inhibitor is bound more tightly [16].

The plots in Fig. 1 may be used to obtain initial (v_0) and steady-state (v_s) velocities as well as k (which is the apparent first-order rate constant for the establishment of equilibrium between $E \cdot I$ and $E \cdot I^*$). The intersection point of two linear curves, as illustrated in Fig. 1 for the curve (▲), can be used to obtain $1/k$ [16]. Such determinations were made for all the curves in Fig. 1 and values substituted in the equation:

$$k_4 = \frac{k \cdot v_s}{v_0} \quad (1)$$

to give a mean value for k_4 of 0.055 min^{-1} , with an s.d. of ± 0.001 ($n = 4$). From the latter, $t_{0.5}$ (half-time) for the isomerization of $E \cdot I^*$ to $E \cdot I$ was calculated to be 12.6 min.

Calculation of K_i values

The progress curves in Fig. 1 can also be used to calculate the

dissociation constant for the initial E·I complex, K_i , and the overall dissociation constant, K_i^* , which is defined as:

$$K_i^* = \frac{[E][I]}{[E \cdot I] + [E \cdot I^*]} = \frac{K_i \cdot k_4}{k_3 + k_4} \quad (2)$$

The K_i (apparent) ($K_{i,app}$) was obtained from the plot of $1/v_0$ against inhibitor concentration ($[I]$) and K_i^* apparent from $1/v_s$ against $[I]$. These values were 20 nM and 11.5 nM respectively.

Using a value of 67 nM [17] for the K_m of the substrate, androstenedione, in the equation:

$$K_{i,app} = K_i \left(1 + \frac{[A]}{K_m}\right) \quad (3)$$

where $[A]$ is the substrate concentration, gives values for K_i of 2.4 nM and K_i^* of 1.4 nM for the thiomethylsteroid (6). Qualitatively, the azidosteroid (5) gave plots similar to those obtained for the thiomethylsteroid (6) in Fig. 1. However, with the former the separation between the two phases was not easily discernible, and it was not possible to obtain a reliable estimate of v_0 and v_s . The data were therefore treated conventionally to obtain a single dissociation constant, K_i , of about 5 nM for the azidosteroid (5).

Spectroscopic examination of the aromatase-inhibitor complexes

The chemical nature of the complexes formed between the thiomethyl and azidosteroids with aromatase were studied by using u.v.-visible spectroscopy. As in other cytochrome P-450 enzymes, the binding to aromatase of its substrate, androstenedione, is marked by the perturbation of the Soret band of haem, giving rise to a 'Type I' spectrum having a peak at 388 nm and a trough at 420 nm [19]. This is shown in curve A, Fig. 3. In contrast, Curves B and C show difference spectra obtained with the thiomethyl and azidosteroids (6 and 5) respectively.

The time-course study in Fig. 4 (curve B) shows that the aromatase-substrate spectrum developed to the extent of 80% of the maximum value within the first 12 s. This was the earliest

Fig. 3. Absorption spectra of aromatase-substrate and aromatase-inhibitor complexes

The following protocol was used to obtain each curve. A 400 μ l sample of aromatase (0.5 μ M, see the Experimental section) was placed in the experimental and reference cuvettes and a baseline recorded. Spectra were recorded 10 min after the addition to the experimental cell of the appropriate ligand: 100 μ M-androstenedione (curve A), 25 μ M-thiomethylsteroid (curve B) or 25 μ M-azidosteroid (curve C).

time at which a reliable absorbance reading could be made using the facilities currently available in our laboratory. Interestingly, however, the absorption spectrum due to the formation of the aromatase-thiomethylsteroid complex developed slowly, reaching a maximum after 6 min. This observation is complementary to the 'slow-binding' profile revealed by the kinetic experiments of Figs. 1 and 2. The time course of the formation of the aromatase-azidosteroid complex gave a plot which was intermediate between curves A and B (Fig. 4) and is not shown here.

The fact that the aromatase-substrate and the two aromatase-inhibitor complexes had their own characteristic absorption profile was exploited to determine spectrophotometrically the reversible nature of the complexes. The phenomenon was most clearly revealed by starting with a set-up in which the experimental and reference cuvettes contained a preformed complex that, on recording, gave a smooth baseline. The displacing ligand was then added to the experimental cell and the development of the new spectrum recorded with time. Fig. 5(a) shows the displacement of the substrate from the aromatase-substrate complex by the thiomethylsteroid, and Fig. 5(b) illustrates the complementary experiment.

The displacement protocol above was extended to the titration of the enzyme with two inhibitors, yielding a series of curves (Fig. 6) that allowed the calculation of apparent spectral dissociation constants, K_s , for thiomethylsteroid and azidosteroid of 6.4 μ M and 6.7 μ M respectively. In view of the tight binding of these inhibitors to aromatase, the titrations were performed using the protocol of Kellis *et al.* [18], in the presence of 20 μ M-androstenedione, and therefore the calculation of 'true' K_s values required the knowledge of the K_m of the substrate under the conditions used in the spectral experiments, which was found to

Fig. 4. Time course of the formation of aromatase-inhibitor complexes

Both the reference and sample cuvettes contained a solution of aromatase (400 μ l, 0.5 μ M) and a baseline recorded. In (a) the sample cell was supplemented with thiomethylsteroid (0.7 μ M final concn.) and the change in absorbance between 429 nm and 393 nm recorded at 6 s intervals for 7 min. In (b) substrate (2.0 μ M final concn.), was used and absorbance change was recorded between 420 nm and 388 nm.

Fig. 5. Reversibility of binding of the aromatase-substrate and the aromatase-inhibitor complexes

(a) A solution of aromatase (400 μ l, 0.5 μ M) saturated with androstenedione (20 μ M final concn.) was placed in the sample and reference cuvettes, and a baseline was recorded. Thiomethylsteroid (3.5 μ M final concn.) was then added in an amount that would give half-maximal spectral displacement, yet be solely saturating, and spectra were recorded every 30 s for 7 min. Only curves A (1 min), B (4 min) and C (7 min) are shown for clarity in both cases. A final spectrum was obtained by adding excess inhibitor (25 μ M final concn.). The experiment was repeated (b), but starting with aromatase (400 μ l, 0.5 μ M) containing thiomethylsteroid (3.5 μ M final concn.) in both cuvettes. Substrate was then added in a concentration that would give half-maximal displacement and be solely saturating (20 μ M final concentration). The final spectrum relates to the addition of excess (100 μ M) androstenedione.

be 130 nm. Using this value in eqn. (3) above gave [20] an approximate K_s for the thiomethylsteroid of 42 nm and for the azidosteroid of 44 nm. In order to allow direct comparison of spectroscopic and kinetic data, the K_i for the thiomethylsteroid was determined using the reconstituted system, and a value of 12 nm was obtained. Thus, in broad terms, these values suggest a similar tight binding of the inhibitor to aromatase in both types of experiments (spectroscopic and kinetic). The data of Fig. 6 are useful, but an important aspect of the present work is the demonstration that the formation of the spectral species in Fig. 4, and the attainment of the maximum inhibition during the kinetic experiments (Fig. 1) are time-dependent processes. It should be noted that the biphasic nature of inhibition observed in Fig. 1 was most readily discernible in the microsomal system and not with the solubilized reconstituted enzyme. However, when the thiomethylsteroid is preincubated with the solubilized reconstituted enzyme for 15 min and the reaction is then initiated by the addition of the substrate, then the same non-linear inhibition profile (i.e. Fig. 2a, curve ●) as seen with the microsomes is obtained.

CONCLUSION

The present work extends our earlier studies [9] and shows that the kinetic profile (Figs. 1 and 2) exhibited by the thiomethyl- and azido-steroids is characteristic of compounds which have been designated as slow-binding inhibitors [16]. This mode of inhibition involves an initial rapid interaction between the enzyme and competitive inhibitor to produce the E·I complex that then undergoes a slow conformational change to produce a tighter complex, EI*, in which the interactions between the enzyme and the inhibitor have been maximized.

The chemical nature of the interaction was further explored by u.v.-visible difference spectroscopy, and this technique corroborated the conclusion drawn from the kinetic experiments. The

complex-formation between the enzyme and the thiomethylsteroid, as revealed by the formation of a unique light-absorbing species, was a time-dependent process (Fig. 4). The final difference spectrum had increased absorption at 429 nm, with a trough at 393 nm, which is characteristic of a 'Type II' spectrum [19] and indicates the formation of a low-spin species, which is attributed to the co-ordination of the steroidal sulphur atom to the haem iron of the cytochrome (Fig. 7). This formulation is based on studies previously performed by Nastainczyk *et al.* [21], who showed that binding of various organic sulphides to several types of liver microsomal cytochrome *P*-450 preparations produced spectra having absorption maxima and minima within a range found for the aromatase-thiomethylsteroid complex in curve B, Fig. 3.

Since our original report [9] that the thiomethylsteroid interacts with aromatase producing a 'Type II' spectrum, related examples of this phenomenon involving a 10 β -thi-irane steroid and 19-thiolandrogens have been described [15,18]. We have now shown that the azidosteroid also interacts with the enzyme by a similar mechanism to produce a spectrum with peak and trough absorbances at 418 and 387 nm respectively. These values are similar to those previously found (maximum at 425 nm and a trough at 391 nm) for the interaction of -NH₂-containing ligands to various cytochrome *P*-450-dependent enzymes (see [19]). The present work, however, demonstrates for the first time that a resonance-stabilized negative charge in the zwitterionic structure of an organic azide can participate in bonding with a metal. Extrapolation from the computer-graphic analysis of the interaction of various C-19 thioalkylsteroids with aromatases in [10] allows us to hypothesize that it is the α -nitrogen in the canonical structure (b) which is most likely to be involved in co-ordination with the haem iron of aromatase:

Fig. 6. Titration curves for the interaction of inhibitors with aromatase

A solution of aromatase ($400 \mu\text{l}$, $0.5 \mu\text{M}$) containing androstenedione ($20 \mu\text{M}$) was placed in the sample and reference cuvettes and a baseline recorded. (a) Thiomethylsteroid was then added sequentially to give final concentrations in the cuvette of 1.0, 1.5, 2.5, 3.5, 4.5 and $6.0 \mu\text{M}$, and spectra were recorded 10 min after each addition. A maximum titre was given by adding excess inhibitor ($25 \mu\text{M}$ for both cases) to fully saturate the enzyme. (b) The same procedure was repeated for azidosteroid, except that the final concentrations in the cuvette were 2.0, 3.0, 4.0, 5.0, 6.5, 8.0 and $10.0 \mu\text{M}$. The plots obtained from the data are displayed to the right of the titration curves. L_{free} is the concentration of free ligand determined as in [18].

Fig. 7. Postulated structures of aromatase-inhibitor complexes

The illustration shows the formation of co-ordinate bonds between the nitrogen of the azidosteroid (5) or the sulphur of the thiomethylsteroid (6) and the iron in the haem *b* prosthetic group (7) of aromatase.

Since the introduction of the term 'slow-binding' inhibition by Williams & Morrison in 1979 ([22] and references cited therein) and its popularization by Morrison & Walsh [16], a large number of potent inhibitors of enzymes have been shown to fall into this

class. For several of the inhibitors, conjectures have been made regarding the nature of chemical interactions which attend the formation of E·I* complexes. The existence of a physiological haem chromophore in aromatase, together with the strategic

location of metal-co-ordinating functional groups in the two inhibitors, have now enabled the identification of the process which may promote tight binding. This, at least in part, is attributed to the tendency of the C-19 heteroatom in the inhibitors (**5** and **6**) to bond with the haem iron of aromatase. The time course in Fig. 4 suggests that the development of a full co-ordination bond is a relatively slow process and, therefore, during the conditions of steady-state kinetics, a range of enzyme-inhibitor complexes may exist which differ in the extent of co-ordination bond formation. Fig. 7 shows the structure of the two inhibitory complexes when the co-ordination bonds are fully developed.

Note added in proof (received 6 December 1990)

Since this paper was originally submitted, a recent review of the field has been published [23].

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