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Evidence that Compound I is the active species in both the hydroxylase and lyase steps by which P450scc converts cholesterol to pregnenolone: EPR/ENDOR/cryoreduction/annealing studies

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Abstract

Cytochrome P450scc (CYP 11A1) catalyzes the conversion of cholesterol (Ch) to pregnenolone, the precursor to steroid hormones. This process proceeds via three sequential monooxygenation reactions: two hydroxylations of Ch first form 22R-hydroxycholesterol (HC) and then 20α,22R-dihydroxycholesterol (DHC); a lyase reaction then cleaves the C20-C22 bond to form pregnenolone. Recent cryoreduction/annealing studies that employed EPR/ENDOR spectroscopy (Davydov et al. 2012 JACS 134 17149) showed that compound I (Cpd I) is the active intermediate in the first step, hydroxylation of Ch. Herein we have employed EPR and ENDOR spectroscopy to characterize the intermediates in the second and third steps of the enzymatic process, as carried out by 77K radiolytic one-electron cryoreduction and subsequent annealing of the ternary oxy-cytochrome P450scc complexes with HC and DHC. This procedure is validated by showing that the cryoreduced ternary complexes of oxy-cytochrome P450scc with HC and DHC are catalytically competent, and during annealing generate DHC and pregnenolone, respectively. Cryoreduction of the ternary complex oxy-P450scc-HC trapped at 77K produces the superoxo-ferrous P450scc intermediate along with minor fraction of ferric hydroperoxo intermediates. The superoxo-ferrous intermediate converts into a ferric-hydroperoxo species after annealing at 145K. During subsequent annealing at 170–180K the ferric-hydroperoxo intermediate converts to the primary product complex with the large solvent kinetic isotope effect that indicates Cpd I is being formed, and ^{1}H ENDOR measurements of the primary product formed in D_2O demonstrate that Cpd I is the active species. They show that the primary product contains Fe(III) coordinated to the 20-O^{1}H of DHC with the ^{1}H derived from substrate, the signature of Cpd I reaction. Hydroperoxo ferric intermediates are the primary species formed during cryoreduction of the ternary complex

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Supporting Information

10 EPR/ENDOR and kinetics figures and information about product analysis. The Supporting Information is available free of charge via the Internet at http://pubs.acs.org.

oxy –P450scc-DHC, and they decay at 185K with a strong sKIE to form low-spin ferric P450scc. Together, these observations indicated that Cpd I also is the active intermediate in the C20,22 lyase final step. In combination with our previous results, this study thus indicates that Cpd I is the active species in each of the three sequential monooxygenation reactions by which P450scc catalytically converts Ch to pregnenolone.

Introduction

The main precursor of all steroid hormones in vertebrates is pregnenolone, which is widely accepted to form from cholesterol via three successive monooxygenation reactions catalyzed by cytochrome P450scc (CYP11A1, cholesterol side-chain cleavage cytochrome P450 or cholesterol hydroxylase/C20,22-lyase).[1–3] During the first step, cholesterol (Ch) is hydroxylated to 22R-hydroxycholesterol (HC), which in the second step is converted to 20α, 22R-dihydrocholesterol (DHC). Cleavage of the C20-C22 bond in the third step produces pregnenolone (Pr) and isocaproic acid. Although it has been assumed that, as with many cytochrome P450 reactions, each step of the Ch transformation uses compound I (Cpd I)[4] as the active intermediate,[5–12] to our knowledge the active oxygen species had not been experimentally established for any one of these steps until we recently began our investigations of this enzyme. Experimental test of these assumptions is of particular importance, as it has been suggested that the peroxo-ferric intermediate is the active species in the desmolase steps catalyzed by CYP 17 and 19.[12]

We have previously demonstrated that application of a cryoreduction/annealing method in combination with EPR/ENDOR spectroscopy can identify the active species in the catalytic cycle of heme-mono oxygenases.[13] In particular, using this approach we recently showed that Cpd I is involved in conversion of Ch to HC catalyzed by cytochrome P450scc.[14] Herein application of this approach gives evidence that Cpd I is the active-oxygen species in P450scc-catalyzed hydroxylation of HC to form DHC, and in cleavage of the DHC C20-C22 bond.

Materials and methods

All purchased materials and reagents were of highest commercial grade available and used without further purification. DHC was synthetized as described previously.[15] Human and bovine recombinant P450scc were expressed, purified, and concentrated as described.[14, 16, 17]

The preparation of ternary oxy-ferrous P450scc-substrate complex was performed as described previously.[14] During oxygenation ~20% of the ferrous P450scc converted to the ferric form; for clarity, the epr signal of the ferric form was subtracted from the EPR spectra of the cryoreduced samples. γ-Irradiation of the oxy-ferrous P450scc samples at 77K and annealing at temperatures over the range 77K–270K were performed as described.[14]

The products DHC and pregnenolone in the cryoreduced samples was determined using GS-MS analysis as described in SI.

Concentration of the cryoreduced ferrous oxy P450scc-substrate complexes was determined by double integration of their EPR signals using 0.2 mM substrate free low-spin ferric human cytochrome P450scc as a standard.

CW EPR spectra at X and Q bands and Q-band CW ENDOR spectra as well as low temperature UV-VIS spectra were collected and analyzed as described.[14]

Results

Effect of substrates and product on EPR/ENDOR spectra of human ferric cytochrome P450scc

To provide a reference for assigning specific states that arise during cryoreduction/annealing of the tertiary oxy-P450scc-substrate complexes, we have studied the effect of the substrates used in this work (HC and DHC) and of the product Pr on EPR and ENDOR spectra of ferric hP450scc. Expressed recombinant human substrate free (SF) ferri P450scc exhibits only a low-spin (S=1/2) EPR signal, g = [2.413, 2.245, 1.921], characteristic of a hexacoordinate aqua-ferric form.[14] The presence of a coordinated water in the ferric hP450scc is confirmed by the observation of its exchangable ^{1}H ENDOR signals with maximum hyperfine coupling of ≈ 8.1 MHz (Fig. S2).[14]

The binary complex of HC with Fe (III) P450scc also shows only a low-spin EPR signal, but with a more rhombic g-tensor, g = [2.44, 2.25, 1.918,], as is the case of the binary complex Fe(III) bovine P450scc-22R HC (Fe(III) bP450scc).[14] By analogy to Fe(III) bP450scc-HC [14] this signal is assigned to the ferric heme with HC bound through the 22R-hydroxyl, consistent with the X-ray structure.[17] The 2D field-frequency pattern of ^{1}H ENDOR signals collected across the EPR envelope of the EPR signal of the complex is considerably different from that for substrate free ferric hP450scc (Fig. S3). The spectrum from the complex collected at the field that corresponds to g_1 = 2.44 shows two resolved ^{1}H EHDOR signals, with A = 4.6 and 8.3 MHz. The latter disappears in deuterated solvent and is assigned to the proton of the 22R-hydroxyl coordinated to Fe(III). The non-exchangeable signal with A = 4.6 MHz is assigned to the H atom of substrate closest to the iron(III), at either the C-22 or C-20 position.[14]

The EPR spectrum of Fe(III) hP450scc in the presence of DHC exhibits two low-spin signals with g = [2.41, 2.25, 1.922] (major) and g = [2.44, 2.25, 1.922] (minor) and a high-spin (S=5/2) signal with g = [8.16, 3.72, 1.65]. The high-spin signal is assigned to the conformational substate with penta-coordinated heme-iron(III). The low-spin signal is characteristic of hexacoordinate aqua-ferric form. The major low-spin conformer of the complex contains coordinated water as shown by the ^{1}H ENDOR spectra which exhibit a signal from exchangeable protons of the bound water that is very similar to that for sf Fe(III) hP450scc (not shown). The reported crystal structure of the complex Fe(III)hP450scc with DHC shows that 22R- and 20- hydroxyls are close to heme iron but without direct interaction.[17] We have also found that low-spin conformers of substrate-free and DHC-bound hP450scc exhibit very similar ^{14}N ENDOR spectra at g_1. *Thus, overall, we conclude that at equilibrium, DHC binds in the heme pocket without coordinating the heme Fe(III).*

Addition of 5 mM Pr has no noticeable effect on the EPR signal (Fig S1), ^{1}H and ^{14}N ENDOR, and UV-VIS spectra (not shown) of ferric hP450scc. The absence of any of these effects indicates that Pr does not bind to the enzyme and/or does not replace the aqua-ligand. In the case of bP450scc, Pr binds weakly, with K_d = 2.9mM.[18]

Cryoreduced ternary complex oxy P450scc-22R-hydroxycholesterol

The spectrum of the cryoreduced complex trapped at 77K displays three distinct EPR signals (Fig. S4). The most intense signal A, which accounts for approximately 60% of reduced oxy-ferroheme centers, has a $g_{||}$ = 2.102 feature characteristic of a superoxo-ferroheme intermediate.[19] Two minor signals have very similar g-tensors, **g** = [2.32, 2.19, nd] and **g** = [2.32, 217, nd] (signals B and B′), characteristic of a ferric-hydroperoxo center.[14, 20, 21] An additional signal with **g** = [2.435, 2.25, nd] is from the ferric contamination formed during oxygenation of ferrous hP450scc-22R HC complex.

Figure 1 presents EPR spectra of the ternary oxy-hP450scc –22R-HC complex after γ-irradiation at 77K followed by annealing of the irradiated sample at the indicated temperatures between 145K and 215K. After annealing the irradiated sample at 145K for 30s, the superoxo ferrous heme intermediate signal A significantly decreases and this process is accompanied by the concomitant increase of the hydroperoxo ferriheme signals B and B′ (Fig. S4). The intermediates B and B′ exhibit proton ENDOR signals with $A_{max} \approx$ 10.5MHz that disappears in deuterated solvent and is assignable to the proton of the hydroperoxo ligand (Fig. 2). During progressive annealing up to 170K the hydroperoxo ferric intermediates B and B′ decay (Fig. 1), with the parallel appearance of a low-spin hexa -coordinated ferri heme species C with **g** = [2.42, 2.20, 1.92]. The decay of the hydroperoxo ferric intermediates B and B′ at 150K, Fig. 3, can be described by a “stretched exponential”,[22] and the decay half-time increases by a factor of over 3.5 in a D_2O/d_3-glycerol mixture (Fig. 3). This significant solvent KIE (sKIE) is expected for the proton assisted conversion of the hydroperoxo species B and B′ into Cpd I. Heterolytic cleavage of the ferriheme hydroperoxo ligand forms the Cpd I, ferryl porphyrin π–cation radical, intermediate upon protonation of the distal hydroperoxo oxygen, with the significant sKIE showing that the rate limiting step is proton delivery (Fig. 3).

The g-values of species C, the primary product of the monooxygenation of HC during cryoannealing, indicate that it may be assigned to either an aqua-ferriheme state or to ferriheme coordinated to the 20-hydroxyl of DHC formed by insertion of the Cpd I oxygen atom into the C20-H bond. The assignment of intermediate **C** to the ferriheme-bound product DHC is confirmed by ^{1}H ENDOR spectra. Species **C** exhibits H ENDOR spectra similar to these for the complex ferric P450scc with the 22R-hydroxyl of HC coordinated to the heme iron (III) (Fig. S5).[14] In particular, at g_1 two features with A of 4.6 MHz and 8.2 MHz can be assigned by analogy to the HC complex respectively to nearby C-H proton and the 20α-hydroxyl proton of the product DHC bound through its hydroxyl to Fe(III).[14]

This assignment is supported by the measurements with a sample prepared in D2O buffer. The 8.2 MHz signal in the primary DHC complex assigned to the 20α C-hydroxyl proton is not only seen in H_2O buffer, but is largely preserved when **C** is generated in D_2O buffer, Fig. 4. As shown previously for hydroxylation of bound cholesterol by the cryoreduced oxy

P450scc[14] this persistence for the primary DHC hydroxylation product implies that Cpd I is the hydroxylating agent.[14] Insertion of an oxygen atom into C-H of 22R-HC by the ferryl ion of Cpd I generates 22R-OH with its hydroxyl group bound to Fe(III), where the hydroxyl proton is the ^{1}H that originates from the 20α C-H of substrate, and not from solvent, so remains a ^{1}H even if the reaction proceeds in D_2O buffer.[20, 23]

As mentioned above, in the equilibrium state of the Fe(III) enzyme in the presence of DHC, neither of the 20α,22R hydroxyls coordinate to the heme iron(III). Thus, the complex between 20α hydroxyl of DHC and Fe(III) in the primary product **C** is expected to dissociate during further relaxation, forming the high spin pentacoordinate ferriheme and free DHC in the distal pocket. However, at relatively high annealing temperatures (>220K), the glycerol/buffer cryosolvent is fluid, and the Fe(III) can bind water or the high excess of substrate HC (>0.5mM) can replace the product DHC, in which case the 22R-hydroxyl of HC can bind. Indeed, during annealing at 225K, intermediate **C** relaxes to the new state with EPR and ^{1}H ENDOR spectra characteristic of the low spin ferric hP450scc - 22RHC complex (Fig. 1, S6, S7)

Confirmation that the cryoreduced ternary ferrous oxy P450scc-22R-HC is enzymatically active, and indeed generates DHC, was obtained from product analysis of the annealed sample. The product analysis showed that the cryoreduced ternary complex annealed at 273K contained 0.1±0.02 mM DHC. Spin quantitation of the EPR signal of the cryoreduced sample annealed at 145K for 10 min and at 180K for 2 min (Fig. 1) revealed that during cryoreduction 0.11±0.03 mM cryoreduced oxyheme centers forms. *These observations together show that the cryoreduced enzyme is competent to quantitatively convert bound 22R-HC into product, thereby validating the conclusions based on spectroscopic measurements.*

Cryoreduced ternary complex oxy P450scc-20,22R-dihydroxycholesterol

EPR spectra of the ternary oxy-hP450scc –DHC complex after cryoreduction at 77K and subsequent annealing of the cryogenerated intermediate(s) at indicated temperatures between 145 and 225K are presented in Figure 5. The spectrum of cryoreduced complex trapped at 77K displays a dominant rhombic EPR signal (A) with g = [2.29, 2.19, nd] and a minor signal B with g = [2.36, 2.21, nd], both g-tensors are typical of hydroperoxo ferriheme intermediates. The intermediate A exhibits a strongly-coupled, solvent-exchangeable ^{1}H ENDOR signal with A_{max} ~ 10 MHz that is assignable to the proton of the hydroperoxo ligand (Fig. 6). The observation of the hydroperoxo ferriheme state as the predominant product of 77K cryoreduction indicates that the majority of the parent ternary oxy complexes possess a hydrogen bonding network that is tied to the precursor dioxygen ligand through an ordered water in the vicinity of the distal oxygen of the O_2 ligand, and that this network supports proton delivery to the cryogenerated peroxo ligand at 77K and below.[13, 14, 20, 24] as seen with oxy P450cam, oxy-heme oxygenase and oxy NOS-Arg.[13, 20, 21, 24, 25]

During progressive annealing from 145K to 185 K, the hydroperoxo ferric intermediate A decays with a parallel increase of signal B. There also appears a weak new EPR signal with **g** = [2.41, 2.25, ~1.91](signal C) that is characteristic of low-spin ferric heme state, and may

be assigned to the primary product of substrate hydroxylation (Fig. 5). Further annealing leads to decay of the signal B with concomitant increase of the product signal C (Fig. 5). The intensity of signal C continues to increase until decay of B is complete (Fig. 5). During this process only small amount of high-spin pentacoordinated ferric P450scc forms. (Fig. S8)

The annealing pattern for the cryoreduced oxy bP450scc – DHC is qualitatively similar although not identical to that for the ternary complex of hP450scc (Fig. S9). Unlike hP450scc the cryoreduced ternary oxy bP450scc –DHC complex trapped at 77K exhibits approximately 3 equally populated conformational substates with g = [2.30, 2.19, nd], [2.33, 2.19, nd] and [2.36, 2.21, nd] characteristic of ferric hydroperoxo intermediates. During progressive annealing to 185K, the first two intermediates decay with concomitant growth of the g_{max} = 2.36 signal. At 210K the g =2.36 species relaxes to two low-spin ferriheme states with close g values of 2.44, 2.24, 1.89 and 2.41, 2.25, 1.91, respectively.

To gain better insight into structural features of the g = 2.36 species and of the low-spin ferriheme states formed during annealing the cryoreduced ternary ferrous oxy P450scc-DHC complex, we have applied H ENDOR spectroscopy. The g tensor of the g =2.36 species is suggestive of a hydroperoxo ferriheme species.[13, 21, 24] However, the unusually large rhombicity of this EPR signal might instead suggest an interpretation in terms of a tetrahedral ferri peroxo acyl intermediate, as proposed by Ortiz de Montellano for this stage of conversion DHC to pregnenolone.[11]

The assignment of the g = 2.36 signal to a hydroperoxo species, not the tetrahedral intermediate, is established by EPR/ENDOR measurements. Firstly, the g = 2.36 species is formed upon cryoreduction of oxy P450scc–DHC trapped at 77K (seen best after annealing at 145 K, Figures 5, and S9), a temperature where any chemical conversion of substrate is unlikely. Secondly, the strong sKIE for the decay of the g =2.36 intermediate and the small sKIE for its formation during relaxation of the g = 2.29 species, are consistent with the hydroperoxo ferriheme assignment of the g =2.36 intermediate. Finally, and definitively, the alternative assignment as an acyl intermediate is ruled out by ENDOR measurements. The g = 2.36 intermediate shows the well resolved strongly coupled H ENDOR signals with Amax ≈ 10 MHz, exchangeable in deuterated solvent (Fig 8), expected for the proton of a hydroperoxo ligand coordinated to heme iron(III). A tetrahedral intermediate would be expected to exhibit ^{1}H ENDOR signals with Amax < 5 MHz because of longer distance between nearest substrate protons and heme iron (III), and these would not exchange in D_2O. As the cryoreduction/annealing process converts the oxy ferroheme of the initial state to the hydroperoxo ferriheme, the peroxo ferriheme thus cannot be the reactive state.

The decay of the g = 2.36 hydroperoxo intermediate shows a large isotope effect, sKIE ~6, at 185K. (Fig. 7). This is expected for conversion of the hydroperoxo ferric heme intermediate into Cpd I through rate-limiting protonation of the distal hydroperoxo oxygen.[13, 20, 21, 26] In contrast the conversion of the g = 2.29 species into the g = 2.36 intermediate shows a much smaller sKIE < 2 (Fig. S10) suggesting a proton-independent structural relaxation of one hydroperoxo ferric conformer into another, more stable one.

Upon proton delivery to the g =2.36, hydroperoxo ferriheme intermediate it can decay by formation of Cpd I or by release of H_2O_2, depending on which oxygen atom of the hydroperoxo ligand is protonated. Protonation of the proximal oxygen and dissociation of H_2O_2 should lead to formation of high-spin pentacoordinate ferriheme species, which is not observed. Protonation of the distal oxygen favors cleavage of the O-O bond to form Cpd I,[20, 21, 23, 27] which commonly does not accumulate in EPR-detectable amounts because it reacts rapidly with bound substrates or radiolytically generated free radicals.[26] Cpd I has been proposed to catalyze oxidative cleavage of the vicinal diol of DHC to form pregnenolone, 4-isocaproic aldehyde and molecule of water.[8, 10, 11, 28] This reaction is expected to form the low-spin aqua-ferriheme species as a primary product. Both the EPR spectrum of the low-spin ferriheme state which forms after loss of the g 2.36 intermediate and orientation-selective ^{1}H ENDOR spectra presented in Fig. 9 are similar to those of the aqua ferriheme center in substrate free ferric P450scc. *Thus, these measurements support the involvement of Cpd I.*

Product analysis of the cryoreduced oxy P450scc-DHC samples annealed at 273K shows that Pr forms during its annealing, thereby validating enzymatic activity of the cryoreduced ternary ferrous oxy P450scc-DHC complex. However, unlike the reaction with HC, which gave a quantitative yield of DHC, the experiments with DHC gave only ~50% yield of Pr, relative to the amount of the cryogenerated reduced oxyheme centers. A possible explanation is discussed below.

Discussion

The present results provides insights into the oxy-heme center in the ternary oxy-P450scc complexes HC and DHC, in addition to the key characterization of the mechanism of the second and third steps in conversion of cholesterol to pregnenolone catalyzed by P450scc.

Structure of oxy-heme center

Cyoreduction of the ternary oxy-P450scc-substrate complexes at 77K generates EPR active states that retain the conformation of the oxy precursor and thus provide sensitive EPR/ENDOR probes of the diamagnetic oxy-ferrous precursor.[13, 14, 20–22, 24, 29–32] The data presented here show that the ternary oxy-P450scc -HC complex in solution exists in at least three different conformational substates that form spectroscopically distinct intermediates upon 77K reduction. EPR spectra show that radiolytic cryoreduction of the dominant conformational substate of the ternary oxy P450scc-HC complex at 77K yields a ferrous superoxide intermediate. In the superoxo ferrous heme species an unpaired electron is localized preferentially on the superoxide ligand and this state is commonly observed in the absence of strong H-bond interaction between the superoxide ligand and its active-site environment.[19]

Cryoreduction of the two minor conformers of the oxy precursor generates two ferric hydroperoxo intermediates with similar EPR spectra. Experiments with a variety of oxyhemoproteins showed that proton transfer to the peroxo ligand formed by cryoreduction of the parent oxy hemoprotein at 77K or below requires the presence in the parent oxy hemoprotein of a hydrogen bonded proton delivery network that includes an ordered water

molecule hydrogen bonded to the terminal oxygen of the bound dioxygen ligand (ref [14] and references therein). This water functions as a proton shuttle to the peroxo ligand generated by cryoreduction.[19, 20, 25, 33–37]

The transferred proton can originate from acid/base groups provided by amino acid residues within the active site, from bound substrate, or from water clusters connected to the active site by a proton delivery network (see [14] and references therein). The nature of the bound substrate can strongly modulate this proton delivery to the cryogenerated peroxo ligand.[21, 31] In contrast, oxy-globins do not have such a bound water, and cryoreduction in these cases generates peroxo-ferric intermediates exclusively.

The EPR data for the cryoreduced oxy–P450scc-HC complex trapped at 77K suggest that in the dominant conformer of the oxy precursor (~70%) the bound substrate screens the dioxygen from H-bonding interaction with the polar distal environment, resulting in the cryroreductive generation of the superoxo state. In contrast, the formation of a hydroperoxo state in the minor conformational substates of the ternary oxy P450scc complex implies that the terminal oxygen of the O_2 ligand H-bonds with a proton delivery network that includes an ordered molecule of water in the active site, enabling effective protonation of a cryogenerated peroxo ferric heme at 77K.

The results of annealing experiments show that the superoxo ferrous species trapped at 77K relaxes to hydroperoxo intermediates at relatively low temperatures, below 145K. This observation indicates that even under the conditions of very limited mobility at T≤ 145K, relatively small rearrangements within the distal pocket allow access of the distal oxygen to the H –bonded proton delivery network, converting the ferric-superoxo sequentially into ferric peroxo, and then ferric hydroperoxo species. Presumably this conformer has a molecule of water incorporated in the vicinity of the dioxygen ligand, and it reorients at temperature above 145K to establish the favorable pathway for proton transfer.

The oxy-hP450scc-DHC exhibits at least two conformational substates with different geometry of the oxyheme moiety, whereas oxy bP450scc-DHC exhibits even three conformational substates with similar populations. In all these conformational substates the cryoreduced oxyheme centers at T= 77K are trapped in the ferri hydroperoxo state, indicating the presence of an ordered molecule water H-bonded with the terminal oxygen of the diatomic ligand as a proton shuttle from the proton delivery array.

Hydroxylation of 22R-hydroxycholesterol

This work demonstrates that the cryoreduced ternary complex of oxy P450scc –HC, like the cryoreduced ternary oxy P450scc-cholesterol complex, [14] is kinetically competent and quantitatively hydroxylates the bound substrate to form DHC during annealing. Controlled annealing of the cryogenerated ferri-hydroperoxo intermediate enables a detailed examination of successive steps in HC hydroxylation by EPR and ENDOR spectroscopy. The cryogenerated ferri-hydroperoxo intermediate **g =2.32** converts completely to the primary product, species **g =2.42**, upon annealing at 145–170K. The hydroperoxo species decay with a large sKIE > 3.5 which indicates that the rate limiting step in this process is the proton-assisted conversion of the hydroperoxo intermediate to Cpd I.

The EPR spectrum and ^{1}H ENDOR pattern for the trapped primary product (g =2.42 intermediate) formed during cryoannealing in H2O and D2O buffers show that this state contains the product of hydroxylation by Cpd I, DHC, bound to the Fe(III) through the 20-OH groupsince the hydroxyl proton originates from substrate through oxygen rebound mechanism during hydroxylation by Cpd I, it remains ^{1}H regardless of the isotopic composition of the solvent. Failure to detect Cpd I by EPR during this process can be accounted for by high rate of reaction with bound substrate, which prevents its accumulation.[26] Finally, the hydroxyl group dissociates at higher temperatures, as expected, given that under equilibrium conditions the 20-OH and 22R-OH hydroxyl groups do not coordinate to the heme iron(III).[17]

Final lyase step: C-C bond cleavage of DHC

Annealing the cryoreduced ternary human oxyP450scc-DHC complex converts the bound substrate into pregnenolone, but with a somewhat lower yield (~50%) than the hydroxylation of HC. This difference may be due to partial decoupling of this process because a population of the ternary oxy complexes is cryotrapped with DHC bound in an unfavorable orientation for reaction. At ambient temperature, rapid rearrangement of the binding mode would allow adequate sampling of favorable configurations for complete coupling. Controlled annealing of the cryoreduced ternary oxy P450scc-DHC complex enables detailed examination by EPR/ENDOR spectroscopy of successive step in C20-C22 bond cleavage. The dominant primary hydroperoxo ferric intermediate trapped at 77K (g_1=2.32 EPR signal) relaxes to a new g_1 =2.36 low-spin ferric heme species upon annealing at 145–175K. The low solvent KIE of this conversion (Fig. S10), and the presence of a strongly-coupled D_2O-exchangeable ^{1}H ENDOR signal in the g_1 =2.36 species, indicate this conversion is associated with the relaxation of the initially formed hydroperoxo ferriheme state to a more stable conformer. Indeed, this g = 2.36 hydroperoxo ferric intermediate is the primary product of 77 K cryoreduction of the oxy bovine P450scc–DHC complex (Fig. S9), and a hydroperoxo intermediate with similar g-tensor components was observed during annealing of the cryoreduced tertiary oxyP450scc-cholesterol complex.[14]

Reaction with substrate DHC by the peroxo ferriheme state is ruled out because cryoreduction and low-temperature annealing produces the g = 2.36 hydroperoxo state. This in turn rules out the possible formation of tertiary peroxyhemiacetal-like intermediate by reaction of the peroxo-ferriheme with DHC, which has been considered, but not favored previously.[11] Moreover, such a tertiary intermediate should not exhibit the strongly coupled exchangeable proton ENDOR signal shown by the g = 2.36 intermediate, which is characteristic of a hydroperoxo ligand.

Further annealing of the g = 2.36 intermediate at T ≥ 180K results in its conversion to a low-spin ferric-heme species whose EPR and ^{1}H ENDOR spectra are very close to these for aqua-ferric P450scc in the presence of saturating amounts of Pr. Taking into account the absence in Pr of functional groups that can coordinate to the heme iron (III), the weak perturbation by bound Pr of the spectroscopic properties of the equilibrium aqua-ferriheme center, and recognizing that a water molecule is a product of the lyase steps,[8] we infer that the low-spin ferric intermediate trapped after complete decay of the g = 2.36 hydroperoxo

species at 185K is the primary product of enzymatic bond cleavage of DHC, in which the products Pr and isocaproic aldehyde remain in the heme pocket, while the molecule of water formed during the process[8] coordinates to the heme Fe(III). The conversion of this hydroperoxo intermediate into the primary product is characterized by large solvent isotope effect (sKIE > 6) Fig. 7, comparable with that for previously reported hydroxylation of Ch,[14] which indicates that the rate-limiting step in this process is also the proton assisted cleavage of the hydroperoxo intermediate and formation of Cpd I.[14]

Taken together the data implicates Cpd I in the final desmolase step of the diol cleavage. This is in good agreement with an earlier hypothesis of radical mechanism for this step of P450scc catalyzed metabolism of cholesterol.[8, 10, 11] According to this hypothesis the C20-C22 bond cleavage step is depicted as proceeding through initial hydrogen abstraction from C-22-OH by Cpd I to form 22-oxy radical and Fe(IV)-OH species. The radical then decomposes to release one carbonyl fragment and carbon radical; the latter is intercepted by the Fe(IV)OH species to yield pregnenolone along with isocaproic aldehyde and the molecule of water bound to the regenerated ferric P450scc. This mechanism also is supported by the fact that C-C bonds are readily cleaved (e.g. by HIO_4, $Pb(OAc)_4$) if each carbon bears as oxygen substituent, as in DHC.[5, 6] The ability of a Cpd I analogue may catalyze oxidative cleavage of a diol was also demonstrated by Sligar and coworkers, who examined the model reaction, cleavage 1-phenyl-1,2 ethanediol by the oxo-(TPP)Cr(IV).[28]

Summary

Cryoreduction/annealing experiments in combination with EPR/ENDOR spectroscopy indicate that Cpd I is the reactive species during P450scc catalyzed hydroxylation of 22R-hydroxycholesterol to 20,22-dihydrocholesterol and in the subsequent C-C bond cleavage of 20,22-dihydrocholesterol to form pregnenolone. In combination with our earlier study that showed Cpd I is the reactive species in the enzymatic hydroxylation of cholesterol to form hydroxycholesterol, the present study therefore shows that Cpd I is the active species in each of the three sequential monooxygenation reactions by which cytochrome P450scc catalyzes the conversion of cholesterol in pregnenolone (Scheme I).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

hP450scc	human cytochrome P450scc(CYP11A1)
bP450scc	bovine cytochrome P450scc
glycerol-d_3	glycerol$(OD)_3$

Ch	cholesterol
HC	22-hydroxycholesterol
DHC	20,22-dihydroxycholesterol
Pr	pregnenolone
Cpd I	compound I
sKIE	solvent kinetic isotope effect

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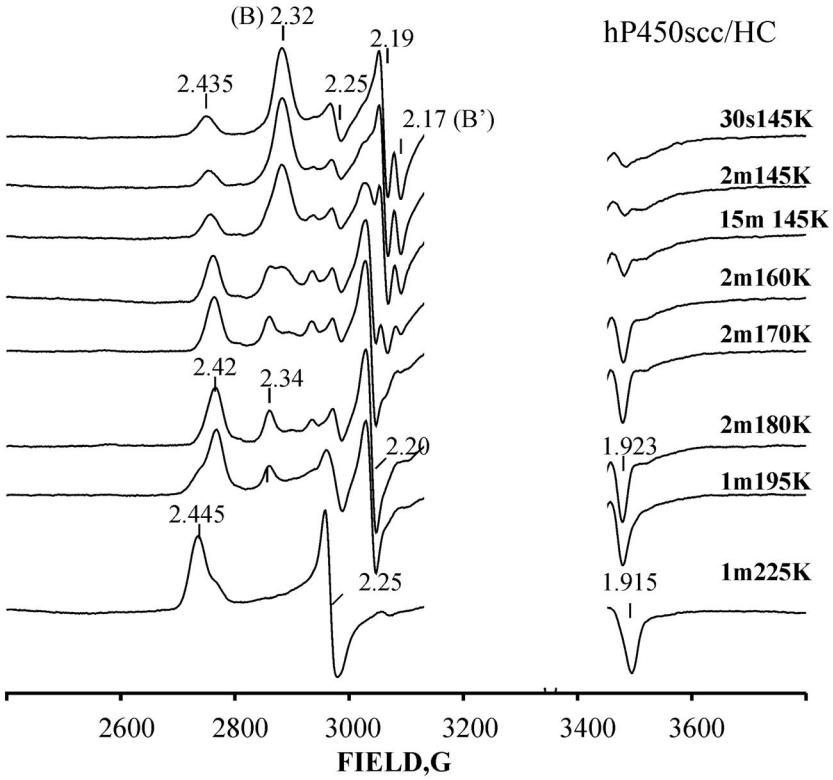


Figure 1.

EPR spectra of cryoreduced ternary oxy ferrous P450scc-22R-HC complex in 50% glycerol/0.1 M KPi pH 7.4 and after annealing at indicated conditions. (For clarity, the EPR signal from ferric contaminant formed during the oxygenation of ferrous P450scc was subtratcted from the EPR signals of the cryoreduced ferrous oxy complex). Instrumental conditions: T=28K; modulation amplitude A=10G; microwave power 10 mW; microwave frequency 9.3. GHz.

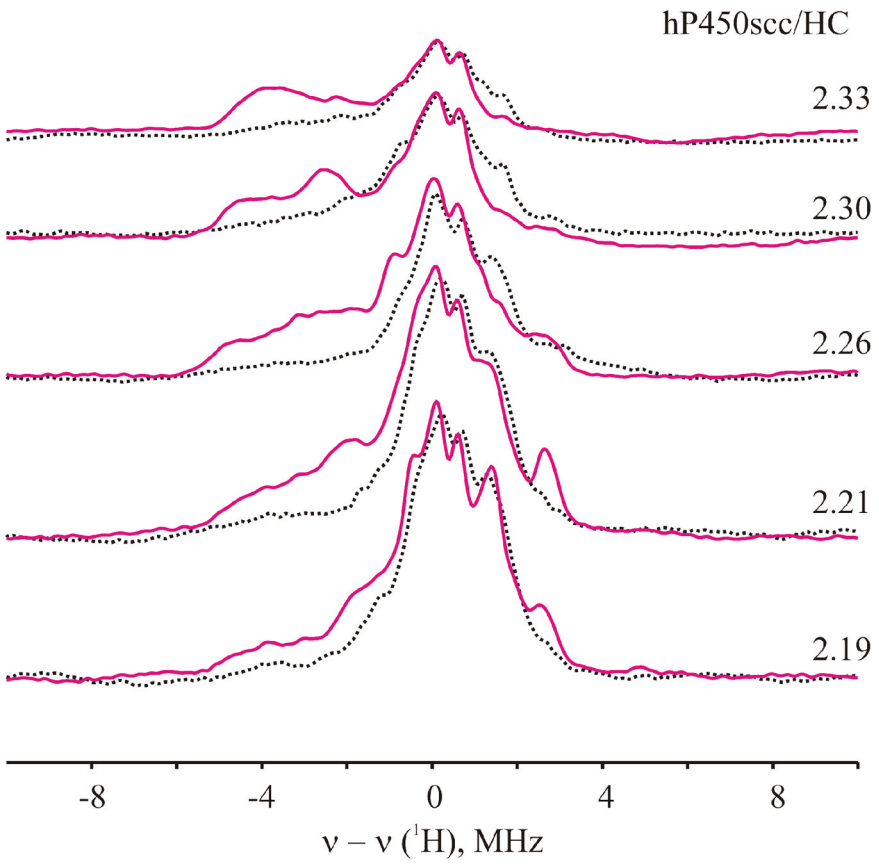


Figure 2.

2D frequency/field 35GHz ^{1}H CW ENDOR spectra of the cryoreduced ternary oxy ferrous P450scc – 22R HC complex in 50% glycerol/H_2O buffer pH 7.4 (red) and 50% d-3 glycerol/D_2O buffer pH 7.0 (dotted black). Instrument conditions: 2K, modulation amplitude 2G; rf power 5W, scan rate 1MHz/s; frequency bandwidth 60kHz; average of 20 scans; microwave frequency, 34.89.

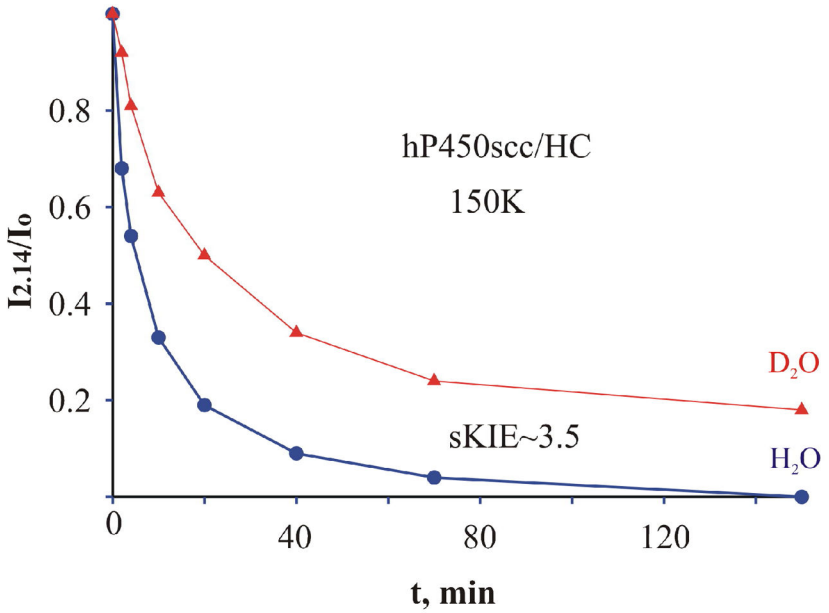


Figure 3.

Kinetics of decay of species A of the cryoreduced ternary oxy ferrous P450scc22R-HC complex at 150K in 50% glycerol/H_2O buffer pH 7.4 (blue) and 50% d-3 glycerol/D_2O buffer pH 7.0 (red).

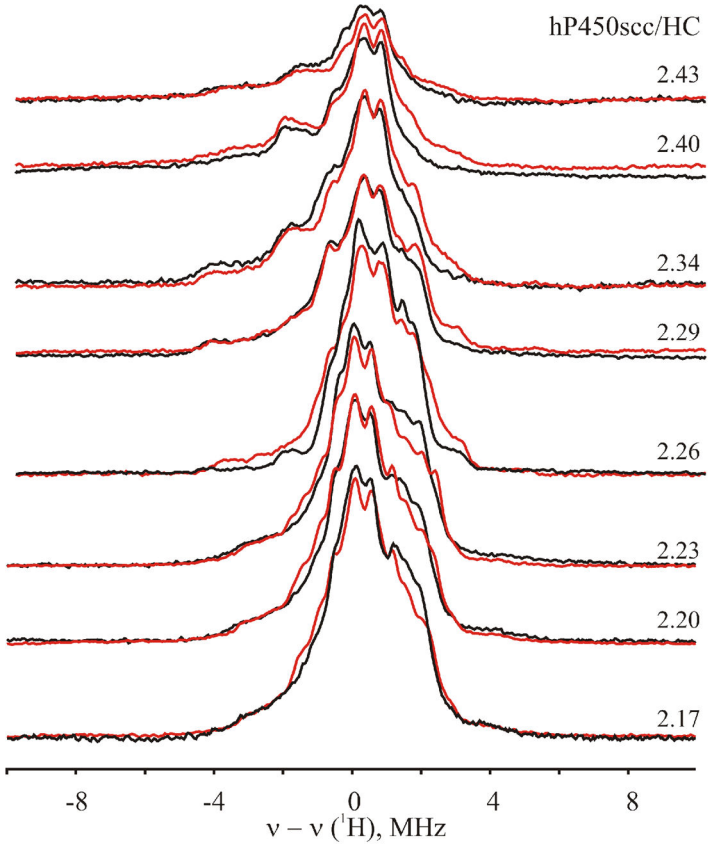


Figure 4.

2D frequency/field 35GHz ^{1}H CW ENDOR spectra of the cryoreduced ternary oxy ferrous P450scc – 22R HC complex annealed at 175K for 1 min in 50% glycerol/H_2O buffer pH 7.4 (red) and 50% d-3 glycerol/D_2O buffer pH 7.0 (black). Instrument conditions as in Fig2

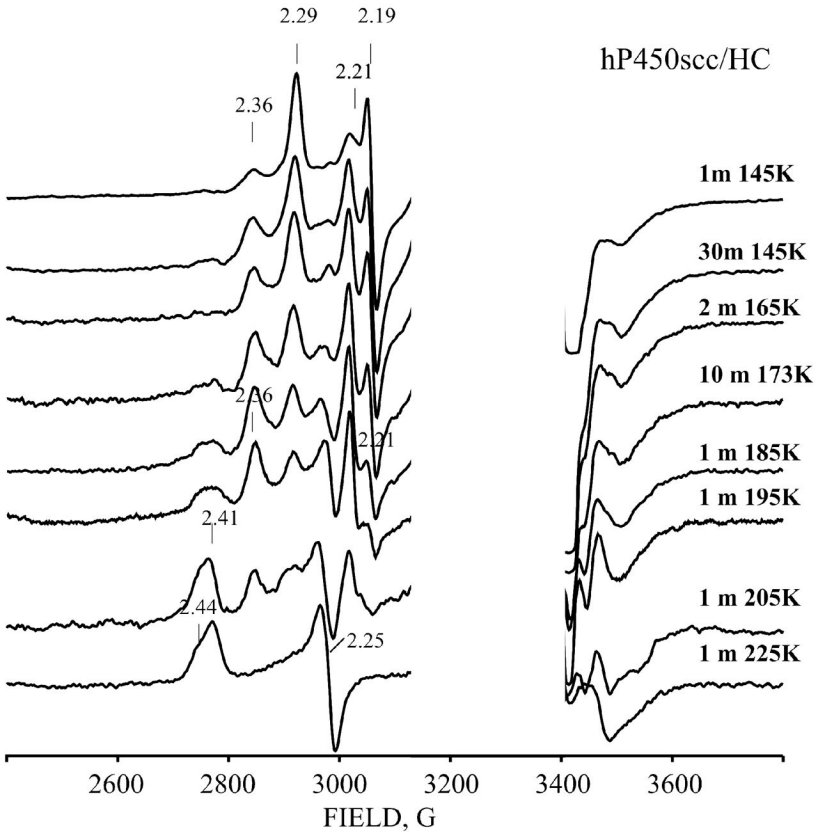


Figure 5.

EPR spectra of cryoreduced ternary oxy ferrous hP450scc-DHC complex in 50% glycerol/0.1 M KPi pH 7.4 and after annealing at indicated conditions. Instrumental conditions: T=28K; modulation amplitude A=10G; microwave power 10 mW; microwave frequency 9.3. GHz.

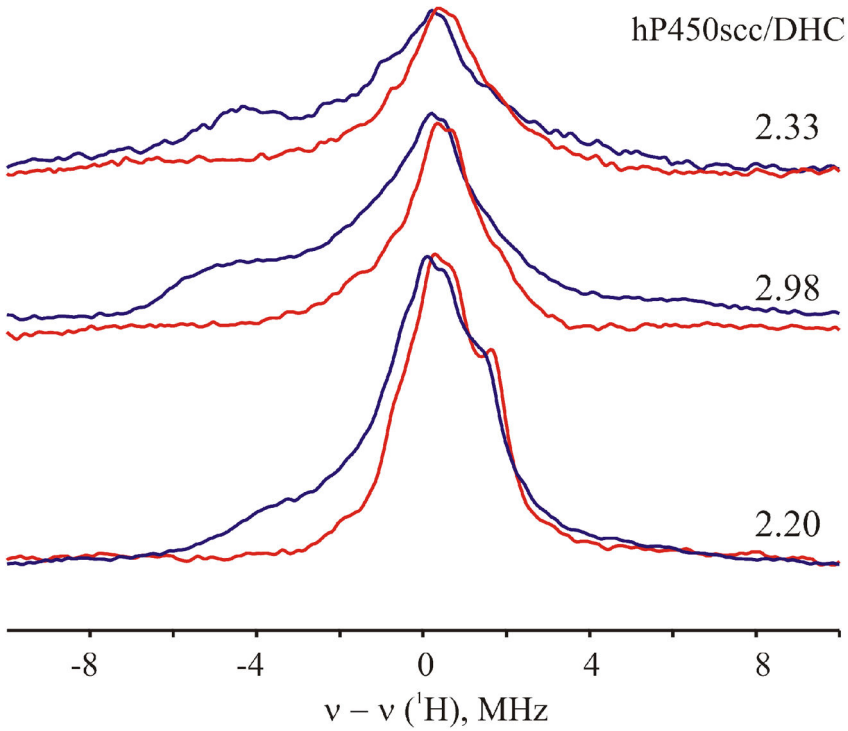


Figure 6.

2D frequency/field 35GHz ^{1}H CW ENDOR spectra of the cryoreduced ternary oxy ferrous P450scc – DHC complex in 50% glycerol/H_2O buffer pH 7.4 (blue) and 50% d-3 glycerol/D_2O buffer pH 7.0 (red). Instrument conditions: T= 2K; modulation amplitude 2G; rf power 5W, scan rate 1MHz/s; frequency bandwidth 60kHz; average of 20 scans; microwave frequency, 34.89.

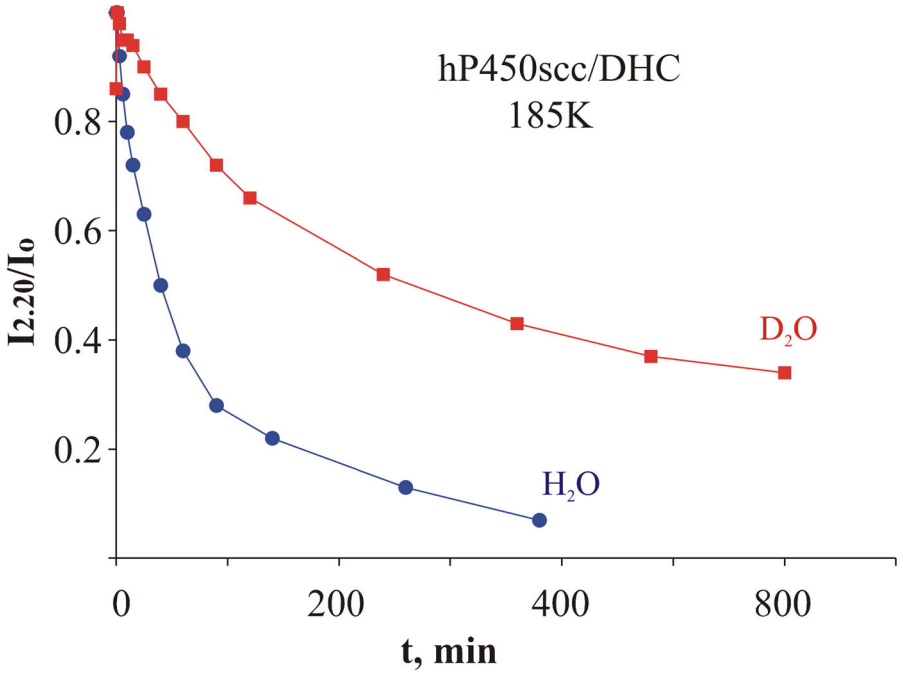


Figure 7.

Kinetics of decay of g =2.36 species of the cryoreduced ternary oxy ferrous P450scc-DHC complex at 185K in 50% glycerol/H_2O buffer pH 7.4 (blue) and 50% d-3 glycerol/D_2O buffer pH 7.0 (red).

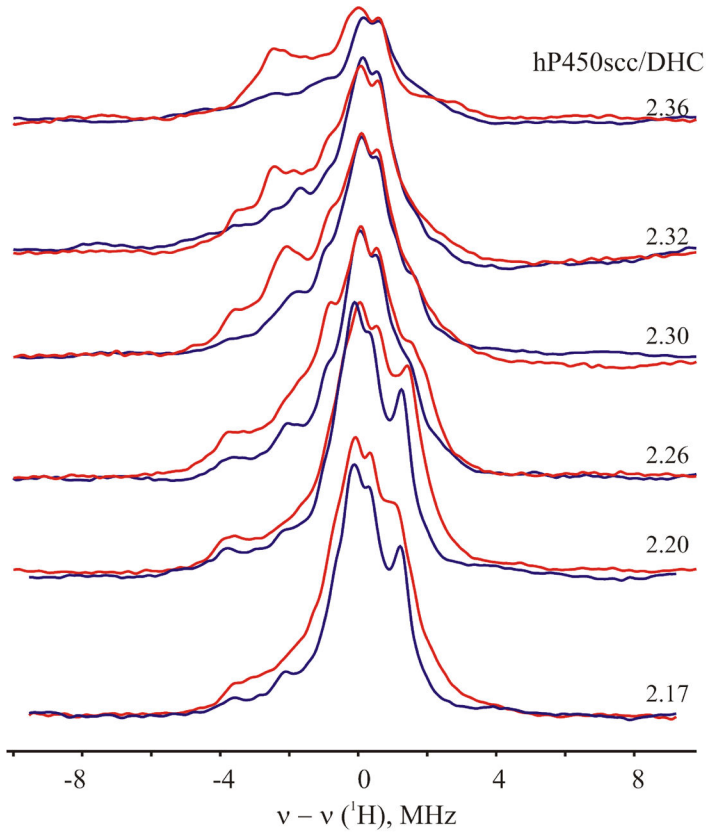


Figure 8.

2D frequency/field 35GHz ^{1}H CW ENDOR spectra of the cryoreduced ternary oxy ferrous P450scc – DHC complex in 50% glycerol/H_2O buffer pH 7.4 (red) and 50% d-3 glycerol/D_2O buffer pH 7.0 (blue) annealed at 180K for 1 min (g =2.36 species). Instrument conditions: T= 2K; modulation amplitude 2G; rf power 5W, scan rate 1MHz/s; frequency bandwidth 60kHz; average of 20 scans; microwave frequency, 34.89.

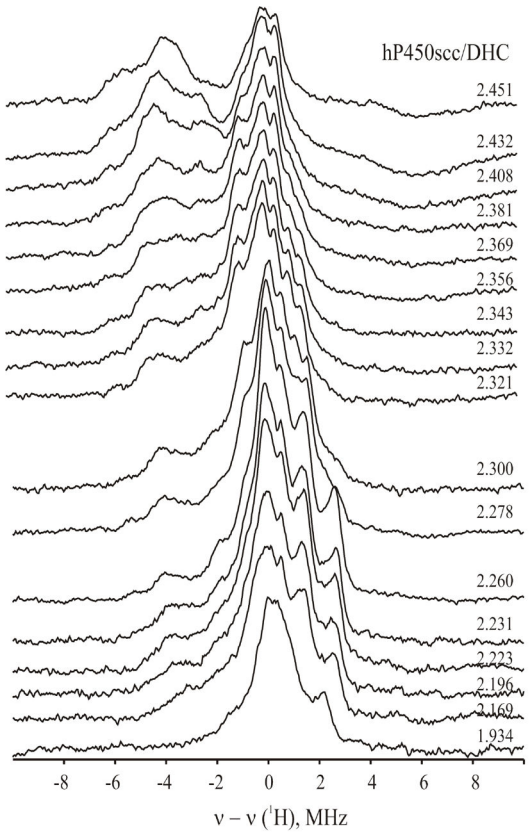
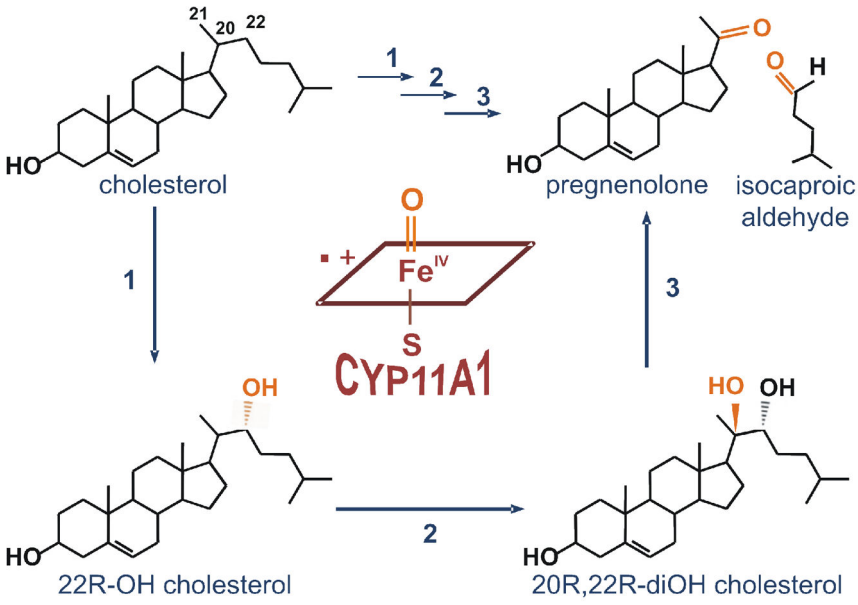


Figure 9.

2D frequency/field 35GHz ^{1}H CW ENDOR spectra of the cryoreduced ternary oxy ferrous P450scc – DHC complex in 50% glycerol/H_2O buffer pH 7.4 annealed at 210K for 1 min. Instrument conditions: T= 2K; modulation amplitude 2G; rf power 5W, scan rate 1MHz/s; frequency bandwidth 60kHz; average of 20 scans; microwave frequency, 34.89.



Scheme 1.

Cpd I as the active species in the metabolism of cholesterol to pregnenolone catalyzed by cytochrome P450scc.