The Involvement of an O_2-Derived Nucleophilic Species in Acyl–Carbon Cleavage, Catalysed by Cytochrome P-450$_{17\alpha}$: Implications for Related P-450 Catalysed Fragmentation Reactions

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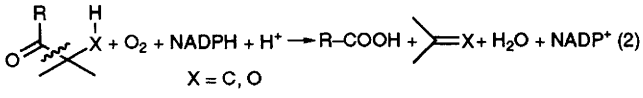
The postulated iron peroxy species involved in acyl–carbon fission by 17α-hydroxylase-17,20-lyase (P-450$_{17\alpha}$) was trapped by an aldehyde analogue, partial **9**, that was cleaved by the enzyme 10 times faster than the side-chain of the natural substrate **1**.

Cytochrome P-450s constitute a large group of enzymes and contain Fe-protoporphyrin IX (haem b) as the prosthetic group. Historically, these enzymes have been associated with hydroxylation reactions[1] which occur according to the stoichiometry of eqn. (1). Our work, directed to the

$$R\text{-}H + NADPH + O_2 + H^+ \rightarrow R\text{-}OH + NADP^+ + H_2O \quad (1)$$

elucidation of the mechanism through which certain methyl groups are removed during the biosynthesis of sterols and steroid hormones, has highlighted that two of the enzymes involved in the process, aromatase[2] and 14α-demethylase,[3] catalyse not only the conventional hydroxylation reaction, but also the oxidation of an alcohol into a carbonyl compound and, more significantly, an acyl–carbon bond cleavage reaction represented by eqn. (2).

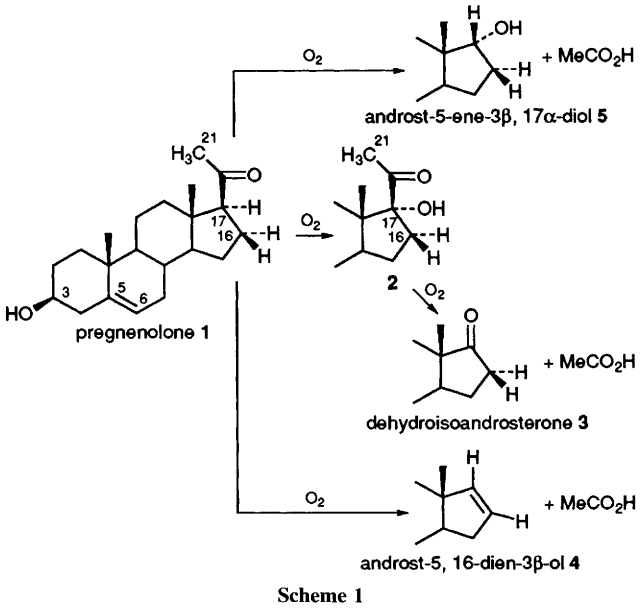


An analogous situation was envisaged for P-450$_{17\alpha}$, which catalyses a hydroxylation reaction and two different types of acyl–carbon bond cleavages, **2** → **3** and **1** → **4**,[4] Scheme 1. Another unusual cleavage,[5] resulting in the formation of **5**, is also thought to be the property of P-450$_{17\alpha}$. Although unambiguous evidence for this characterisation is not yet available, isotopic studies on the biosynthesis of **5** have been informative in showing that this compound is formed by a stepwise process from a 20-hydroperoxy intermediate.[6]

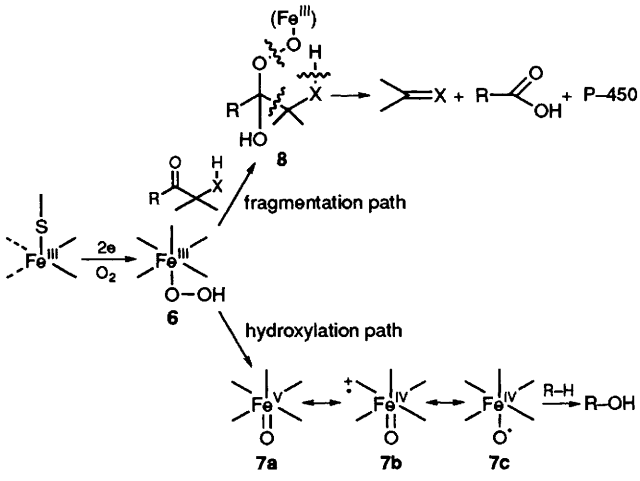
The ability of these enzymes to catalyse different generic reactions at the same active site has been rationalised in terms of the model of Scheme 2.[2d] The main assumption underlying the model is that in the catalytic cycle of P-450s, the Fe^{III}-OOH species is generated for conversion into the oxo-derivative **7** that promotes hydroxylation by a free-radical mechanism.[7] However, when the target C-atom of the substrate contains a carbonyl functionality, the iron peroxide is trapped producing the adduct **8**. The mechanism through which the latter then fragments has been debated elsewhere.[1c,2e]

Of the hormonal multifunctional enzymes, P-450$_{17\alpha}$ displays the greatest degree of promiscuity. We have exploited this feature to probe the behaviour of the enzyme towards an analogue in which the ketone group of the physiological substrate was replaced by a more electrophilic aldehyde functionality, as in the structure of type **9**.

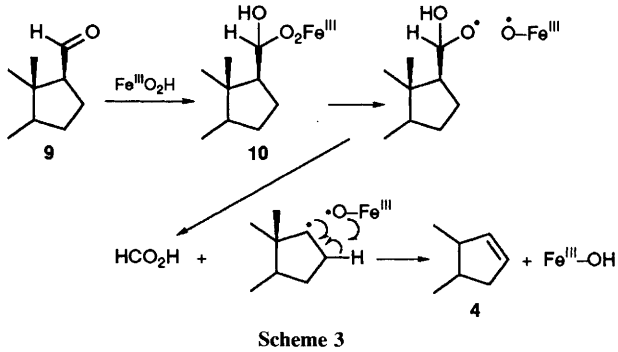
Two isotopomers of 3β-hydroxyandrost-5-ene-17β-carbaldehyde (partial **9**) labelled at C-3 and C-20 with either ^2H or ^3H, were prepared from 21-acetoxypregnenolone as follows: the latter was oxidised with Jones reagent[8] and the resulting diketone treated with isotopic sodium borohydride. Following the hydrolysis of the reduced material with methanolic KOH, the vicinal diol was cleaved with periodic acid and the aldehyde **9** separated from the contaminating 3α-epimer by preparative TLC. For the biological work the following enzymes were purified to homogeneity, to give single bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis:



Scheme 1



Scheme 2†



Scheme 3

Table 1 Product profile from the incubation of pregnenolone (**1**) and the aldehyde (**9**)

Substrate[a]	Incubation conditions	nmol Product[b] per 100 nmol substrate incubated		
		DIA (**3**)	17OH-Preg (**2**)	Diene (**4**)
Pregnenolone, **1**	No cyt. b_5	16	21	0
	Plus cyt. b_5[c]	44	9	19
	Bovine testes microsomes[d]	47	26	0
Aldehyde, **9**	No cyt. b_5	4	0	75
	Plus cyt. b_5[c]	5	0	71
	Bovine testes microsomes[d]	0	0	37

[a] Substrate (120–550 nmol) was incubated with Pig P-450$_{17\alpha}$ (2 nmol), NADPH–cytochrome-P-450 reductase (3 units), phosphatidylcholine (300 μg), $NADP^+$ (5 mg), glucose-6-phosphate (20 mg), glucose-6-phosphate dehydrogenase (10 units) and 4 cm^3 of 50 mmol dm^{-3} potassium phosphate buffer pH 7.2. [b] Products purified by silica gel TLC (benzene–acetone 10:1). [c] When present, 8 nmol of cytochrome b_5 was added. [d] Substrate (200 nmol) was incubated with calf testes microsomes (400 mg protein) and the NADPH generating system above, in 10 cm^3 of phosphate buffer pH 7.2.

cytochrome P-450$_{17\alpha}$ from neonatal pig testes,[4a] NADPH–cytochrome P-450 reductase[9] and cytochrome b_5 from pig liver.[10] The incubation of the physiological substrate, pregnenolone, with P-450$_{17\alpha}$ and the reductase, gave dehydroisoandrosterone as well as 17α-hydroxypregnenolone (Table 1). Supplementation of the incubation mixture with cytochrome b_5 led to the additional formation of the 5,16-diene **4** as reported previously.[4b] The requirement of cytochrome b_5 for the formation of the 5,16-diene **4** in a reconstituted system has been used to rationalise the observation that pig testes microsomes convert pregnenolone into the diene because of the abundance of cytochrome b_5 in this tissue.[11]

The incubation of the aldehyde **9** with the P-450$_{17\alpha}$–reductase system gave the diene **4** as the predominant product, and in the process the side chain was released as formic acid; cytochrome b_5 was not required for this conversion. The identity of the 5,16-diene **4** in the proceeding experiment was established by mass spectrometry and of the formic acid by conversion into the *p*-bromophenacyl derivative. Unlike the pig testes microsomes (results not shown), the corresponding preparations from calf testes did not convert pregnenolone into the diene, but the latter was effectively produced when aldehyde **9** was used as the substrate.

Michaelis–Menten parameters for pregnenolone, 17-hydroxypregnenolone and the aldehyde were obtained using purified P-450$_{17\alpha}$ and the reductase in the presence or absence of cytochrome b_5. It was found that the aldehyde was cleaved at a rate that was 8–11 times faster (in the absence of cytochrome b_5) and 3–4 times faster (in the presence of cytochrome b_5) than the rate of any other reaction **1** → **4**, **1** → **2** or **2** → **3** catalysed by the enzyme, using the physiological substrate.

The facile conversion of the aldehyde **9** into the diene **4** may be rationalised by the mechanism previously proposed by us[2] and supported by others,[12,13] in which the initial reaction is the formation of the peroxide adduct,[2b,12,13] which then undergoes fragmentation[1c,2d] by the sequence of Scheme 3. That the aldehyde was a better substrate for P-450$_{17\alpha}$ than the physiological substrates may be attributed to the greater reactivity of its carbonyl group towards nucleophiles than would be expected from the C-20 ketone in the physiological pregnene side-chain.

It is to be noted that the main biological function of P-450$_{17\alpha}$ is to promote hydroxylation using the oxo-derivative **7**, and it is this property that is completely suppressed by the aldehyde. By trapping the peroxide **6** the aldehyde is exclusively directed towards the cleavage pathway. This observation provides further support for the view that in multifunctional P-450s the electronic structure of the sensitive C-atom of the substrate determines the course of the overall reaction, by selecting one of the compatible oxygen species, **6** or **7**. The channelling of the peroxide intermediate either towards the oxoderivative or adduct formation is not confined to the steroidal enzymes but may also be demonstrated with detoxifying P-450s,[13] and has been implicated in the reaction catalysed by nitric oxide synthase.[14]

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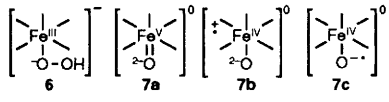
Footnote

† *In Scheme 2*: in accordance with the accepted conventional of inorganic chemistry, while assigning charge to iron in the structures in Scheme 2, it is implicit that the pair of electrons forming the coordination bond resides with the ligand and not shared with the metal. It is to be noted that some workers prefer to make this feature explicit with respect to the oxygen ligand and also show the overall charge on the complex, in which case structures **6**, **7a**, **7b** and **7c** will need to be written as:



6 **7a** **7b** **7c**

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