Mechanistic Studies on Pregnene Side-chain Cleavage Enzyme (17α-Hydroxylase-17,20-lyase) using ^{18}O

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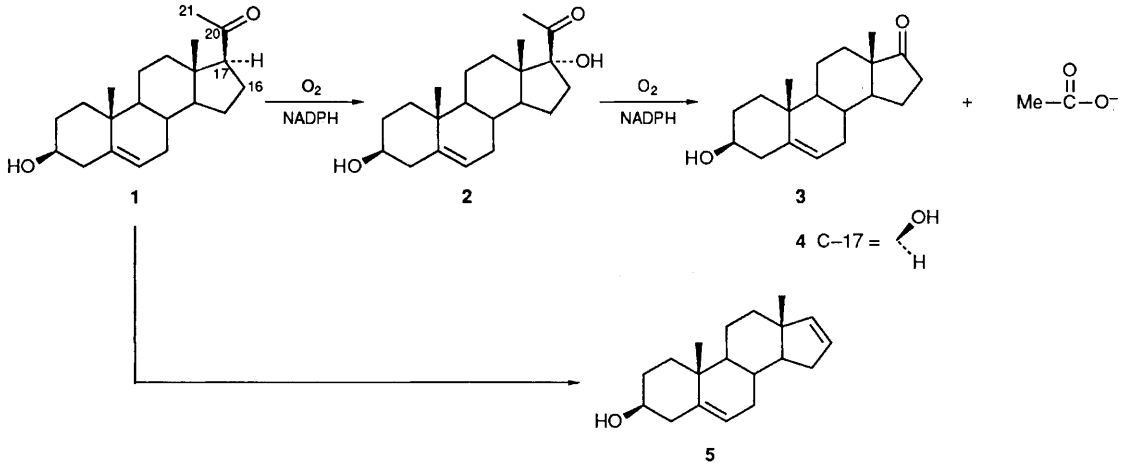
In the conversion of pregnenolone **1** into dehydro*iso*androsterone **3** and 3β-hydroxyandrosta-5,16-diene **5** an atom of oxygen from O_2 is incorporated into the side chain released as acetate; during the conversions the C-21 methyl hydrogens of the precursor are not disturbed, the status of 16α and 17α hydrogen atoms during the conversions is also defined.

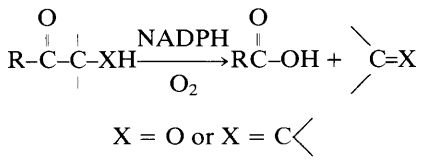
Recent mechanistic studies on sterol and steroid hormone biosynthesis together with advances in the purification of enzymes involved in the process have highlighted that certain P-450 dependent oxygenases are involved not only in the conventional hydroxylation process, [eqn. (1)], but also in

$$R\text{–}H + O_2 + NADPH \rightarrow ROH + H_2O + NADP \quad (1)$$

other generic reactions, most noteworthy of which are the cleavage of C–C bonds.[1–3] An example of this is 17α-hydroxylase-17,20-lyase (also known as P-$450_{17\alpha}$), which catalyses the removal of the pregnenolone side chain **1** (Scheme 1) to produce dehydro*iso*androsterone **3**. The enzyme not only promotes[4] the initial hydroxylation reaction to give 17α-hydroxypregnenolone **2** but also its further conversion into **3**. The enzyme also possesses another activity,[5] which culminates in the formation of the Δ^{16}-compound **5**. The C–C bond



Scheme 1 Reactions catalysed by 17-hydroxylase-17,20-lyase



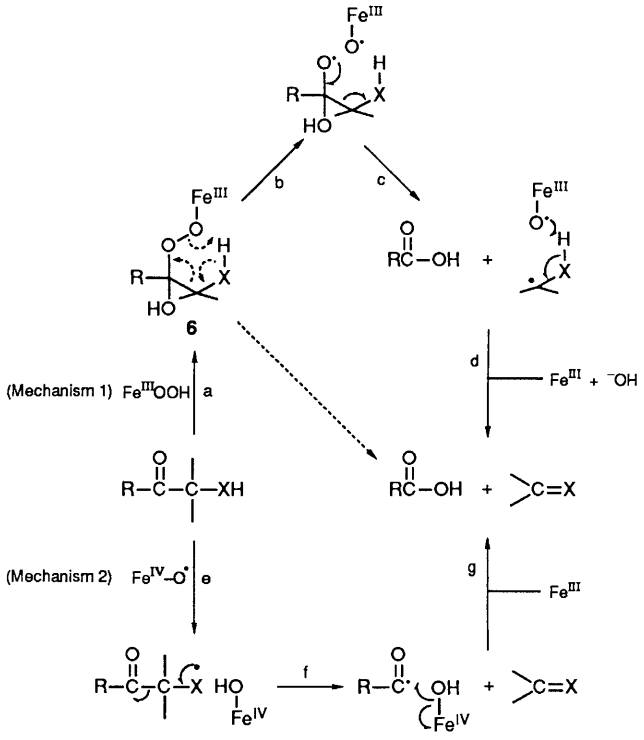
Scheme 2

cleavage reactions in the aforementioned transformations may be generally represented by Scheme 2. Although a large number of mechanisms are possible for the rationalisation of Scheme 2, the two most likely options use either Fe^{III}–OOH or Fe^{IV}–O as presented in Scheme 3. In order to test the validity of our generalisation we have extended the approach previously used for aromatase[6] to the pregnene side-chain cleavage process.

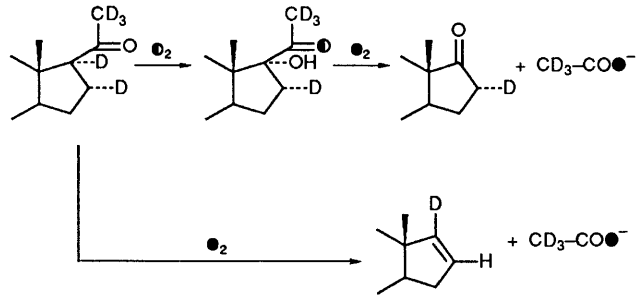
[21-$^2H_3$]17α-Hydroxypregnenolone† (0.3 μmol ml^{-1}) was mixed with a tracer quantity of the corresponding species containing ^3H at C-21, and incubated under $^{18}O_2$ with a microsomal enzyme preparation (10 mg of protein per ml) obtained from neonatal pig testes[4] in the presence of an NADPH regenerating system for 30–120 min. The incubation mixture was processed to obtain the C-20 and C-21 of the precursor as acetate[7] and from the measurement of ^3H used as the tracer, it was found that under the incubation conditions *ca.* 65% of the precursor had undergone side-chain cleavage. The acetic acid, following conversion to benzyl acetate,[6] was analysed by GC–MS to show that the predominant species contained three deuterium atoms and one atom of ^{18}O (*m/z* 155). By comparing the intensities of molecular ion peaks due to $^2H_3CCOOBzl$ and $^2H_3C^{18}OOBzl$ it was found that the incorporation of 0.91 atom of ^{18}O into the acetate occurred in the side-chain cleavage reaction. These results are in accord with the general mechanism for P-450 dependent lyases proposed by us[3] and the incorporation of an atom of molecular oxygen into acetate during the formation of the 17-keto-steroid as originally predicted by Lynn and Brown.[7]

In order to evaluate the status of ^{18}O in dehydro*iso*androsterone **3**, a similar incubation, under $^{18}O_2$, was performed now using [16α-^2H]17α-hydroxypregnenolone† so that the product formed during the biosynthesis could be discerned by the presence of a deuterium atom at C-16. After the termination of the incubation the medium was reduced with $NaBH_4$ to stabilise the C-17 carbonyl oxygen of **3** by its conversion into androstenediol **4**. The analysis of the steroid fraction by GC–MS revealed the absence of the Δ^{16}-steroid and showed that a single deuterium-containing species of androstenediol **4** was present in expected amounts and was to the extent of 97% labelled only with ^{16}O.

Next, a similar incubation under $^{18}O_2$ was performed using [16α, 17α, 21-$^2H_5$]pregnenolone.† This labelling pattern was chosen to obtain quantitative information on the incorporation of ^{18}O into the side-chain released as acetate as well as the steroid nucleus. It was found that the major isotopomer of acetate contained three deuterium atoms and 92% of such molecules were also labelled with one atom of ^{18}O (*m/z* 155). The GC–MS analysis of the steroid-containing fraction (following reduction with $NaBH_4$) showed the presence of two main products, the Δ^{16}-sterol **5** and androstenediol **4** in a ratio of *ca.* 8:1. We have already seen that in the cleavage of the side-chain of 17α-hydroxypregnenolone when the exclusive steroid formed is dehydro*iso*androsterone, the released acetate contained 0.91 atom of ^{18}O. The fact that a similar level of incorporation of ^{18}O into acetate is found in the cleavage of



Scheme 3 Two classes of mechanism for the cleavage of C–C bonds. The decomposition of the adduct **6** may occur by a concerted process (→) or a stepwise radical mechanism as shown.



Scheme 4 The status of C-16, C-17 and C-21 hydrogen atoms of the substrate and also of O_2 during the lyase catalysed reaction. ○, is the C-20 carbonyl oxygen and ◐$_2$ denotes the oxygen used in the first step and incorporated into the 17α-hydroxyl group. ●$_2$ is the oxygen that participates in the C–C bond cleavage step in the formation of dehydro*iso*androsterone and also the Δ^{16}-steroid.

the side-chain of pregnenolone when two pathways operate leading to the formation of **3** and **5** indicates that the cleavage reactions in both cases occur by mechanisms that are characterised by the introduction, somehow, of an atom from O_2 into the carbonyl carbon of pregnenolone.

Returning to the isotopic analysis of the steroids obtained from the incubation of [16α, 17α, 21-$^2H_5$]pregnenolone it was found that two, single, deuterium-containing isotopomers of **4** were present; one containing ^{16}O (*m/z* 291) and the other ^{18}O (*m/z* 293). The ratios of the intensities of the two peaks, 291:293, showed that in the conversion of pregnenolone into **3**, 0.64 atom of ^{18}O is incorporated into its C-17 from $^{18}O_2$. This aspect has been investigated previously[8,9] and the incorporation of 0.39 and 0.55 atom of ^{18}O were reported. The reason for this less than stoichiometric incorporation of ^{18}O is at present unknown although the possibility exists that this may be due to the exchange of the C-17 carbonyl oxygen with the oxygen of water during the incubation and work-up conditions. The mass spectrum of the Δ^{16}-steroid **5** had the molecular ion peak at 273 showing the presence of only one

† [16α, 17α-$^2H_2$]Pregnenolone was prepared by the method[13] previously described but using $^2H_2$. [16α-^2H]17α-Hydroxypregnenolone was prepared from [16α, 17α-$^2H_2$]pregnenolone by the method of Gardner *et al.*[14] Throughout, deuterium at C-21 and C-17α was incorporated by exchange[10] using CH_3O^2H and KO^2H.

deuterium atom. In order to explore the position of the ^2H, the experiment was repeated using [17α, 21-$^2H_4$]pregnenolone when the Δ^{16}-steroid **5** was found to contain 0.83 atom of deuterium thus confirming that the deuterium originally present at 17α of the precursor is retained in the Δ^{16}-steroid.[10] It thus follows that in the experiment conducted with [16α, 17α, 21-$^2H_5$]pregnenolone the deuterium atom removed from ring D in the formation of the 16,17-double bond must be the one present at C-16α in the precursor. An interlocking experiment using [16α-^2H]pregnenolone gave the Δ^{16}-steroid **5**, which was devoid of any deuterium label thus strengthening the inference drawn above. The loss of the 16α-^2H of pregnenolone in the formation of the Δ^{16}-steroid convincingly demonstrated above was anticipated by Osawa *et al.*[11] Together these data on the Δ^{16}-steroid shed light on two aspects. Firstly, that the 16,17-double bond of the latter is formed by the removal of the 16α-hydrogen and the 17β-side chain of pregnenolone thus showing that the reaction occurs by a *trans*-scission process. Secondly, the retention of the 17α-H of pregnenolone in the Δ^{16}-steroid shows that the latter is not formed *via* the intermediary of a 17α-hydroxy compound. This conclusion supports an earlier suggestion[10] but is at variance with another.[12]

The main finding of the work is that in the lyase-catalysed conversion of 17α-hydroxypregnenolone into **3** one atom of oxygen from O_2 is incorporated into the released acetate. A similar incorporation of oxygen into acetate occurs when pregnenolone is converted into the Δ^{16}-steroid **5** and dehydro-*iso*androsterone **3**. It is also shown that an oxygen atom from O_2 is incorporated into C-17 of the latter; this oxygen atom should be the one that was introduced as the 17-hydroxy group in the conversion **1** → **2**. Salient features of the isotopic results are summarised in Scheme 4. Cumulatively these results support our earlier suggestion that the two reactions for the cleavage of the side-chain of pregnenolone are examples of the general process shown in Scheme 2 and that these conversions may be rationalised by at least two broad mechanisms. These use either an iron–peroxy or an iron–oxo intermediate known to be formed in the P-450 dependent enzymic reaction.

In the mechanism using the peroxy species the crucial primary event is its reaction with the carbonyl group to produce an adduct **6**, Scheme 3, that decomposes with the incorporation of one atom of oxygen from O_2 into the released acid, RCO_2H. In principle the fragmentation of the adduct **6** can occur by a heterolytic, homolytic or a cyclic pathway. The choice between these processes is dictated by geometrical considerations together with the acidity of the X–H bond. In the conversion of 17α-hydroxypregnenolone **2** into dehydro-*iso*androsterone, the adduct of the type **6** is likely to decompose by an ionic mechanism because of the presence at the X–H position of a readily ionisable –OH bond. However, in the formation of the Δ^{16}-steroid since the pK_a of the C-16 carbon acid is expected to be high (>40) it is advantageous to use a homolytic process (Mechanism 1) converting the adduct **6** into ferroxy and alkoxy radicals (reaction b). The formation of the product from these species occurs by predictable reactions involving fragmentation (reaction c) and disproportionation (reaction d). The Mechanism 2 uses the iron–oxo intermediate, in the Fe^{IV}–$\dot{O}$ form, which is implicated in the normal hydroxylation process. In this case the initial reaction is a hydrogen abstraction to give the radical species (reaction e), which undergoes disproportionation (reaction f) to furnish the product. The relative merits of these two options and our preference for the mechanism(s) involving an adduct of the type **6** are debated elsewhere.[1,3]

We thank the SERC and the Wellcome Trust for grants in support of this work and Miss Ann Roberts for valuable technical assistance. This is a contribution from the SERC Molecular Recognition Centre, Southampton University.

Received, 16th October 1990; Com. 0/04659K

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