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STEROL METABOLISM

XV. REARRANGEMENT OF CHOLESTEROL 20α-HYDROPEROXIDE BY BOVINE ADRENAL CORTEX MITOCHONDRIAL CYTOCHROME P-450 PREPARATIONS

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SUMMARY

Cholesterol 20α-hydroperoxide was converted by bovine adrenal cortex mitochondrial cytochrome P-450 preparations at pH 8 to 20α,21-dihydroxycholesterol and 20α,22*R*-dihydroxycholesterol. The rearrangement reaction previously followed by gas chromatography was followed spectrally by means of induced difference spectra. Cholesterol 20α-hydroperoxide did not give an induced difference spectrum with cytochrome P-450 nor did 20α,22*R*-dihydroxycholesterol, but the product 20α,21-dihydroxycholesterol gave a strong Type II induced difference spectrum, thus permitting direct observation of the rearrangement of cholesterol 20α-hydroperoxide to 20α,21-dihydroxycholesterol. A spectral dissociation constant K_s could be derived from the spectral binding data for 20α,21-dihydroxycholesterol. These results are discussed in light of our suggestion that cholesterol 20α-hydroperoxide may be an intermediate in the biosynthesis of pregnenolone from cholesterol.

INTRODUCTION

We have previously demonstrated the enzymic rearrangement of cholesterol 20α-hydroperoxide to 20α,21-dihydroxycholesterol and 20α,22*R*-dihydroxycholesterol by adrenal cortex mitochondria and suggested that mitochondrial cytochrome P-450 be implicated in the reaction[1]. In order to explore this possibility we sought to examine the binding of cholesterol 20α-hydroperoxide to cytochrome P-450 by means of induced difference spectra. The present study deals with our results in this effort.

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Systematic nomenclature for steroids given trivial names in the text include: cholest-5-en-3β-ol, cholesterol; 3β-hydroxypregn-5-en-20-one, pregnenolone; 3β-hydroxycholest-5-ene-20α-hydroperoxide, cholesterol 20α-hydroperoxide; cholest-5-ene-3β, 20α, 21-triol, 20α, 21-dihydroxycholesterol; cholest-5-ene-3β, 20α, 22R-triol, 20α, 22*R*-dihydroxycholesterol; cholest-5-ene-3β, 20α-diol, 20α-hydroxycholesterol.

EXPERIMENTAL

Samples of cholesterol 20α-hydroperoxide, 20α-hydroxycholesterol, 20α,21-dihydroxycholesterol, and 20α,22*R*-dihydroxycholesterol used in this study were of the highest purity as evinced by melting point, infrared absorption spectra, thin-layer and column chromatography, and gas chromatography.

Cytochrome P-450 preparations were obtained by means already described in detail from acetone-dried bovine adrenal cortex mitochondria (P-2 fraction)[1]. The cytochrome P-450 content of the preparation was measured by the method of OMURA AND SATO[2] using 91 $mM^{-1} \cdot cm^{-1}$ as the molecular absorption difference between the 450- and 490-nm bands in the reduced CO-treated difference spectrum. A revision of this factor to 50 $mM^{-1} \cdot cm^{-1}$ has been suggested[3]. Accompanying cytochrome P-420 in our preparations was evinced by absorption at 420 nm in the reduced CO-treated difference spectrum but the levels were only a few percent of those of cytochrome P-450.

1 vol. of the P-2 cytochrome P-450 fraction was diluted as required (approx. with 5 vol.) of 0.005 M Tris–HCl buffer at pH 8.0 (or at pH 6.0, 7.0, 9.0, 10.0) so as to give 2 mg/ml of total protein in a volume of 2.5 ml per cuvette. Comparison studies were also made using 0.02 M phosphate buffer at pH 7.5 to match our previous work[1]. Induced difference spectra were examined by adding a 20-μg sample (in 10 μl of ethanol) of 20α,21-dihydroxycholesterol, 20α,22*R*-dihydroxycholesterol, or cholesterol 20α-hydroperoxide to the sample cell and 10 μl of ethanol to the reference cell and the two cells were scanned against one another after 15 min over the 350–500-nm region with a Cary Model 14 spectrophotometer equipped with a 0–0.1 absorbance slide wire. Addition of graded amounts of 20α,21-dihydroxycholesterol (1–200 μl of a 0.221 μg/μl solution in ethanol), with a 15-min delay after addition before recording the difference spectrum, afforded binding data which established that at low sterol concentrations the absolute difference in absorbance ($\Delta A = A_{418\,nm} - A_{388\,nm}$) between

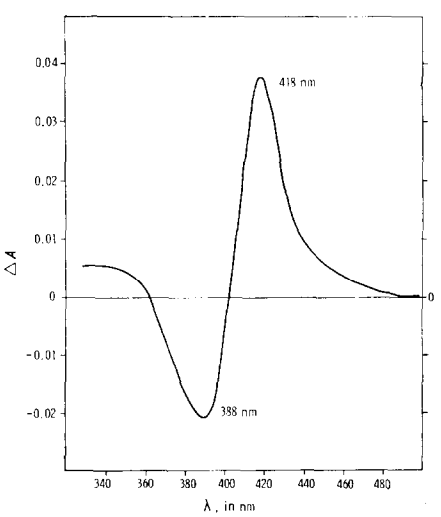

Fig. 1. Induced difference spectrum of 20α,21-dihydroxycholesterol (20 μg) with adrenal cortex mitochondrial cytochrome P-450 (5 mg, 3.75 μmoles) in 0.005 M Tris–HCl (pH 8.0) at 25°.

the peak at 418 nm and the trough at 388 nm in the induced difference spectrum (Fig. 1) increased linearly with concentration up to 1.6 μM (0.40 μM solutions giving ΔA 0.010), but curved hyperbolically thereafter.

Kinetic study of the rearrangement of cholesterol 20α-hydroperoxide by cytochrome P-450 was performed using different sterol concentrations (40–100 μM final sterol concentration), added in 10-μl volumes of ethanol. A similar volume of ethanol was added to the reference cell preparation.

Thin-layer and gas chromatographic analyses of the reaction mixtures were performed using previously described techniques[4,5]. The sterol products were recovered from the test solutions by extraction with methylene chloride, evaporation of solvent under vacuum, and redissolving in a minimum volume of methylene chloride for chromatography.

RESULTS

20α,22R-Dihydroxycholesterol did not give an induced difference spectrum with adrenal cortex mitochondrial cytochrome P-450 at 20 μM sterol concentration, but 20α,21-dihydroxycholesterol gave a strong Type II (ref. 6) induced difference spectrum (Fig. 1) with a peak at 418 nm and a trough at 388 nm. Cholesterol 20α-hydroperoxide also did not give an induced difference spectrum showing the characteristic selective absorption bands of either Type I or Type II spectra[6]. Rather the difference spectrum obtained was characterized by a generalized increase in positive absorption, possibly due to turbidity, toward low wavelengths in the substrate-treated cell.

The Type II induced difference spectrum obtained with 20α,21-dihydroxycho-

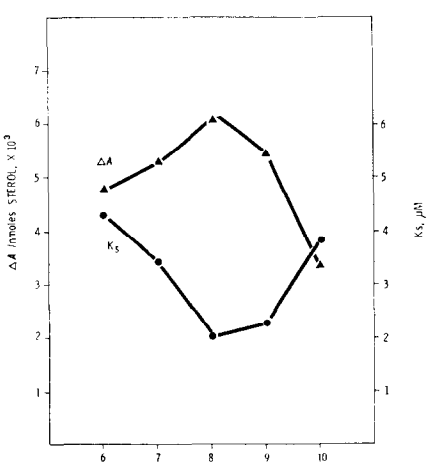

Fig. 2. Dependency on pH of the absolute absorbance difference ($\Delta A = A_{418\text{nm}} - A_{388\text{nm}}$) and of the spectral dissociation K_s.

lesterol was pH-dependent as regards the absolute difference in absorption ($\Delta A = A_{418\,\text{nm}} - A_{388\,\text{nm}}$) between the peak at 418 nm and the trough at 388 nm, and maximum absorption difference obtained at pH 8 (Fig. 2). A double reciprocal plot of binding data obtained at different pH gave straight lines from which the spectral dissociation

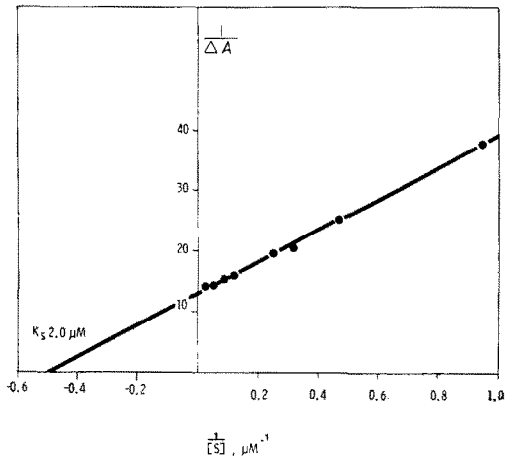

Fig. 3. Double reciprocal plot of absorbance differences ($\Delta A = A_{418nm} - A_{388nm}$) in the induced difference spectrum *versus* 20α,21-dihydroxycholesterol concentration.

constant[6], K_s, could be calculated (Fig. 3). The K_s values were also pH-dependent, with minimum K_s 2.0 μM at pH 8.0 (Tris–HCl buffer). Using the 0.02 M phosphate buffer at pH 7.5 of our previous studies[1], a K_s value of 2.7 μM was obtained.

The initial difference spectrum of cholesterol 20α-hydroperoxide did not show any selective absorption, but after a few minutes a Type II induced difference spectrum slowly developed, superimposed on the generalized background spectrum initially obtained (Fig. 4). By substracting an interpolated background absorption from each curve obtained over the time studied, it was possible to construct a graph (Fig. 5) from which the initial reaction rate could be derived, thereby giving a measure of the

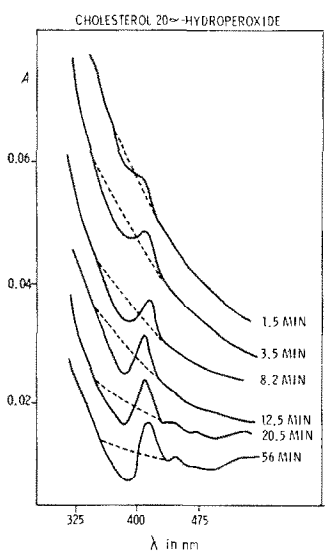

Fig. 4. Changes in the induced difference spectrum obtained with cholesterol 20α-hydroperoxide (100 μg) and cytochrome P-450 (5 mg protein, 3.75 μmoles) in 0.005M Tris–HCl (pH 8.0).

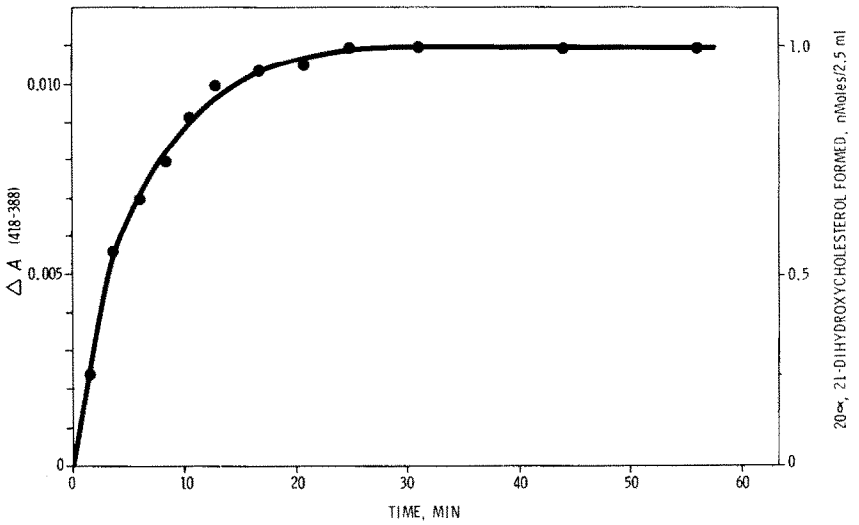

Fig. 5. Course of the rearrangement reaction of cholesterol 20α-hydroperoxide to 20α,21-dihydroxycholesterol moderated by cytochrome P-450.

specific activity of the cytochrome P-450 preparation. Gas and thin-layer chromatography of the reaction mixture established that the 20α-hydroperoxide had been consumed and that the sole reaction products were 20α,21-dihydroxycholesterol and 20α,22*R*-dihydroxycholesterol as previously found to be the case in incubations under slightly different conditions[1].

DISCUSSION

Difference spectra induced on addition of steroid substrate to cytochrome P-450 preparations are considered to represent initial substrate–enzyme binding prior to oxidation[6,7]. Decay of the induced difference spectrum has been associated with 21-hydroxylation of select substrates using adrenal cortex microsomal cytochrome P-450 (ref. 7).

Difference spectra of two types have been induced by steroids and by other agents with adrenal cortex mitochondrial[8–11] and adrenal and hepatic microsomal (refs. 6, 7, 12, 13) cytochrome P-450. Absolute absorption spectra[10], difference absorption spectra[3], and electron spin resonance spectra[11] of adrenal cortex mitochondrial cytochrome P-450 suggest that the cytochrome as isolated be a mixture of two forms, one corresponding to the Fe^{3+} in a high spin (5/2) state, the other to a low spin (1/2) state[14]. Interaction with a suitable substrate appears to cause a shift in the amount of one spin state of the iron atom or the other[10], leading to the characteristic induced difference spectra of Type I (high spin) or Type II (low spin)[6,13], depending on as yet undiscovered details of substrate–enzyme interaction.

We regard the spectral changes attendant on interaction of cholesterol 20α-hydroperoxide with cytochrome P-450 preparations as representing kinetically the rearrangement of the 20α-hydroperoxide to the 20α,21- and 20α,22*R*-dihydroxycholesterols, moderated by cytochrome P-450 or by an enzyme closely associated with it.

In that 20α,22*R*-dihydroxycholesterol demonstrably formed of the reaction does not give an induced difference spectrum, its formation is not reflected in the spectral changes noted (Fig. 4). Either 20α,22*R*-dihydroxycholesterol must fail to bind with the cytochrome P-450 or else it must be bound so as not to perturb the cytochrome P-450 Fe^{3+}, thus not to give an induced difference spectrum. The previously recognized concerted reaction properties[15–17] of the overall side-chain cleavage reaction of cholesterol to give pregnenolone, characterized by tightly bound intermediates and release only of the terminal product pregnenolone[18], support only the second proposition.

Accordingly, it appears that a putative substrate may (i) not be bound at all to cytochrome P-450, (ii) be bound so as to perturb the Fe^{3+} and thereby induce difference spectra of Types I or II, or (iii) be bound at a remote site so as not to alter the spin state of or perturb the iron atom and thereby not to induce difference spectra. In that cholesterol 20α-hydroperoxide is actively metabolized in these systems and one of the products exhibits Type II induced difference spectra, the hydroperoxide must be regarded as being bound by the cytochrome P-450 under conditions where interaction with the Fe^{3+} does not occur or as being bound to another protein in close association with the cytochrome P-450. Thus we recognize that both cholesterol 20α-hydroperoxide and 20α,22*R*-dihydroxycholesterol may be bound by cytochrome P-450 at sites for which interactions altering the spin state of the Fe^{3+} do not obtain.

Cholesterol, the natural substrate in adrenal cortex mitochondrial biosynthesis of pregnenolone, gives a Type I (high spin) induced difference spectrum with cytochrome P-450 (ref. 9). The terminal product pregnenolone gives a Type II spectrum[9,10], as does 20α-hydroxycholesterol[9,11,19]. The recognized important intermediate 20α,22*R*-dihydroxycholesterol[20,21] does not give an induced difference spectrum. Conversion of cholesterol to 20α,22*R*-dihydroxycholesterol to pregnenolone thus is formally accompanied by a change from a cytochrome P-450 of high spin state iron through intermediates which do not disclose a spin state change to a low spin state associated with the released product. The indicated sequence of events may actually measure changes in the cytochrome P-450 iron atom in consequence of the bioconversion of cholesterol to pregnenolone.

20α,21-Dihydroxycholesterol is not a recognized biosynthesis intermediate, and we regard its Type II difference spectrum as supporting our hypothesis. 20α-Hydroxycholesterol, although implicated as an intermediate in pregnenolone biosynthesis, also exhibits a Type II difference spectrum and accordingly is grouped with pregnenolone and 20α,21-dihydroxycholesterol rather than with cholesterol or the suspected intermediates cholesterol 20α-hydroperoxide and 20α,22*R*-dihydroxycholesterol.

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