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SPECTROSCOPIC EVIDENCE FOR THE FORMATION OF A TRANSIENT SPECIES DURING CYTOCHROME P-450$_{scc}$ INDUCED HYDROPEROXYSTEROL-GLYCOL CONVERSIONS

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SUMMARY: Spectroscopic analysis of the interaction of the epimeric 20-hydroperoxy derivatives of cholesterol with bovine adrenocortical cytochrome P-450$_{scc}$ preparations suggested the formation of a transient species. The intermediate was detected at 4°C and characterized by a minimum at 412 nm in the difference spectrum.

Cytochrome P-450$_{scc}$ catalyses the side-chain cleavage of cholesterol (I) through three consecutive oxidation cycles on the C20-C22 bond to yield pregnenolone (II) as the final product (1). The two intermediates, (22R)-22-hydroxycholesterol (22R-OH) and (20R,22R)-20,22-dihydroxycholesterol (20R,22R-glycol) are tightly bound to P-450$_{scc}$ and accordingly are difficult to detect under steady state turnover conditions of the reconstituted P-450$_{scc}$ enzyme system (2,3). Together with cholesterol, the 22R-OH and the 20R,22R-glycol have been identified as natural constituents of purified adrenocortical P-450$_{scc}$ (4). The 20R,22R-glycol is also obtained upon incubation of purified P-450$_{scc}$ with (20S)-20-

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Abbreviations:

20S-OOH, (20S)-20-hydroperoxycholesterol; (20R)-OOH, (20R)-20-hydroperoxy-20-isocholesterol; 20R,22R-glycol, (20R,22R)-22,22-dihydroxycholesterol; 20R,21-glycol, (20R)-20,21-dihydroxy-20-isocholesterol; 20R-OH, (22R)-22-hydroxycholesterol; P-450$_{scc}$, cytochrome P-450 which catalyzes the side-chain cleavage of cholesterol.

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hydroperoxycholesterol (20S-OOH) while the epimeric (20R)-20-hydroperoxy-20-isocholesterol (20R-OOH) is converted to the (20R)-20,21-dihydroxy-20-isocholesterol (20R,21-glycol) by this enzyme (Scheme I) (5). In contrast with other hydroperoxide-driven P-450 mediated hydroxylations, the intramolecular 20-OOH/glycol conversions proceed quantitatively at equimolar hydroperoxide/P-450$_{scc}$ concentration (5,6). These rapid, stereo-specific interactions most likely result from the unique combined substrate/oxygen-donor role of the 20-hydroperoxysterols thus providing a model system in which detectable amounts of putative radical intermediates may be expected to accumulate.

In this spectrophotometric study we report on the interaction between the epimeric 20-OOH's with both P-450$_{scc}$(CH) and substrate free P-450$_{scc}$(SF). The presence of a spectral intermediate during the early phase of the transformations is presented, providing direct evidence for the existence of a transient species in P-450 mediated peroxidative hydroxylations.

MATERIALS AND METHODS

Bovine adrenals were obtained from a local slaughterhouse and adrenocortex mitochondrial cytochrome P-450$_{scc}$(CH) was purified by the method of Suhara et al. (7). The purified enzyme, isolated as a soluble lipoprotein, retains its activity remarkably well, even during a prolonged storage at -30°, probably due to the stabilizing effect of the enzyme-bound substrates cholesterol, 22R-OH and 20R,22R-glycol (4). These sterols, at least in part, account also for the mixed spin state of the purified P-450 preparation. The endogenous sterols may be removed from the enzyme by their conversion to pregnenolone upon reconstitution of the P-450$_{scc}$ system. After chromatography a complete low spin, sterol free P-450 (SF) preparation was obtained, as shown by methylene chloride extraction and gas chromatographic analysis. The heme content of our P-450 preparations varied between 8-9 nmol per mg of protein based on a value of $\varepsilon 450-\varepsilon 490 = 91\ mM^{-1}\ cm^{-1}$ for the difference spectrum of the reduced-CO-complexed minus the reduced preparation (8). All preparations were free of P-420 and their homogeneity was confirmed by NaDodSO$_4$ slab gel electrophoresis.

The epimeric 20-hydroperoxysterols were isolated from air-aged cholesterol as previously described (5), 22R-OH, 20R,22R-glycol and 20R,21-glycol were prepared by established methods (9-11). UV-visible difference spectra were recorded as repetitive

scans on a Cary model 219 spectrophotometer. Sterols were dissolved in methanol and added to P-450$_{scc}$ in 50 mM phosphate buffer pH 7.4, by means of a microsyringe. The final methanol concentration was kept below 2% and did not contribute to the observed spectral changes. All manipulations were performed at 4°C. Sterol products were extracted from the incubation mixtures with methylene chloride and analyzed together with reference sterols on Brinkman silica gel chromatoplates developed 3 times in hexane-ethyl acetate (3:1) (4,5).

RESULTS

(20R)-20-Hydroperoxy-20-isocholesterol

When 20R-OOH is added in stoichiometric amounts to a 2 μM P-450$_{scc}$(CH) solution a difference spectrum with a characteristic trough at 416 nm develops. Superimposed on this spectrum a reverse type I difference spectrum (λ_{max} 414 nm; λ_{min} 388 nm) evolves due to the formation of the 20R,21-glycol (Fig. 1). The latter spectrum is also observed when the reaction product

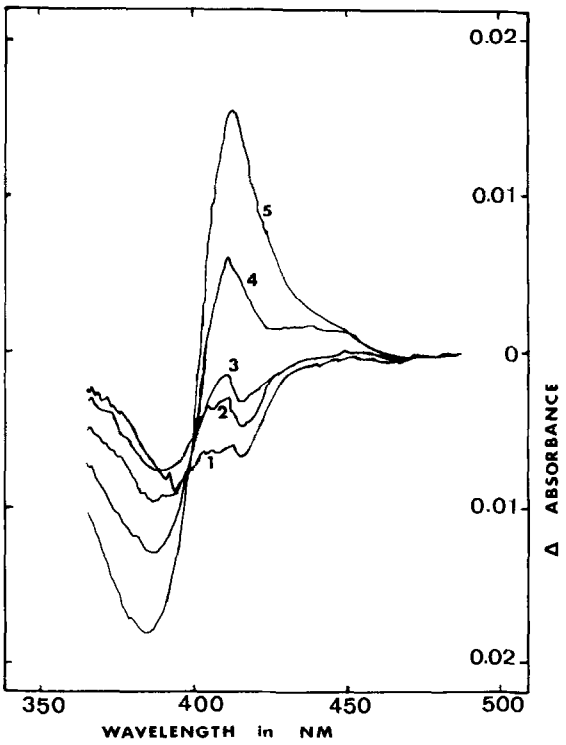


FIGURE 1. Difference spectra obtained after addition of a stoichiometric amount of the 20R-OOH to a 2 μM solution of P-450$_{scc}$ (CH) in 50 mM phosphate buffer, pH 7.4. Scans were recorded (1) immediately, (2) 80 s, (3) 200 s, (4) 400 s and (5) 480 s after addition of the hydroperoxide.

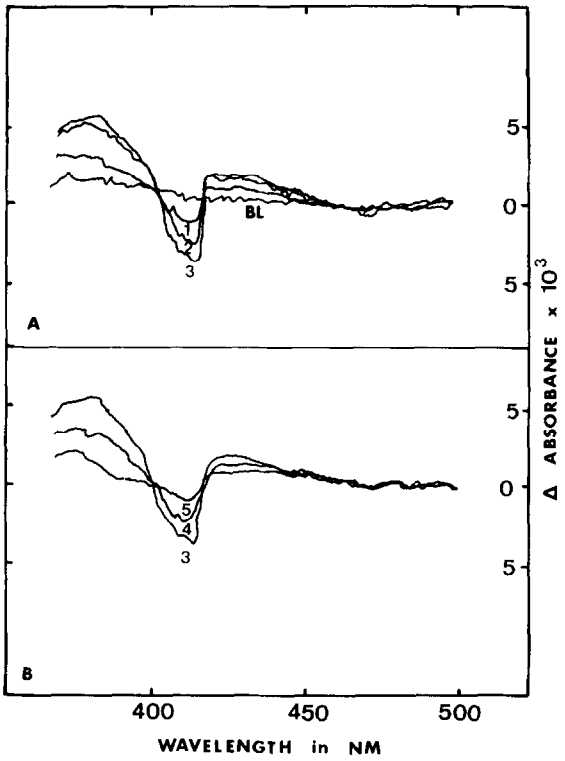


FIGURE 2. Difference spectra obtained after addition of a stoichiometric amount of the 20R-OOH to a 2 μM solution of P-450$_{scc}$ (CH) which was previously satured with a twofold excess of the 20R,21-glycol. (A) Formation of the spectral intermediate: scans were recorded, (1) immediately, (2) 40 s, (3) 80 s after addition of the hydroperoxide. BL: Baseline. (B) Return to the initial state: scans were recorded at (3) 80 s, (4) 200 s, (5) 400 s after addition of the 20R-OOH.

(20R,21-glycol) is added directly to the P-450$_{scc}$(CH) solution. In order to obtain more information on the initial spectral development, the 20R-OOH was also added to a solution of P-450$_{scc}$(CH) which was previously satured with a twofold excess of the 20R,21-glycol. Only the earlier phase of the biphasic spectral phenomenon is now observed revealing a hypochromy, reaching a maximum amplitude at 412 nm in about 80 s, followed by a much slower return to the original baseline (Fig. 2).

If 20R-OOH is added to a sterol free P-450$_{scc}$(SF) preparation, the early trough at 416 nm escapes detection and a type I difference spectrum (λ_{max} 404 nnm; λ_{min} 424 nm) develops

immediately as the result of rapid product formation. The final spectrum is identical to the one obtained by direct addition of the 20R,21-glycol. A clear isobestic point is absent among the repetitive scans and the maximum shifts from 388 nm to 404 nm during the reaction. Repetition of the experiment with a P-450_{scc}(SF) preparation saturated with the 20R,21-glycol prior to addition of the 20R-OOH evolves as in figure 2, except that the maximum amplitude is reached within 1 min.

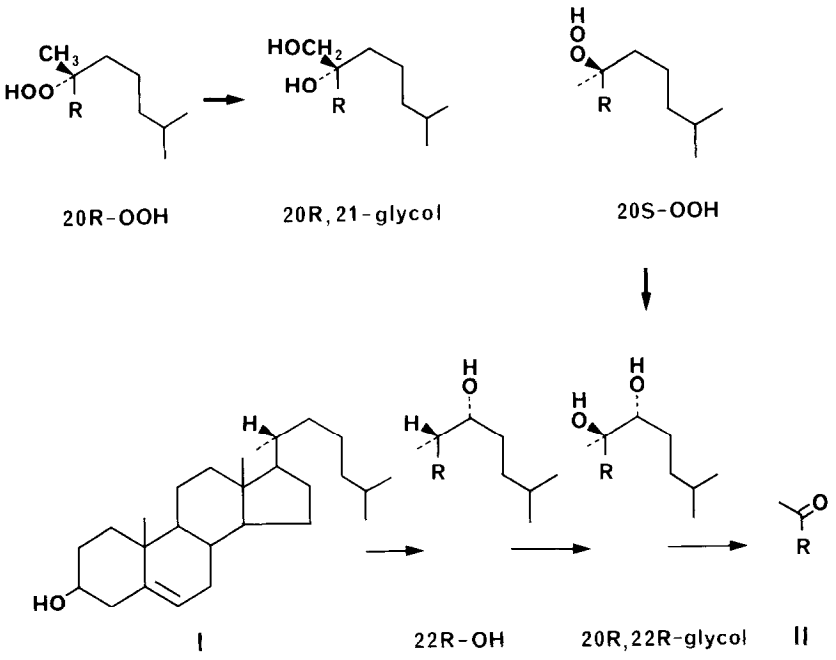
(20S)-20-Hydroperoxycholesterol

The reaction of 20S-OOH with either P-450_{scc}(CH) or (SF) preparations is very rapid, too fast to be followed by our present technique. The reaction with P-450_{scc}(SF) almost instantaneously gives a stable type I difference spectrum due to 20R,22R-glycol formation (λ_{max} 388 nm; λ_{min} 420 nm), whereas interaction of the 20S-OOH with P-450_{scc}(CH) gives a weak reversed type I difference spectrum which evolves again without a clear isobestic point. The final spectra were again identical to those obtained by direct addition of the 20R,22R-glycol to the enzyme preparations.

TLC analysis of the hydroperoxysterol/P-450 incubation mixtures confirmed the quantitative conversions of the 20R-OOH and 20S-OOH substrates and revealed the 20R ,22R- and 20R,21-glycols as the sole sterol products by their characteristic purple color response to sulfuric acid.

DISCUSSION

The addition of either the 20R-OOH or the 20S-OOH to P-450_{scc}(CH) or (SF) results in the rapid evolution of a type I or a reversed type I difference spectrum which reaches a final spectrum identical to that obtained with the corresponding glycol products. The absence of an isobestic point and, in some cases,



SCHEME I. The 20S-OOH posesses the natural 20α-configuration (Fischer projection) of the cholesterol side-chain, which is retained during its P-450$_{scc}$ catalyzed conversion to the 22R-glycol. The latter product together with the 22R-OH are the intermediates of the P-450$_{scc}$ mediated side-chain cleavage of cholesterol (I) to pregnenolone (II). The side-chain of the 20R-OOH is in the unnatural 20β-configuration, which is likewise retained during its interaction with P-450$_{scc}$ to give the 20R,21-glycol.

the clear biphasic spectral evolution observed during the reaction, suggest a complex multistep phenomenon. The complexity of these intramolecular hydroxylations is particularly evident during the reaction of the 20R-OOH with P-450$_{scc}$(CH). This suggests that the detectability of the intermediate decreases with the expected ease of interaction between the hydroperoxy substrate and the active site of the P-450$_{scc}$. Thus, addition of the 20S-OOH, with the natural 20α-side-chain configuration (Scheme I), to a P-450$_{scc}$(SF) preparation results in an almost instantaneous reaction. The presence of endogeneous substrate on the enzyme slows the reaction rate substantially as evinced by the slower evolution of the product-induced difference spectrum.

The use of the 20R-OOH, with the unnatural 20β-iso-side-chain configuration, and the inert 21-methyl group as a hydroxylation target, leads to a further inhibition of the reaction. Here again, the presence of endogeneous sterols in the enzyme preparation constitutes an additional hindrance to the interaction, thus permitting the detection of a transient species during the early phase of the reaction (Fig. 1). The presence of this intermediate is even more evident when the product-induced spectral changes are suppressed by saturation of the enzyme with the 20R,21-glycol prior to addition of the 20R-OOH substrate (Fig. 2). Possible species responsible for the spectral perturbation are the ferryl-atomic oxygen complex $(FeO)^{3+}$, a sterol-oxy radical formed upon cleavage of the O-O bond of the 20-hydroperoxy group, or a sterol-C. radical formed upon hydrogen abstraction by the $(FeO)^{3+}$ prior to the terminal hydroxylation step (5,12).

These 20R/S-OOH induced oxydations exhibit the same stereospecificity as the hydroxylations involved in the $P\text{-}450_{scc}$ mediated metabolism of cholesterol and accordingly are likely to proceed through the same intermediate activated oxygen and/or substrate radical species. Since the rate limiting step of the NADPH-driven hydroxylations is rather at the electron- than the oxygen-transfer level (6), $(FeO)^{3+}$ or related intermediate hydroxylating species elude detection under physiological turnover conditions of the enzyme. In our 20R/S-OOH - $P\text{-}450_{scc}$ model system electron transport is not a limiting factor, which might explain why detectable concentrations of an intermediate are obtained. We are presently conducting EPR studies combined with chemical analysis of the decomposition products formed during the early phase of the reaction in order to learn more about the nature of this intermediate.

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