The Mechanism of Cytochrome P-450 Dependent C–C Bond Cleavage: Studies on 17α-Hydroxylase-17,20-lyase

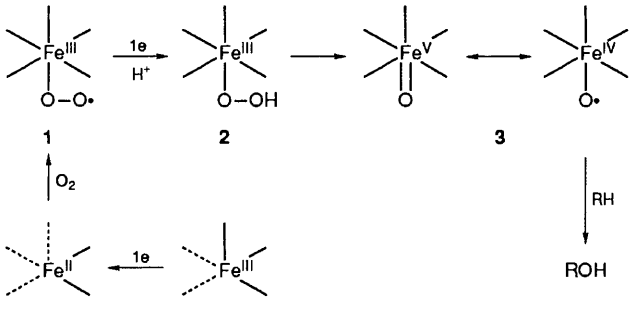
David L. Corina, Sharon L. Miller, J. Neville Wright and Muhammad Akhtar*

Department of Biochemistry, School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, UK

It is shown that formation of 17α-hydroxyandrost-5-en-3β-ol from pregnenolone occurs solely at the expense of the cleavage of the C-17–C-20 bond of the precursor and is best rationalised by invoking the participation of an Fe^{III}–OOH intermediate in the acyl–carbon fission process.

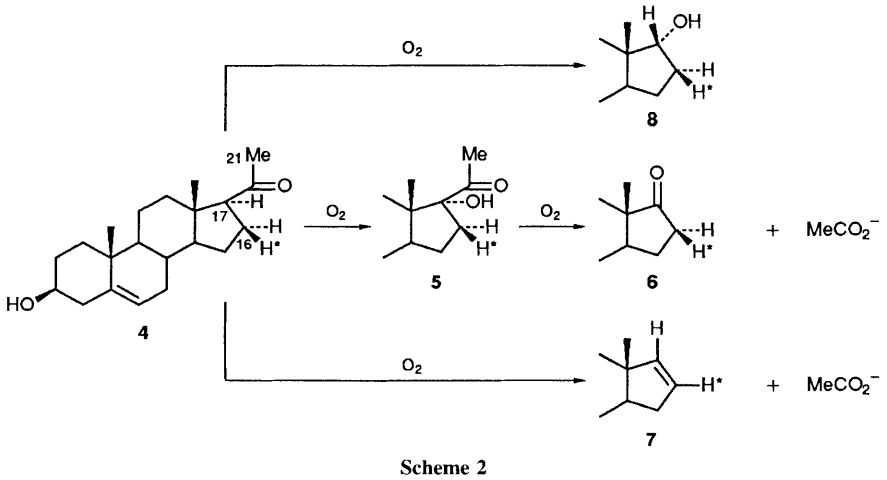
There is now general consensus[1] that the activated form of oxygen involved in P-450 dependent hydroxylation reactions is the iron–monooxygen species **3** that is produced by the sequence of reactions shown in Scheme 1. Our work on aromatase[2,3] and 14α-demethylase[4] showed that these P-450 enzymes catalyse not only the hydroxylation reaction but also the conversion of alcohols into carbonyl compounds and, more importantly, a C–C bond cleavage reaction. We suggested that the C–C bond cleavage reaction in these enzymic transformations is catalysed by a mechanism which uses[1c,2–4] Fe^{III}–O–OH **2**.

Recently,[5] experimental evidence was provided to show that a similar mechanism may also operate in the side-chain cleavage reaction(s) catalysed by 17α-hydroxylase-17,20-lyase ($P\text{-}450_{17\alpha}$). The latter enzyme acts on the pregnene nucleus **4** and promotes, first the hydroxylation of the 17α position **4** → **5** and then, a cleavage reaction which produces the 17-keto steroid **6** and releases the side chain as acetate.[6] The enzyme is believed to possess another activity[7] which culminates in the formation of the Δ^{16}-steroid **7**. During the course of a study on the mechanisms of these reactions, we have observed the accumulation of a third metabolite, 17α-hydroxyandrost-5-en-3β-ol **8**. This was originally isolated from pig testes[8] and its formation during incubations *in vitro*[9] was also shown but the mechanistic significance of these observations at that time was not obvious. In order to unravel the genesis of this compound, biological reactions with a variety of deuteriated pregnenolone samples under $^{18}O_2$ were performed and the results interpreted to provide strong support for the involvement of the Fe^{III}–OOH species **2** in the C–C bond cleavage reaction catalysed by P-450 enzymes (Scheme 3).

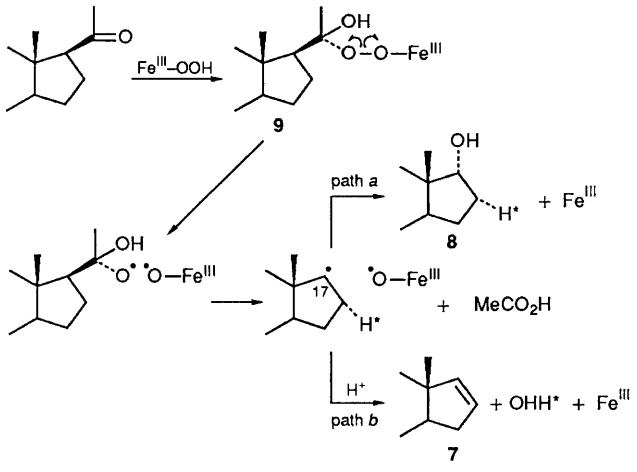


Scheme 1

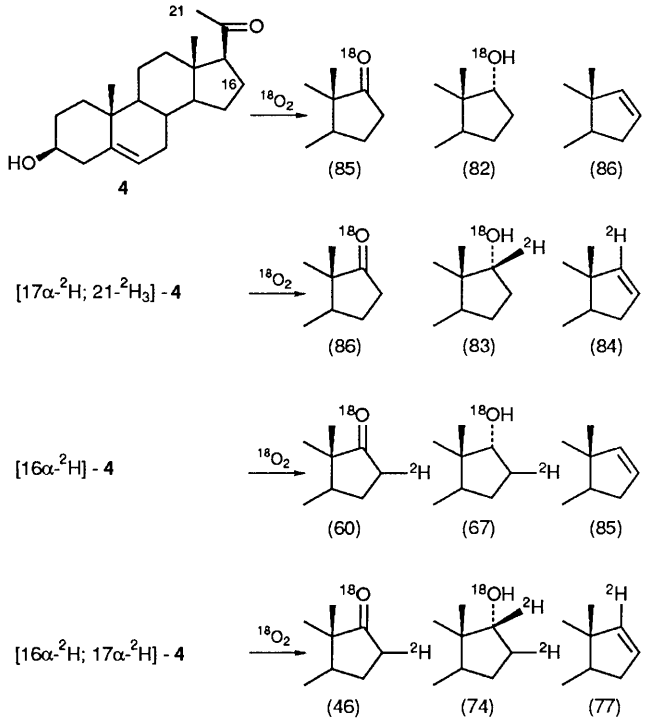
Pregnenolone samples (3 μmol), variously labelled with deuterium at C-16α, C-17α and C-21, were incubated[5] under $^{16}O_2$ or $^{18}O_2$ with a microsomal preparation obtained from neonatal pig testes (100 mg of protein) in 10 ml of 60 mmol dm^{-3} potassium phosphate buffer, pH 7.25, in the presence of an NADPH generating system. The incubation mixture, following freeze-drying, was extracted with methanol and the mixture of steroids was isolated either directly or following reduction with $NaBH_4$. In initial studies, GC–MS analysis was carried out on the steroids without derivatisation; however, subsequently it was found that the chromatographic resolution was improved following trimethylsilylation and the results in Scheme 4 were obtained using such a protocol. It was found that in all these types of analyses [(*i*) without $NaBH_4$ treatment, (*ii*) following $NaBH_4$ reduction, (*iii*) after silylation of the latter], in addition to the two expected products, Δ^{16}-steroid **7** and dehydroisoandrosterone **6** (or its 17β-hydroxy counterpart), a third steroid was present which had the same behaviour as authentic 17α-hydroxyandrosten-5-en-3β-ol **8**. When the latter metabolite was produced from [17α-^{2}H; 21-$^2H_3$]pregnenolone under $^{18}O_2$, it was found to retain the C-17 deuterium of the precursor and incorporate 0.83 atoms of ^{18}O. A similar retention of the C-17 hydrogen in **8** was previously found[9] when the steroid was produced under $^{16}O_2$. The experiment shows that the 17α-hydroxyandrogen **8** was not formed from the cleavage of the side-chain of 17α-hydroxypregnenolone **5** since, if this were the case, the product **8** would be devoid of deuterium. Further studies with



Scheme 2



Scheme 3



Scheme 4 The composition of the predominant isotopic species produced from variously deuteriated pregnenolone samples incubated under $^{18}O_2$. The figure under each structure denotes the percentage of the isotopomer when the sum of all the species of the compound is taken as 100.

[16α-^{2}H]pregnenolone and [16α-^{2}H; 17α-^{2}H]pregnenolone provided the all important information that the formation of the 17α-hydroxyandrogen **8** was attended by the retention of the 16α-deuterium and the incorporation of an atom of oxygen from $^{18}O_2$ (Scheme 4). Together, these features highlight that **8** was not formed from the Δ^{16}-steroid **7** by an hydration mechanism. The distribution of isotopes in the other two steroid products **6** and **7** was as reported previously.[5,10]

The most important conclusion to be drawn from these results is that the 17α-hydroxylation is directly linked to the cleavage of the C-17–C-20 bond and that the overall process corresponds to an inversion of configuration. The results forcefully highlight that the conversion must occur by a stepwise process. Although the nature of the intermediates which may participate in the conversion is not directly revealed by our experiments, chemical considerations dictate that these should be radical species, as shown in Scheme 3. The key feature of the mechanism is the involvement of the Fe^{III}–OOH species to produce the adduct **9** which homolyses to produce acetate and the C-17 radical species. The formation of the 17α-hydroxyandrogen **8** from the C-17 radical occurs by an oxygen rebound process (path *a*) while the same carbon radical, by the loss of the C-16α hydrogen (path *b*), furnishes the Δ^{16}-steroid **7**. As far as we are aware, the above example provides the first experimental evidence, using a natural substrate, to suggest that C–C bond cleaving P-450 enzymes may operate *via* a radical mechanism.

It is, however, possible to envisage a mechanism in which the 17α-hydroxy-androgen **8** is formed through the involvement of Fe^{IV}–O$^•$ that is implicated in the normal hydroxylation process. For example, Fe^{IV}–O$^•$, through the abstraction of a C-21 hydrogen, may generate the C-21 carbon radical which would fragment to the C-17 radical, ultimately producing the 17α-hydroxyandrogen **8**. This scenario requires that the side chain is initially expelled as ketene and then converted to acetate by hydration. Such a mechanism, however, lacks generality and is not applicable to the formation of the Δ^{16}-steroid **7** since it is known[5] that in this case the acetate released from the side chain contains all the three deuterium atoms originally resident at C-21 of the precursor and incorporates 1 atom of oxygen from O_2. Whether the C^2H_2HCOOH isotopomer is produced in amounts stoichiometric to that of the 17α-hydroxyandrogen **8** is not yet known. Notwithstanding this minor reservation, the labelling pattern found in the 17α-hydroxyandrogen **8** at present provides the strongest support for the participation of a peroxy adduct in a carbon–carbon cleavage process. The conclusion is valid whether the formation of the 17α-hydroxyandrogen **8** is catalysed by 17α-hydroxylase-17,20-lyase or another enzyme. Should the three reactions (Scheme 2) turn out to be the property of the same enzyme, then the justification for invoking the participation of a peroxy adduct in the other two transformations **4** → **6** and **4** → **7** will be very strong indeed.

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