Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1023–1028

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

MECHANISM OF 17α-HYDROXYLASE/17,20-LYASE - AN INITIAL GEOMETRIC PERSPECTIVE FOR THE LYASE OF THE C(17)-C(20) BOND OF C_{21} STEROIDS.

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Received 29 October 1997; accepted 25 March 1998

ABSTRACT Using the novel 'substrate-heme complex' approach[1], the mechanism of 17α-Hydroxylase/17,20-Lyase (P-$450_{17\alpha}$), in particular the lyase of the C(17)-C(20) bond, is considered from a geometric perspective. The results of the study appear to suggest that the final oxidative step in the lyase of the C(17)-C(20) bond involves the use of a ferroxy attacking species as opposed to peroxy or a mixture of ferroxy and peroxy, an observation which is consistent with results previously obtained with Aromatase[1].

In the treatment of hormone dependant cancers such as prostate cancer, inhibitors of the enzyme complex 17α-Hydroxylase/17,20-Lyase (P-$450_{17\alpha}$) have been shown to be of benefit, for example the antifungal compound Ketoconazole until recently was considered a hopeful candidate in the treatment of prostate cancer. P-$450_{17\alpha}$ mediates the conversion of the C_{21} steroids to the C_{19} steroids via a series of radical reactions[2,3,4]. The initial reaction involves α-hydroxylation of the C(17) position of, for example, pregnenolone in the Δ5 pathway to give 17α-hydroxypregnenolone. The hydroxysteroid then undergoes further oxidation to yield the products dehydroisoandrosterone (or androstenedione from progesterone in the Δ4 pathway) and acetate, each step requiring a mol of O_2 and NADPH[2]. The species involved in the hydroxylation steps is thought to be the ferroxy radical (Fe^{IV}-O•), however, the nature of the attacking group involved in the C-C lyase steps has not been clearly established, although, as with another P-450 enzyme, Aromatase (AR), it is believed to be either the ferroxy radical once again or a peroxy radical (Fe^{III}-O-O$^-$).

In the absence of the crystal structure of the overall enzyme complex, most of the studies on the mechanism of P-$450_{17\alpha}$ have revolved around chemical points of view, and the rôle of the active site of P-$450_{17\alpha}$ and the rest of the enzyme has not generally been considered, mainly due to lack of knowledge concerning the position of the heme and therefore the iron. We have sought to elucidate the probable position of the heme of the individual components of P-$450_{17\alpha}$ with respect to the substrate backbone, and have produced 'substrate-heme complexes' as representations of the active site of 17α-Hydroxylase[5] (using progesterone) and 17,20-Lyase[6] (using 17α-hydroxypregnenolone). We have recently reported a similar approach for AR[7], using which we have successfully discussed the mode of action of several AR inhibitors and studied the mechanism of the lyase of the C(10)-

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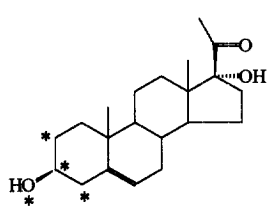
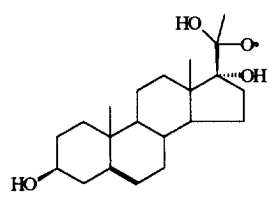
0960-894X/98/$19.00

PII: S0960-894X(98)00154-1

C(19) bond in androstenedione[1]. We now seek to apply this general approach to study the mechanism of P-450$_{17\alpha}$, in particular 17,20-lyase. We have thus constructed molecular model representations of both the ferroxy and the peroxy equivalents of the substrate-heme complexes [as well as the structures shown in Figure 1] using the previously reported approach[1,7] and the molecular modelling software Alchemy III[8] (using atoms/fragments/groups available within the Alchemy structure libraries). In the construction of the substrate-heme complexes, we hypothesised that the attacking oxygen species must be positioned within approximate attacking distance (and angle) to the C(20) carbonyl such that attack on the C(20) carbonyl group can take place. We therefore attached the terminal oxygen of the Fe^{III}-O-O$^-$ and/or Fe^{IV}-O• species to the C(20) carbonyl carbon atom of 17α-hydroxypregnenolone and carried out an initial minimisation of the 'complex' until the gradient fell to below 10^{-5} (resulting, in general, in 300 or more iterations per structure), the C(20) sp^2 carbon was therefore converted to a sp^3. This then resulted in both ferroxy and peroxy heme-based 17,20-Lyase substrate-heme complexes (Figures 2 & 3). Conformational analysis was performed on flexible parts of the substrate-heme complex i.e. about the Fe-O• and O-C(20) in the case of the ferroxy substrate-heme complex or the O-O and O-C(20) bonds in the peroxy based substrate-heme complex (using the systematic search method with energy window of 5Kcal/mol and bond rotations of 30-60°) using the conformational analysis software Powersearch[8]. The low energy conformers produced were retained for further study. It should be noted that the theoretical study reported here has only taken note of the interactions between the substrate and the porphyrin, whilst 'ignoring' the remainder of the protein.



17α-Hydroxy pregnenolone



3β-Hydroxy-androst-5-ene-
17α-hydroxy-17β-carboxylate radical

Figure 1. Substrate and a proposed intermediate of the 17,20-Lyase enzyme.

In considering the possible mechanisms put forward by Akhtar et al[2,3,4], we hypothesise that the hydrogen abstraction from the C(17)-αOH [after the initial attack on the C(20)=O, and thus leading to the formation of the C(17)=O] is a crucial step. Thus, the basis of this theoretical study involves the determination of the feasibility of the hydrogen abstraction step from a geometric point of view. The substrate for the lyase step (17α-hydroxypregnenolone), was therefore superimposed onto the substrate-heme complexes previously mentioned, using points on the A-ring of pregnenolone [shown

in Figure 1 with *]. The distance between the C(17)-αOH group (in particular the position of the hydrogen atom) with respect to the Fe^{III}-O-O$^{\cdot}$ and Fe^{IV}-O$^{\bullet}$ species was then determined.

From the consideration of the peroxy substrate-heme complex (Figure 2), we observe that, as would be expected, the 'extra' oxygen causes an upward movement of the steroid backbone (with respect to the porphyrin), compared to the ferroxy substrate-heme complex (Figure 3). Fitting the two complexes (using the Fe and nitrogen atoms on the heme moiety of each complex) we discovered that the difference in geometry between the steroid backbones [more specifically the C(3)-OH groups] of the ferroxy and the peroxy complexes was some 6.8Å. From this result it would seem unlikely that both ferroxy and peroxy radicals are involved in the lyase of the C(17) to C(20) bond, as this would require the steroidal intermediates to reposition themselves during the course of the reaction such that the ferroxy oxygen of Fe^{III}-O-O$^{\cdot}$ could carry out H abstraction from the hydroxyl of 17α-hydroxypregnenolone during the production of the C(17)=O of dehydroisoandrosterone, as suggested by Robichaud et al[2]. Also, the movement required from ferroxy to peroxy would need to be in an approximate vertical plane, as such a 'ladder arrangement' of hydrogen bonding groups would be required so as to stabilise the enzyme-substrate complex. If such repositioning did occur, we would need to presume the existence of numerous hydrogen bonding sites about the active site, and although multiple substrate binding sites have been observed in a homology based study[9], the existence of the required 'short ladder' arrangement of hydrogen bonding sites has not. Therefore, we conclude that the involvement of a mixture of ferroxy and peroxy radicals is unlikely in the lyase of the C(17)-C(20) bond of pregnenolone (or progesterone).

On fitting the substrate 17α-hydroxypregnenolone onto the peroxy substrate-heme complex, we observe that the H atom of the 17α-hydroxy group is now placed some 3.4Å away from the appropriate oxygen (Figure 4). A similar distance (3.6Å) is also observed for the 17α-hydroxy-20,20-diol-pregnane (a simplified form of the 3β-hydroxy-androst-5-ene-17α-hydroxy-17β-carboxylate radical - which has been suggested as a trap for the iron peroxide[2]), however, both of these distances are considered to be too large for the pivotal H abstraction step to occur. The involvement of a peroxy radical in the carbon-carbon bond cleavage mechanism therefore appears to be unlikely, unless there is an extensive movement of the steroid backbone such that the 17α-hydroxy group can approach the ferroxy radical.

During the catalytic cycle of the P-450 enzymes, a complex is formed consisting of [Fe^{IV}-O$^{\bullet}$-substrate-P-450]. It could be argued that the protein architecture of the cytochrome P-450s would be flexible enough to accommodate such movements by the substrate. This explanation cannot be

accepted, however, since the application of such logic would ultimately lead to the conclusion that rational drug design (in particular structure-activity relationship determination) was futile and non-productive since the range of movement required for the intermediates is such that almost any compound could possess the potential to inhibit a particular enzyme through the flexing. As such, the validity of the numerous structure-activity studies would be put into doubt.

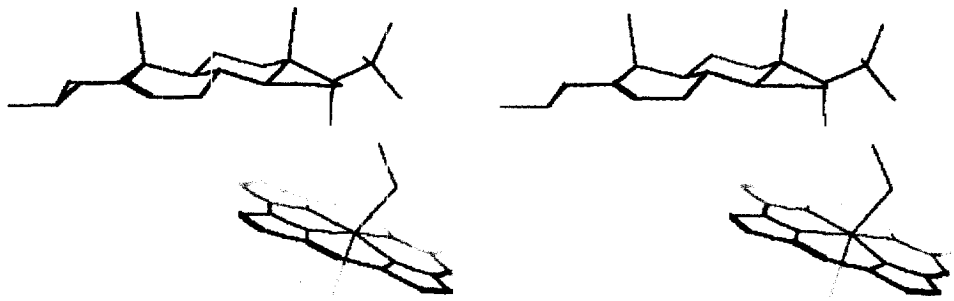


Figure 2. Peroxy substrate-heme complex of 17,20-Lyase.

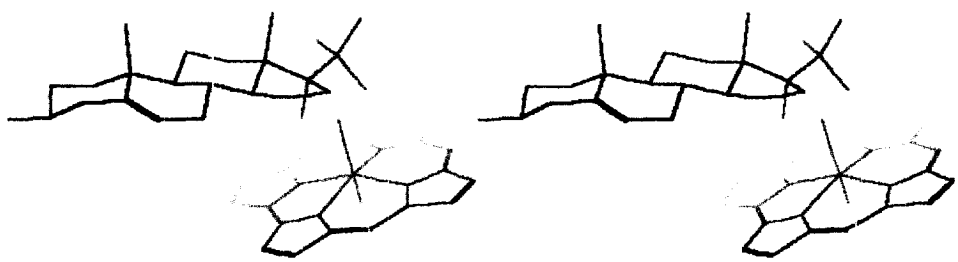


Figure 3. Ferroxy substrate-heme complex of 17,20-Lyase.

In the postulated mechanism involving the ferroxy attacking species, the H abstraction step appears to be a favourable process with the ferroxy oxygen being positioned some 1.4Å away from the C(17)-OH group (Figure 5) of 17α-hydroxypregnenolone. Indeed, of the two possible mechanisms, the ferroxy based mechanism appears to be the most probable since the C(20) containing side chain is also positioned close enough (1.7Å) such that the Fe-OH formed, as a result of H abstraction from C(17)-OH, can be postulated to combine with the C(20) containing radical forming the acetate[2].

Using the ferroxy-based substrate-heme complex [in particular the short distance observed between the C(20)=O of the steroid and the Fe^{IV}-O• to be close enough for attack by the ferroxy radical, resulting in the synthesis of by-products], it is possible to take into account all the different products produced by the P-$450_{17\alpha}$ enzyme complex. For example, in the mechanism for the formation of the Δ16 steroid, it is postulated[2] that there is an initial peroxy attack on the C(20)=O group which breaks

by the remaining ferroxy radical, resulting in the formation of Fe-OH and androst-5,16-diene-3β-ol. It is possible (although we have no direct experimental data) to postulate an alternative mechanism which uses the ferroxy radical and which results in the expected products (Figure 6). Indeed, using the ferroxy radical attacking species of both the 17α-hydroxylase and 17,20-lyase substrate-heme complexes previously reported, it is possible to hypothesise reaction mechanisms which give rise to all the observed products[2] [and which involve distances approaching bond lengths and are therefore appropriate for the reaction to take place]. For example, it has been observed that an 17α-hydroxy androgen can be produced as a result of the action of P-$450_{17\alpha}$ enzyme on progesterone - no explanation has yet been forwarded for this observation. However, using the ferroxy based substrate-heme complex, we suggest that the initial step in this observation is an attack of the C(20)=O by the Fe^{IV}-O• resulting in the cleavage of the C(17)-C(20) bond and the production of a C(17) radical which, in forming the planar structure, causes the C(17)α-H to move in an approximate vertical direction. This then allows attack of the C(17) position from the α face resulting in the 17α-hydroxy androgen (Figure 7).

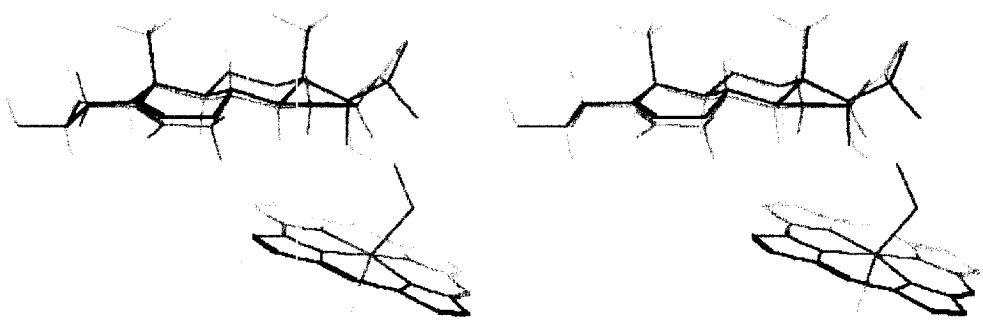


Figure 4. 17α-Hydroxy pregnenolone fitted onto the peroxy based substrate-heme complex of 17,20-Lyase.

In conclusion, the approach of the substrate-heme complex has allowed us to consider the mechanism of the C(17)-C(20) bond cleavage, the conclusion of which appears to suggest that ferroxy radical attack is involved in the 17,20-Lyase step of the P-$450_{17\alpha}$.

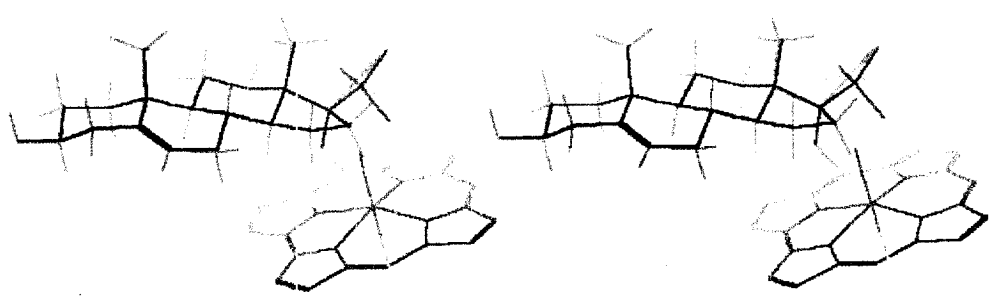


Figure 5. 17α-Hydroxy pregnenolone fitted onto the ferroxy based substrate-heme complex of 17,20-Lyase.

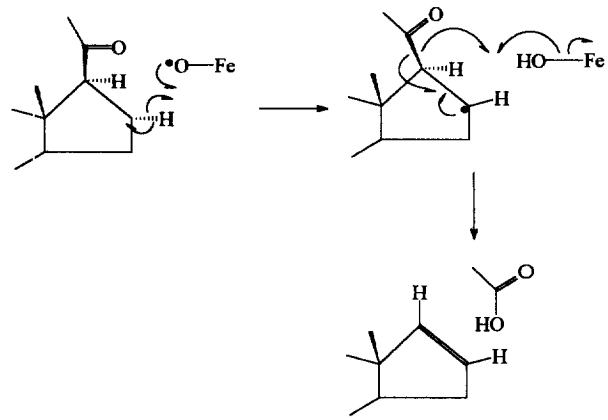


Figure 6. Hypothetical mechanism for the formation of androst-5,16-diene-3β-ol using the ferroxy attacking radical.

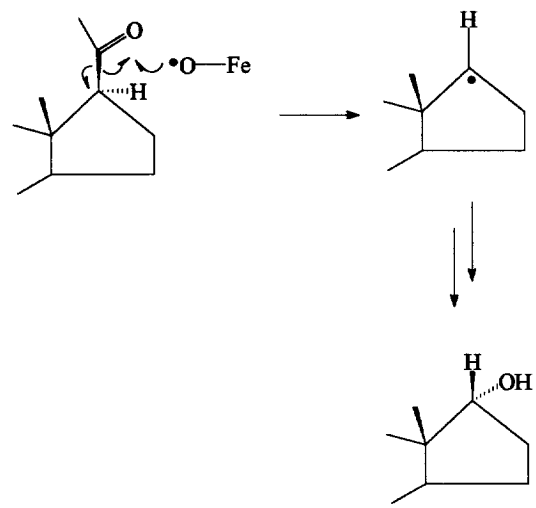


Figure 7. Hypothetical mechanism for the formation of 17α-hydroxy androgens using the ferroxy attacking radical.

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