MECHANISM OF CHOLESTEROL SIDE-CHAIN CLEAVAGE: ENZYMIC REARRANGEMENT OF 20β-HYDROPEROXY-20-ISOCHOLESTEROL TO 20β,21-DIHYDROXY-20-ISOCHOLESTEROL

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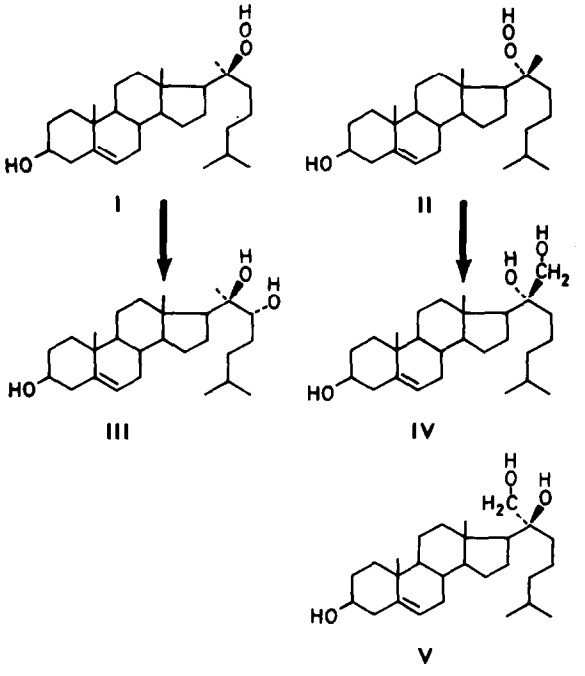
1. Introduction

Previous studies suggested that 20α-hydroperoxycholesterol* (I) is converted by adrenal cortex mitochondria to 20α,22R-dihydroxycholesterol (III) and 20α,21-dihydroxycholesterol (V) [1]. Although the enzymic formation of the alleged 20α,21-glycol (V) could not be accounted for as part of a normal pathway in steroidogenesis, the stereospecific formation of the 20α,22R-glycol (III) suggested I as an intermediate in the biosynthesis of pregnenolone. However detailed studies of these conversions were not possible because of difficulties in preparing the 20-hydroperoxysterol in sufficient yield. Several formulations for the physiological role of I in the oxidative side-chain cleavage of cholesterol to pregnenolone were proposed in the light of these findings. The suggested mechanisms included a conversion of I to either a 20α,22R-glycol (III), a 20-oxy radical or a 20,22-dioxetane as intermediates leading to scission of the C_{20}–C_{22} carbon–carbon bond [1–4]. The first pathway would imply that both oxygen atoms of III are derived from a single molecule of O_2. This possibility has recently been precluded by the ^{18}O studies of Burstein et al. which indicated that the oxygen atoms of the C_{20}- and C_{22}-hydroxyl groups of III originated from separate molecules of oxygen [5,6]. In contrast Kraaipoel et al. observed the incorporation of O from water into the glycol III during the biosynthesis of pregnenolone from cholesterol [7]. Such discrepancies have revived our interest in the metabolic role of the hydroperoxide–glycol conversion.



* In order to prevent confusion the Fisher nomenclature is used throughout, for the C_{20} configuration of these sterols. Systematic nomenclature for trivial names includes: 20α-hydroperoxycholesterol (I), (20S)-20-hydroperoxycholest-5-en-3β-ol; 20β-hydroperoxy-20-isocholesterol (II), (20R)-20-hydroperoxy-20-isocholest-5-en-3β-ol; 20α,22R-dihydroxycholesterol (III), (3β,20R,22R)-3,20,22-trihydroxycholest-5-ene; 20β,21-dihydroxy-20-isocholesterol (IV), (3β,20R)-3,20,21-trihydroxy-20-isocholest-5-ene; 20α,21-dihydroxycholesterol (V), (3β,20S)-3,20,21-trihydroxycholest-5-ene.

In the present paper we report the isolation and characterization of both 20α-hydroperoxycholesterol (I) and its 20β-isomer, 20β-hydroperoxy-20-iso-cholesterol (II). Incubation of I with an acetone-dried bovine adrenocortical mitochondrial preparation gave 20α,22R-dihydroxycholesterol (III) in quantitative yield as the sole product. A second glycol, previously reported to be formed during this incubation, was shown instead to be 20β,21-dihydroxy-20-iso-cholesterol (IV) from the incubation of II with the enzyme system. In the earlier work II had been present in the preparation of I but had escaped our attention. Conformational analysis of the sterol side-chain of the epimeric molecules suggests the identical spatial position of the 22-OH of III and the 21-OH of IV in the enzyme-bound form. These results and other considerations lead us to propose that the mechanism of enzymic side-chain cleavage of cholesterol involves three consecutive in situ oxidations via a ferryl-atomic oxygen complex of cytochrome *P*-450 to give pregnenolone as the final product.

2. Experimental

2.1. *Adrenocortical enzyme preparation*

Acetone-dried bovine adrenocortical mitochondria were prepared as previously described [1]. The acetone powder was homogenized in 0.01 M Tris (pH 7.5) and centrifuged at 30 000 × *g* for 1 h. The supernatant was freeze dried, homogenized at −30°C in anhydrous diethyl ether, collected by filtration, dried on a vacuum and stored at −60°C. The resulting powder was used throughout these studies.

2.2. *Isolation of the epimeric 20-hydroperoxy derivatives of cholesterol (I,II)*

The 20-hydroperoxycholesterol (80 mg) was isolated from 500 g of a 15 year old sample of cholesterol as previously described [8]. GC analysis after borohydride reduction of this hydroperoxide suggested the presence of both the 20α- and 20β-isomers. Therefore the material was repeatedly chromatographed on a 60 × 1 cm column of Sephadex LH-20 in methylene chloride containing 1% of methanol to give small amounts of the pure 20α- and 20β-hydroperoxy derivatives of cholesterol (I,II: 25 mg and 35 mg respectively). The separation of the isomers could be monitored by GC since decomposition in the flash heater zone gave small amounts of the 20-hydroxycholesterols with retention of the configuration about C_{20}. The 20-hydroxy epimers are readily separated by GC [9] and their ratio in the decomposed 20-hydroperoxy samples gave a direct measurement for the ratio of their parent 20-hydroperoxysterol epimers.

2.3. *Incubation conditions and product analysis.*

The adrenocortical enzyme preparation (250 mg) was dissolved in distilled water (5 ml), flushed with nitrogen and kept deoxygenated under a nitrogen atmosphere. The sterol hydroperoxides (5 mg) were introduced as DMSO solution (50 μl). The mixture was stirred at 30°C for 20 min and frozen in liquid nitrogen. The water was removed by freeze drying and the residue was extracted with methylene chloride. The total extract was chromatographed on a 60 × 1 cm column of Sephadex LH-20, developed in methylene chloride containing 1% methanol, a system which well resolves such oxidized sterols [9]. Fractions of 2.5 ml were collected and 5 μl aliquots were analyzed by silica gel TLC. The appropriate glycol fractions were combined and repurified by preparative TLC. Mass spectra of the persilylated sterol glycols were taken on an AEI MS-30 combined GC-MS. Proton spectra were recorded on a Bruker instrument adapted for Fourrier analysis.

3. Results and discussion

The epimeric 20-hydroperoxycholesterols I and II were isolated as described above. They were characterized by borohydride reduction to the known 20α- and 20β-hydroxycholesterols which were in all respects (TLC, GC, MS) identical with the authetic 20-hydroxysterols. In addition to their differing mobilities on Sephadex LH-20, I and II were distinguished by their proton spectra. The C_{18} signal of II at δ 0.83 (*s*, 3H) was hidden under one of the signals of the C_{26} + C_{27} protons at δ 0.88 (*d*, 6H, J = 6.3 Hz). In the 20α-hydroperoxide I the C_{18} singlet was shifted upfield by 0.04 ppm to δ 0.79 which resolves the signal from the C_{26} + C_{27} doublet. The C_{21} singlet of both isomers I and II, at δ 1.25, was

displaced downfield as compared to cholesterol. The C_{19} protons gave the usual shift at δ 1.02.

After incubation of I with the adrenal enzyme powder, preparative silica gel TLC gave 3 mg of the 20α,22R-dihydroxycholesterol III as the sole product. This glycol exhibited the same chromatographic mobilities (TLC and GC) as a synthetic sample, and the mass spectrum of the persilylated derivative was identical to that of the authentic sterol confirming our earlier findings [1].

Incubation of the 20β-hydroperoxy-20-iso-cholesterol II (5 mg) was conducted in the same manner to yield a single product, slightly more polar than the 20α,22R-glycol III on silica gel TLC. Previously this product was believed to originate from the 20α-hydroperoxide I and its structure was mistakenly described [1] as 20α,21-dihydroxy-cholesterol V. We now have established this glycol as 20β,21-dihydroxy-20-isocholesterol IV, derived from the 20β-hydroperoxide II. The structure was assigned upon comparing its chromatographic and spectral properties with those of the synthetic 20,21-glycol epimers IV and V. The authentic glycols were obtained from the Grignard addition of the sterol side-chain onto 3β,21-dihydroxypregn-5-ene-20-one which gave V as the major product [1]. After crystallization of V from aqueous methanol the mother-liquor was shown to contain V together with the 20β-isomer IV. Although the 20,21-glycol epimers have similar chromatographic properties, the corresponding 3,21-dibenzoates are readily separated by silica gel TLC in benzene. In this system the 20α-isomer V is less polar. The assigned configurations were confirmed by transforming the two glycols to the known 20α- and 20β-hydroxysterols [1]. The 3,21-dibenzoates of the epimers IV and V are also distinguished by their NMR spectra. The signal of the C_{21} protons of IV at δ 0.94 (*s*, 2H) was hidden under one of the signals of the C_{26} + C_{27} protons at δ 0.88 (*d*, 6H, J = 6.3 Hz). In the 20α-isomer V the C_{21} singlet was shifted downfield by 0.03 ppm to δ 0.97 which resolves the signal from C_{26} + C_{27} doublet. The C_{18} singlet gave a similar shift for both isomers at δ 0.91 and the C_{19} singlet was observed at δ 1.09.

The hydroperoxide–glycol conversions appear to be associated with cytochrome *P*-450$_{scc}$, the terminal oxidase for the side-chain cleavage of cholesterol [1,10]. The enzymic nature of these rearrangements

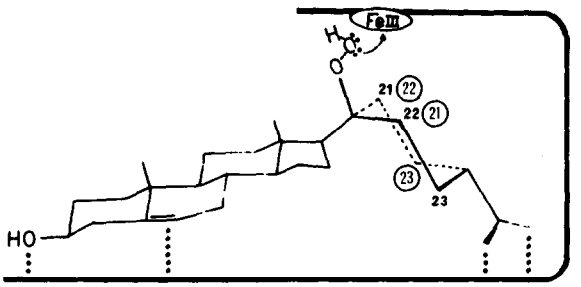


Fig.1. Proposed composite structure of 20α- and 20β-hydroperoxycholesterols I and II, bound to the cytochrome P-450$_{scc}$ enzyme through their ring systems and side-chain terminal methyl groups. The 20-hydroperoxy groups of I and II are depicted in the same conformation to permit interaction with the heme iron, thus placing the C_{22} of I and the C_{21} of II in the same spatial position. Accordingly the bold numbers refer to the carbon atoms of I, the circled numbers to those of II.

is well established since no glycol formation could be observed during the thermal decomposition of the hydroperoxysterols nor during their incubations with heat-denatured enzyme preparations [1,11]. The stereospecificity of the rearrangements may be explained by assuming that both substrates I and II bind to the same *P*-450$_{scc}$. Association of the side-chain terminal methyl groups with specific binding sites and of the 20-hydroperoxy group with the heme iron, would place the C_{22} of I and the C_{21} of II in the same spatial configuration (fig.1). Interaction of the 20-hydroperoxy group with the ferric ion followed by cleavage of the 0–0 bond may yield the

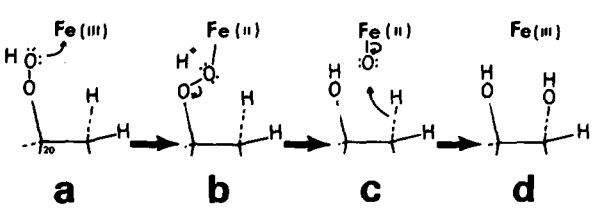


Fig.2. The enzymic rearrangement of the 20α-hydroperoxide I to the 20α,22R-glycol III and of the 20β-hydroperoxide II to the 20β,21-glycol IV is proposed to involve the following reaction sequence: After binding to the active site of P-450$_{scc}$ (a), the 20-OOH group reacts with the ferric heme ion to form a ferrous-hydroperoxysterol complex (b). Cleavage of the 0–0 bond gives the ferryl ion complex (c) which reacts with the C_{22}–bond in I or the C_{21}–H bond in II to yield the 20α,22R- or 20β,21-glycols III and IV respectively (d).

highly reactive ferryl ion complex Fe(II)–Ö:, in the same manner as in the ferric ion-hydrogen peroxide-catechol model system of Hamilton [12–14]. In the final step the ferryl-atomic oxygen could attack the C_{22}–H in I or the C_{21}–H in II, to give the 20α,22R-glycol III and the 20β,21-glycol IV respectively (fig.2).

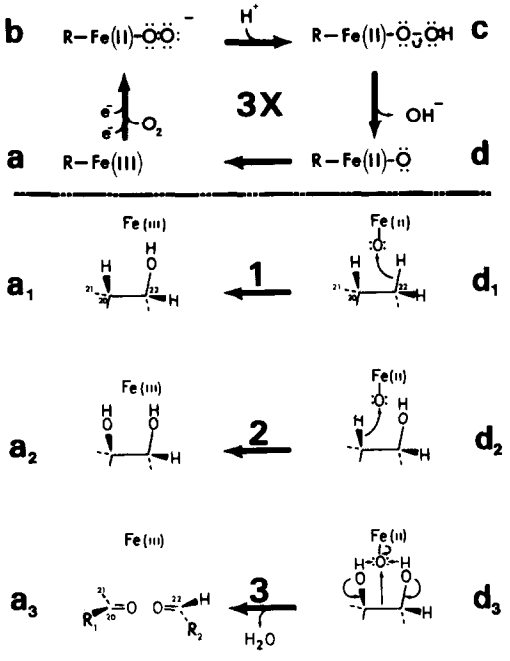


Fig.3. Proposed model for side-chain cleavage through three consecutive in situ attacks of a ferryl ion complex (d) on the C_{22}–H, C_{20}–H and C_{20}–C_{22} bonds of enzyme-bound cholesterol. In the first cycle cholesterol (R) binds to *P*-450$_{scc}$ to give complex (a). After a one-electron reduction the ferrous heme ion binds a molecule of oxygen, which upon further one-electron reduction forms the ferrous-superoxide complex (b). Protonation stabilizes this complex in the hydroperoxide form (c), thus facilitating cleavage of the 0–0 bond to yield the reactive ferryl–oxygen complex (d). In the first cycle (1) the oxygen atom attacks the C_{22}–H bond (d_1) to give enzyme-bound (22R)-22-hydroxycholesterol (a_1). In the second cycle (2) due to hindrance of the 22R-OH, the ferryl-oxygen attack is directed towards the C_{20}–H (d_2) to give the enzyme-bound 20α,22R-glycol III (a_2). In the third cycle (3), since both carbons near the activated oxygen atom are hydroxylated, the ferryl–oxygen stabilizes by abstracting two electrons of the C_{20}–C_{22} bond (d_3), causing its rupture to give pregnenolone (R_1-), caproic aldehyde (R_2-) and water, together with regenerated oxidized *P*-450 (a_1). The pregnenolone, having lost the enzyme binding groups of the sterol side-chain, dissociates from the enzyme surface, making the site available for the next cholesterol molecule.

The selective formation of III from the 20α-hydroperoxide I, together with the results of ^{18}O studies [5,6,15], suggests that the reaction of I with oxidized *P*-450$_{scc}$ gives a natural type of enzyme–substrate complex, even though I is not per se a natural intermediate.

Accordingly we propose that the side-chain cleavage of cholesterol proceeds through such a ferryl ion complex, by three consecutive in situ oxidations of the C_{22}–H, C_{20}–H and C_{20}–C_{22} bonds to give pregnenolone as the final product (fig.3). The formation of a ferrous–superoxide complex as an intermediate in *P*-450 catalyzed hydroxylations is generally accepted [16,17]. Abstraction of a hydrogen atom would stabilize the hydroperoxy form, thus facilitating cleavage of the 0–0 bond to the ferryl ion complex Fe(II)–Ö: which may be depicted in many resonance forms [12,13]. The involvement of this species as the oxidizing intermediate in biological oxidations was proposed over two decades ago by George [18]. Evidence that such a species is capable of hydroxylating an organic substrate was provided by the Hamilton model system [12,13]. Our work suggests that such ferryl–oxygen intermediates may indeed be involved in hydroxylase and desmolase activities mediated by cytochrome *P*-450.

Acknowledgement

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