Enzyme-Bound Sterols of Bovine Adrenocortical Cytochrome P-450$_{scc}$†

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ABSTRACT: Bovine adrenocortical cytochrome P-450 capable of cleaving the side chain of cholesterol was purified to homogeneity by the method of Suhara et al. [Suhara, K., Gomi, T., Sato, H., Itagaki, E., Takemori, S., & Katagiri, M. (1978) *Arch. Biochem. Biophys. 190*, 290]. The substrate-bound form of the enzyme preparation was shown to contain in addition to cholesterol (1.2–3.0 mol/mol of P-450) 0.4 mol of (22*R*)-22-hydroxycholesterol, 0.1 mol of (20*R*,22*R*)-20,22-dihydroxycholesterol, and a trace amount (0.005 mol) of (20*S*)-20-hydroxycholesterol per mol of P-450$_{scc}$. This relatively large concentration of (22*R*)-22-hydroxycholesterol is in accord with the hypothesis that the major pathway leading to side-chain cleavage proceeds through initial hydroxylation at the 22 position. The presence of these sterols as native constituents of cytochrome P-450$_{scc}$ supports their role as enzyme-bound intermediates in the biosynthesis of pregnenolone from cholesterol.

The adrenocortical enzyme system which cleaves the side chain of cholesterol to give pregnenolone and isocaproic aldehyde consists of a cytochrome P-450 (P-450$_{scc}$)[1] and an electron transport system. This conversion requires NADPH as a source of reducing equivalents and 3 mol of molecular oxygen/mol of cholesterol (Shikita and Hall, 1974). The classical scheme depicts the side-chain cleavage as proceeding through sequential 20*S*- and 22*R*-hydroxylations to form (20*R*,22*R*)-20,22-dihydroxycholesterol, followed by scission of the C-20,22 bond by a desmolase activity as the final step (Boyd & Simpson, 1968).

Difficulties encountered in detecting the putative oxygenated sterol intermediates in active cholesterol metabolizing enzyme systems led to early suggestions that the side-chain cleavage might be concerted and that no discrete intermediates are released from the enzyme–substrate complex prior to pregnenolone formation (Hayano, 1962; Hall & Koritz, 1964; Boyd & Simpson, 1968). Consistent with this concept is the postulate that the true intermediates are not stable products but rather are transient, reactive radical, or ionic species (Luttrell et al., 1972; Hochberg et al., 1974). However, kinetic studies by Burstein et al. (1970) indicated that the 20(*R*),22(*R*)-glycol is an intermediate in the enzymic side-chain cleavage of cholesterol. Subsequently detailed analysis of the early kinetics of cholesterol conversion to pregnenolone proved initial oxygen attack at C-22 followed by hydroxylation at C-20 to yield the 20(*R*),22(*R*)-glycol (Burstein & Gut, 1976). Electrofocusing and substrate competition studies by Duque et al. (1978) confirmed that the hydroxylase and desmolase activities leading to side-chain cleavage of cholesterol reside in a single cytochrome P-450 species.

Our own work on the rapid and highly stereospecific 20-hydroperoxy–glycol conversions mediated by P-450$_{scc}$ provided a model in which the epimeric hydroperoxysterols serve both as substrate and as source of the activated oxygen species, and our findings with this system led to the proposal that the side-chain cleavage of cholesterol involves three consecutive in situ oxidations through a ferryl–atomic oxygen complex of P-450$_{scc}$ to give the 22(*R*)-hydroxy and 20(*R*),22(*R*)-glycol derivatives of cholesterol as enzyme-bound intermediates with pregnenolone as the final product (van Lier & Rousseau, 1976; van Lier et al., 1977, 1980). The enzyme-bound nature of the intermediates would imply their existence in a steady-state cholesterol metabolizing system at levels which, in total, are no greater than the available cytochrome P-450$_{scc}$ heme sites. In the present study we have purified P-450$_{scc}$ from bovine adrenals and identified the (22*R*)-22-hydroxycholesterol and the (20*R*,22*R*)-20,22-dihydroxycholesterol as the only major oxidized sterols present.

Experimental Procedures

Reference steroids were purchased from Steroloid Inc. (Pawling, NY). (22*R*)-22-Hydroxycholesterol and (20*S*)-20-hydroxycholesterol were prepared by established methods (Tsuda & Hayatsu, 1959; Mijares et al., 1967). The (20*R*,22*R*)-20,22-dihydroxycholesterol was the generous gift of Dr. E. Forchielli, Syntex Research Center. All solvents and reagents were of the highest analytical grade available and used without further purification.

Bovine adrenals were obtained from a local slaughterhouse, and adrenocortex mitochondrial cytochrome P-450$_{scc}$ was purified to homogeneity (Suhara et al., 1978; Takikawa et al., 1978). The cytochrome enrichment of the preparation was monitored by the absorbance ratio A_{414}/A_{280} of the glycerol-induced low-spin form and analyzed for homogeneity by $NaDodSO_4$ slab gel electrophoresis (Haugen & Coon, 1976). Ultraviolet absorption spectra were obtained on a Cary Model 219 spectrophotometer. The heme concentration of the preparation was adjusted to 10 μM with 0.1 M phosphate buffer, pH 7.4, by using a value of $\Delta\epsilon_{450} - \Delta\epsilon_{490} = 91$ mM^{-1} cm^{-1} for the difference spectrum of the reduced–CO-complexed minus the reduced preparation (Omura & Sato, 1964). Samples of 10 nmol of P-450 (1 mL) were extracted with 1 mL of methylene chloride, and the phases were separated by centrifugation. The organic layer was collected, concentrated in a stream of nitrogen, and applied as a single spot onto a silica gel chromatoplate (E. Merck, Darmstadt). Chromatoplates were developed 3 times in hexane–ethyl acetate (3:1) in the usual ascending manner, and resolved sterols were detected by their color responses to 50% aqueous sulfuric acid spray upon heating to 110 °C.

Gas chromatographic (GC) and mass spectral (MS) analyses were performed on the trimethylsilyl derivatives of

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[1] Abbreviations used: P-450$_{scc}$, mitochondrial cytochrome P-450 capable of cholesterol side-chain cleavage; TLC, thin-layer chromatography; GC, gas chromatography; MS, mass spectroscopy; 20(*R*),22(*R*)-glycol, (20*R*,22*R*)-20,22-dihydroxycholesterol.

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the sterols. A 3:2:2 mixture of Me_3Si-imidazole–*N*,*O*-bis-(Me_3Si)acetamide–Me_3SiCl (TRI-SIL TBT, Pierce Chemical Co.) was used for silylation of all sterol hydroxyl groups (60 min; 80 °C), whereas a special formulation of bis(Me_3Si)-trifluoroacetamide catalyzed with 1% Me_3SiCl (BSTFA with 1% Me_3SiCl, Pierce Chemical Co.) was used to silylate primary and secondary hydroxyl groups only (60 min; 80 °C). Gas chromatography was conducted using a Hewlett-Packard Model 402 gas chromatograph equipped with a 1.5 m long × 3 mm i.d. glass column packed with 3% SP-2250 on 100–120-mesh Supelcoport (Supelco) or with 3% OV-17 on 100–120-mesh Gas-Chrom Q (Applied Science Laboratories). The columns were operated at 280 °C (SP-2250) or at 250 °C (OV-17) with nitrogen as the carrier gas. Gas chromatographic mobilities are given in terms of retention times (r_T) relative to cholesterol as unity. Mass spectra were taken on a Model MS-30 double-beam spectrometer (AEI Co., U.K.). The sample beam was connected via a membrane separator (250 °C) to a Hewlett-Packard Model 5710A gas chromatograph equipped with a 2 m long × 2 mm i.d. glass column packed with 3% SP-2401 on 100–120-mesh Supelcoport (Supelco). The column was operated at 230 °C with helium as the carrier gas. Spectra of resolved sterols were obtained at 24 eV with a resolution of 1000 and a scanning speed of 10 s/decade.

For gas chromatographic and mass spectral analysis, a larger sample of cytochrome P-450_{scc} (100 nmol in 10 mL of buffer) was extracted with 10 mL of methanol–methylene chloride, 1:1 (v/v). The extraction was repeated twice with 5 mL of methylene chloride, and the combined organic layers were concentrated under vacuum. The residue was dissolved in methylene chloride and applied as a 6 cm wide streak on a 20 × 20 cm analytical silica gel chromatoplate. A mixture of reference sterols consisting of cholesterol and the 20(*S*)- and 22(*R*)-hydroxy and 20(*R*),22(*R*)-dihydroxy derivatives of cholesterol was applied on both edges of the chromatoplate, leaving ample space and a division in the silica gel to prevent cross contamination with the enzyme extract. After a 3-fold migration in hexane–ethyl acetate, 3:1 (v/v), the reference sterols were visualized by sulfuric acid spray and heating, and the appropriate sterol zones of the enzyme extract were marked. After the chromatoplate had been carefully cleaned of acid-sprayed silica gel, the four sterol zones were collected and extracted 3 times with 0.5 mL of ethyl acetate. For quantitative determinations one-tenth of the recovered cholesterol sample was added as an internal standard to each of the recovered hydroxysterol zones. After evaporation of the ethyl acetate extracts in a stream of nitrogen, the mixtures were silylated with TRI-SIL TBT and 2–5-μL aliquots injected onto the 3% OV-17 column operated at 250 °C. Peak heights for resolved sterol components were measured and compared against a calibration curve constructed with authentic sterols.

The cholesterol content of the cytochrome P-450_{scc} was determined by using 4-cholesten-3-one as an internal standard without derivatisation. To this end 10 nmol of 4-cholesten-3-one was added to 5 nmol of the purified enzyme preparation. After extraction in the usual manner, the lipid residue was taken up in 10–25 μL of ethyl acetate, and 2-μL aliquots were analyzed by gas chromatography on a SP-2250 column at 280 °C. The cholesterol content was calculated by comparing the height of the cholesterol peak with the peak height of the internal standard.

Results

Cytochrome P-450_{scc} was isolated in a mixed spin state with ~50% low spin at pH 7.4 (Figure 1). The high- and low-spin

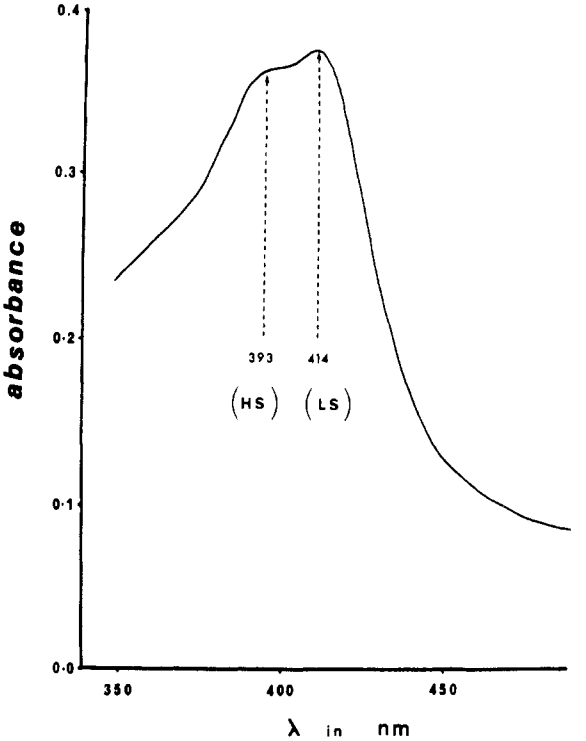

FIGURE 1: Absorption spectrum of 2 μM solubilized adrenocortical cytochrome P-450_{scc} in 0.1 M phosphate buffer, pH 7.4. Maxima at 393 and 414 nm indicate mixed high/low spin states (HS/LS) of the heme iron resulting from the presence of the enzyme-bound HS-inducing substrate cholesterol and 20(*R*),22(*R*)-glycol and the LS-inducing (22*R*)-22-hydroxycholesterol.

forms are characterized by their absorption maxima at 393 and 414 nm, respectively, with a slightly higher ε value for the low-spin form (Takikawa et al., 1978). The specific content of P-450_{scc} -heme was 9.0 nmol/mg of protein [lit. 7–9 nmol (Orme-Johnson et al., 1979); 12.3 nmol (Suhara et al., 1978)] with an A_{414}/A_{280} ratio of 0.70 in the glycerol-induced low-spin form. Takikawa et al. (1978) report an A_{393}/A_{280} ratio of 0.83 for the cholesterol-bound high-spin form of their purified cytochrome P-450_{scc}. The homogeneity of our preparation was confirmed by $NaDodSO_4$ slab gel electrophoresis which showed a single protein band. Five different cytochrome P-450_{scc} samples of 10 nmol each were extracted and analyzed by TLC. Upon heating of the acid-sprayed chromatoplates, we visualized two polar sterols in addition to the expected cholesterol spot. The two newly recognized sterol constituents exhibited the same mobility and the same characteristic color response to sulfuric acid as authentic (22*R*)-22-hydroxy- and (20*R*,22*R*)-20,22-dihydroxycholesterol (Figure 2). Purification of the polar sterol fraction by TLC followed by GC analysis of the silylated products further confirmed their presence among the cytochrome P-450_{scc} lipids (Figure 2). No other sterol products were detectable by TLC analysis, whereas the 22(*R*)-hydroxy and 20(*R*),22(*R*)-dihydroxy derivatives of cholesterol were detected consistently in each of the different enzyme preparations.

Cholesterol. A methylene chloride extract of 10 nmol of purified cytochrome P-450_{scc} was analyzed by TLC and GC and shown to contain cholesterol as the major sterol component (R_f 0.59, magenta color response to sulfuric acid; r_T = 1.0 on 3% OV-1). The structure was confirmed by MS analysis (GC–MS) which gave a fragmentation pattern identical with that of authentic cholesterol, with characteristic ions at *m*/*e* 386 (M, base peak), 371 (M – CH_3, 42%), 368 (M – H_2O, 75%), and 353 (M – CH_3 and H_2O, 48%).

(22R)-22-Hydroxycholesterol. Highly purified bovine

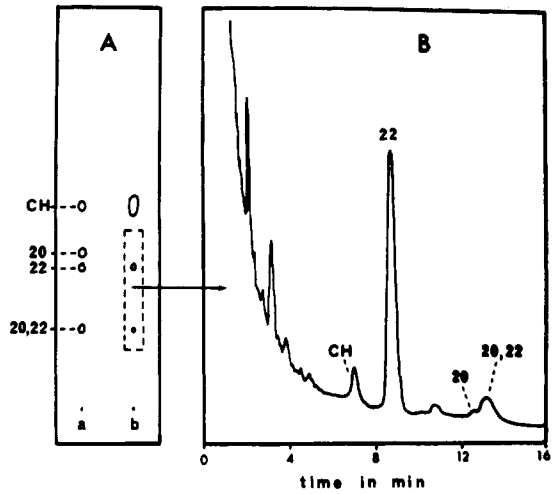

FIGURE 2: Chromatographic analysis of the sterol fractions recovered from highly purified adrenocortical cytochrome P-450$_{scc}$. (A) TLC analysis on silica gel developed 3 times in hexane–ethyl acetate (3:1) of (a) a mixture of reference sterols including cholesterol (CH), (20*S*)-20-hydroxycholesterol (20), (22*R*)-22-hydroxycholesterol (22), and (20*R*,22*R*)-20,22-dihydroxycholesterol (20,22) and (b) a methylene chloride extract of 5 nmol of cytochrome P-450$_{scc}$. (B) GC analysis on 3% OV-17 of the silylated (TRI-SIL TBT) polar sterol fraction of 5 nmol of cytochrome P-450$_{scc}$ after TLC purification. For the quantitation of the hydroxy derivatives of cholesterol, TLC bands corresponding to the individual sterols were recovered, which were devoid of cholesterol.

adrenocortical mitochondrial cytochrome P-450$_{scc}$ (100 nmol) was extracted, and four sterol fractions were separated by TLC. The material recovered from the R_f 0.40 zone was in all regards identical with authentic (22*R*)-22-hydroxycholesterol. TLC analysis of 10% of the material was conducted in the usual manner and revealed (22*R*)-22-hydroxycholesterol as a single spot (R_f 0.40, blue-green color response to sulfuric acid). The remaining material was silylated with TRI-SIL TBT and analyzed by gas chromatography which confirmed the presence of the 3,22-disilyl derivative of (22*R*)-22-hydroxycholesterol as the major sole product (r_T = 1.25 on 3% OV-1). The assigned structure was confirmed by MS analysis which gave a fragmentation pattern identical with that of the authentic 3,22-disilyl derivative with characteristic ions at *m/e* 531 (M – CH_3, 48%) and 441 (29%) due to subsequent loss of a Me_3SiO group, at *m/e* 475 (M – C_5H_{11}, 22%) and at 385 (31%) due to cleavage of the C-22,23 bond and subsequent loss of a Me_3SiO group, and at *m/e* 343 (100%) (Burstein et al., 1975).

(20S)-20-Hydroxycholesterol. Although TLC analysis did not reveal the presence of (20*S*)-20-hydroxycholesterol, the corresponding zone (R_f 0.45) on the chromatoplate of the resolved cytochrome P-450$_{scc}$ lipids was collected for analysis by the more sensitive gas chromatographic method. Half the sample was silylated with 10 μL of BSTFA and a 5-μL aliquot was injected on the GC column. The presence of a small amount of the 3-monosilyl derivative of (20*S*)-20-hydroxycholesterol was evident (r_T = 1.79 on 3% OV-1). The sample was contaminated with the (22*R*)-22-hydroxycholesterol due to the relative small difference in mobility between the 22- and 20-hydroxysterols on the preparative chromatoplate. Silylation of the other half of the sample with TRI-SIL TBT gave the 3,20-disilyl derivative of (20*S*)-20-hydroxycholesterol (r_T = 1.33 on 3% OV-1). The retention times of both the 3-mono- and 3,20-disilyl derivatives of (20*S*)-20-hydroxycholesterol were the same as those of the authentic sterol.

(20R,22R)-20,22-Dihydroxycholesterol. The third and most polar zone recovered from the preparative TLC corresponded to the 20(*R*),22(*R*)-glycol (R_f 0.24). The sterol was recovered as described above and 10% of the material was analyzed by TLC, revealing the 20(*R*),22(*R*)-glycol as a single spot (R_f 0.24, purple color response to sulfuric acid). The remaining material was silylated with BSTFA and analyzed by GC which gave the single peak of the 3,22-disilylated 20(*R*),22(*R*)-glycol (r_T = 1.89 on 3% OV-1 and r_T = 1.75 on SP-2250). The assigned structure was confirmed by MS analysis of the 3,20,22-trisilyl derivative prepared with TRI-SIL TBT, which gave a spectrum identical with that of the synthetic sterol. Characteristic peaks included the ion at *m/e* 461 (M – $C_6H_{12}Me_3SiO$, 37%) due to cleavage of the C-20,22 bond and ions at *m/e* 370 (25%) and 281 (100%) due to subsequent loss of two Me_3SiO groups, the fragment at *m/e* 173 (88%) representing the $C_6H_{12}Me_3SiO$ side chain and the ion at *m/e* 289 (56%) representing the $C_8H_{13}(Me_3SiO)_2$ side-chain fragment due to cleavage of the C-17,20 bond.

Quantitative Determinations. The relative levels of the oxidized sterols present in methylene chloride extracts of cytochrome P-450$_{scc}$ were estimated by gas chromatography of sterol preparations after initial thin-layer chromatography, using endogeneous cholesterol as an internal standard. The cholesterol concentration in turn was determined by GC analysis of the methylene chloride extract prior to chromatographic purification, in the presence of 4-cholesten-3-one as an internal standard. The calibration curves for the various reference sterols were linear over the range examined. Two different cytochrome P-450$_{scc}$ preparations were analyzed in this manner and shown to contain 3.02 and 1.75 nmol of cholesterol, 0.44 and 0.37 nmol of (22*R*)-22-hydroxycholesterol, 0.005 and 0.002 nmol of (20*S*)-22-hydroxycholesterol, and 0.11 and 0.10 nmol of (20*R*,22*R*)-20,22-dihydroxycholesterol per nmol of P-450$_{scc}$, respectively. Two additional P-450$_{scc}$ preparations were shown to contain 1.25 and 2.75 nmol of cholesterol per nmol of P-450$_{scc}$.

Discussion

The present finding that (22*R*)-22-hydroxycholesterol and (20*R*,22*R*)-20,22-dihydroxycholesterol are natural constituents of highly purified cytochrome P-450$_{scc}$ supports the role of these sterols as intermediates in the bioconversion of cholesterol to pregnenolone. Although these sterols have previously been isolated from large quantities of bovine adrenals (Dixon et al., 1970), we have shown for the first time their association with cytochrome P-450$_{scc}$ of the adrenal mitochondria. Our lipid enzyme extracts were devoid of other cholesterol derivatives, including the 22*S*-hydroxy epimer and the three possible 20,22-glycol isomeric products, all of which are well resolved in the chromatographic systems employed. The 20,22-dehydro- and 20,22-epoxycholesterols which have been suggested as possible intermediates in cholesterol metabolism (Kraaipoel et al., 1975a,b,c) were also absent. The synthesis and characterization of both epimeric 20,22-dehydrocholesterols as well as all four possible 22,22-epoxy isomers has been described (Koreeda et al., 1975; Byon & Gut, 1976; Bannai et al., 1976; Morisaki et al., 1977), and, if present in our extracts, these would have been detected in our analytical procedures. The absence of such sterols in our enzyme preparations was to be expected since none of the dehydro- and epoxysterols can serve as a substrate for pregnenolone formation (Burstein et al., 1976; Morisaki et al., 1976a). The only additional oxidized sterol which could be detected in trace amounts was the (20*S*)-20-hydroxycholesterol, whereas the 20*R* isomer and the analogous 20-hydroperoxy derivatives were absent. The rapid conversion of this (20*S*)-20-hydroperoxycholesterol to (20*R*,22*R*)-20,22-dihydroxycholesterol had led

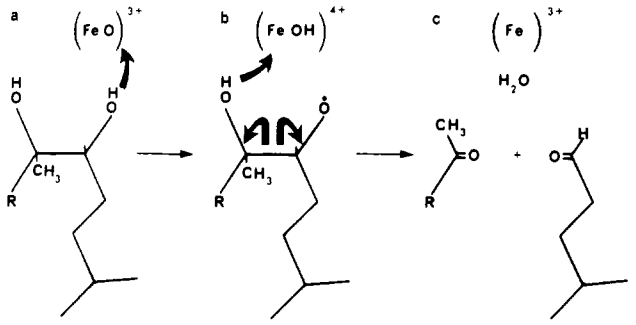

FIGURE 3: Proposed mechanism for the desmolase step in cholesterol side-chain cleavage. The bioconversion of cholesterol has previously been suggested to involve three consecutive in situ oxidations through a ferryl–atomic oxygen complex $(FeO)^{3+}$ of P-450_{scc} (van Lier & Rousseau, 1976). The final C-20,22 bond cleavage step is depicted as proceeding through initial hydrogen abstraction (a), to give a 22-oxy radical and a ferryl hydroxy radical species (b), which stabilize via a concerted reaction to give pregnenolone a molecule of water, isocaproaldehyde, and regenerated P-450_{scc} (c).

us previously to postulate a role for the 20-hydroperoxide as an intermediate in pregnenolone biosynthesis (van Lier & Smith, 1970a,b,c; van Lier et al., 1972). However, mass spectral analysis of the 20,22-glycol formed during incubation of cholesterol with adrenal enzyme preparations in the presence of mixtures of $^{18}O_2$ and $^{16}O_2$ indicated that the glycol arose by random selection from the available oxygen atom pool. This excluded the pathway through an intramolecular rearrangement of a 20-hydroperoxide and supported the notion of sequential hydroxylations of cholesterol for formation of the 20,22-glycol (Burstein et al., 1975).

The 22(*R*)- and 20(*S*)-hydroxy and 20(*R*),22(*R*)-dihydroxy derivatives of cholesterol exhibit extremely high affinity for cytochrome P-450_{scc} with the (22*R*)-22-hydroxycholesterol showing the strongest binding. The dissociation constant of (20*S*)-20-hydroxycholesterol is an order of magnitude greater than that for (22*R*)-22-hydroxycholesterol and by a factor of 2 smaller than that of the 20(*R*),22(*R*)-glycol (Orme-Johnson et al., 1979). Since all three oxidized cholesterol derivatives are rapidly converted to pregnenolone by the reconstituted cytochrome P-450_{scc} system (Morisaki et al., 1976b), the presence of enzyme-bound (20*S*)-20-hydroxycholesterol at less than 1% of the 22*R*-hydroxylated sterols supports the notion that initial 20*S*-hydroxylation of cholesterol constitutes only a minor pathway in pregnenolone biosynthesis (Burstein et al., 1970, 1976).

Cholesterol has only a weak affinity for the specific P-450_{scc} binding site and most of the sterol appears to be bound at random to the heme protein (Orme-Johnson et al., 1979). Together with the abundant availability of cholesterol in the adrenal, this might explain the variations found in the cholesterol levels of the purified P-450_{scc} preparations. The spin state of the cytochrome P-450_{scc} is influenced by many factors (Kido et al., 1979), and although cholesterol is a high-spin inducer, the amount of cholesterol associated with the enzyme appears unrelated to the ratio of high/low spin states of the purified enzyme (Orme-Johnson et al., 1979). The 20(*R*),22(*R*)-glycol is also a high-spin inducer whereas the (22*R*)-22-hydroxycholesterol is a strong low-spin inducer (Orme-Johnson et al., 1979). It seems thus likely that the spin state ratio of purified P-450_{scc} reflects, at least to some extent, the amount of enzyme-bound hydroxylated sterols present. Oxidized sterol levels in our cytochrome P-450_{scc} preparations allow for ~50% saturation of the active sites available, and a similar percentage can be anticipated during the steady-state metabolism of cholesterol. Our observations thus are in agreement with the hypothesis that the side-chain cleavage of cholesterol proceeds through two distinct enzyme-bound intermediates, e.g., the 22(*R*)-hydroxy and 20(*R*),22(*R*)-hydroxy derivatives of cholesterol which are formed by two consecutive attacks of a ferryl oxide $(FeO)^{3+}$ species on the enzyme-bound cholesterol substrate. A third ferryl oxide attack on the enzyme-bound 20(*R*),22(*R*)-glycol has been suggested to represent the desmolase activity (van Lier & Rousseau, 1976). In view of evidence that the ferryl oxide–substrate interaction in cytochrome P-450 mediated hydroxylations involves initial hydrogen abstraction (Groves et al., 1978), it seems likely that this final desmolase step proceeds through hydrogen abstraction of the 22(*R*)-OH group. Cleavage of the C-20,22 bond than could be envisaged via a concerted ferryl hydroxy radical attack on the 20(*R*)-OH group (Figure 3). Recently, we have stabilized the oxyferro complex of P-450_{scc} by using mixed solvents and subzero temperatures (Larroque & van Lier, 1980). Studies to visualize the reactive ferryl oxide intermediates by using similar subzero techniques are presently in progress in our laboratories.

Acknowledgments

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Polyglutamyl Derivatives of Tetrahydrofolate as Substrates for *Lactobacillus casei* Thymidylate Synthase†

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ABSTRACT: Tetrahydropteroylpolyglutamates containing up to seven Glu residues were tested as substrates for *Lactobacillus casei* thymidylate synthase. The K_m values decreased from 24 μM for the monoglutamate to 1.8 μM for the triglutamate. Addition of residues 4, 5, 6, and 7 did not decrease the K_m further. When monoglutamate and polyglutamate substrates were simultaneously incubated with the enzyme, the rate observed was characteristic of the polyglutamate even when the monoglutamate concentration was 44 times that of the polyglutamate. Iodoacetamide treatment inhibited the enzyme to the same extent with monoglutamate and polyglutamate substrates. Addition of 0.3 M NaCl doubled the rate obtained with the polyglutamate substrate whereas the rate with the monoglutamate was inhibited 25%. $MgCl_2$ stimulated the reaction only 10% with the polyglutamate substrate compared with 80% stimulation obtained with the monoglutamate. Inhibition by fluorodeoxyuridylate was similar with both mono- and polyglutamate substrates; however, with the phosphonate derivative of fluorodeoxyuridine, the polyglutamate substrate enhanced inhibition 5- to 8-fold.

Tetrahydrofolic acid (H_4PteGlu)[1] is commonly found in tissues in the form of poly(γ-glutamyl) derivatives (Baugh & Krumdieck, 1971). However, most studies on folate requiring enzymes employ H_4PteGu$_1$ as substrate because of its ready availability. Folate enzymes generally show a higher afffinity for the polyglutamates than for H_4PteGlu$_1$ (Baggott & Krumdieck, 1979; Cheng et al., 1975; Coward et al., 1974; Curthoys & Rabinowitz, 1972; Kisliuk et al., 1974; Mackenzie & Baugh, 1980; Matthews & Baugh, 1980). In view of the importance of thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45) in thymine deoxynucleotide biosynthesis and as a target for chemotherapeutic agents (Danenberg, 1977), we undertook a detailed study of the substrate activity of H_4PteGlu derivatives containing one through seven glutamyl residues for *Lactobacillus casei* thymidylate synthase.

Experimental Procedures

Glutamyl derivatives of pteroic acid were prepared by solid-phase peptide synthesis (Krumdieck & Baugh, 1969) and their authenticity was verified as described (Kisliuk et al., 1974). In addition PteGlu$_5$ was analyzed by high-performance liquid chromatography by Dr. Michael Archer (Reed & Archer, 1976) and found to be 94% pure.

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[1] Abbreviations used: PteGlu, folic acid; H_2PteGlu, 7,8-dihydrofolic acid; H_4PteGlu, 5,6,7,8-tetrahydrolfolic acid; CH_2H_4PteGlu, the methylene counterpart; FdUMP, 5-fluoro-2′-deoxyuridylic acid; H_4PteGlu$_{x-y}$, combination of folic acids varying from x to y in degree of polymerization.

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