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Reaction of Superoxide Radical with Quinone Molecules

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Abstract

When the superoxide radical $O_2^{\cdot -}$ is generated on reaction of KO_2 with water in dimethyl sulfoxide, the decay of the radical is dramatically accelerated by inclusion of quinones in the reaction mix. For quinones with no or short hydrophobic tails, the radical product is a semiquinone at much lower yield, likely indicating reduction of quinone by superoxide, and loss of most of the semiquinone product by disproportionation. In the presence of ubiquinone-10, a different species (I) is generated, which has the EPR spectrum of superoxide radical. However, pulsed EPR shows spin interaction with protons in fully deuterated solvent, indicating close proximity to the ubinquinone-10. We discuss the nature of species I, and possible roles in the physiological reactions through which ubisemiquinone generates superoxide by reduction of O_2 through bypass reactions in electron transfer chains.

Keywords

Superoxide; quinone; EPR; bc_1 complex; reactive oxygen species

INTRODUCTION

Many studies have focused on the generation of intermediate semiquinone radicals in intrinsic membrane proteins such as complexes I (NADH-ubiquinone oxidoreductase), II (succinate-ubiquinone oxidoreductase), and III (the cytochrome (cyt) bc_1 complex) of mitochondria and bacteria, and cyt bo_3 quinol oxidase from *Escherichia coli*.[1–9] Their main role is to serve as intermediates in the normal electron transfer chains, but these and complementary studies have also drawn attention to a role in pathology. In addition to its normal redox function, the semiquinones can participate in the one-electron reduction of O_2 to generate superoxide anion ($O_2^{\cdot -}$), the source of reactive oxygen species of importance in protein dysfunction, DNA damage, cellular pathology and aging.[10–14] A donor antioxidant role for the quinone couple is generally assumed.

Direct oxidation of hydroquinone by oxygen is slow, and spin-forbidden.[15] The redox reaction between semiquinone radicals and oxygen producing superoxide can be very rapid, but it is strongly dependent on occupancy of $SQ^{\cdot}$ at its site of generation, and the redox potential of the $Q/SQ^{\cdot}$ couple involved, and hence on the stability constant and factors modulating disproportionation.[13a-c] Superoxide radicals generate secondary radicals which are dangerous[13c], and excessive concentrations lead to the development of pathological

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states leading to carcinogenesis, atherosclerosis, chronic inflammation, and neurodegenerative disease.[11,14,16] The evolutionary history of enzymes involving intermediate semiquinone radicals dates back to the anaerobic era of the biosphere, but since the advent of cyanobacterial oxygen evolution some 2.5 billion years ago, aerobic organisms have adapted to the presence of oxygen by minimizing these deleterious reactions.[17] However, when function in the respiratory chain is compromised, radical intermediates can accumulate, leading to superoxide production and pathological effects. Since the reaction leading to superoxide production is reversible,

$$SQ^{\cdot -} + O_2 \rightleftharpoons Q + O_2^{\cdot -} \qquad \text{eq. 1}$$

quinones could play a protective role in consuming superoxide.[18]

Natural quinones are amphipathic, with a polar headgroup and hydrophobic tail. Biological functions of the isoprenoid tails are less discussed than the redox activities of the head groups. However, they vary significantly, and likely have evolved to fit particular physiological roles. Most obviously, the tail serves as a ‘lipid anchor’ to keep these molecules in the membrane. The strongly hydrophobic character and low solubility in water serve to partition quinones almost exclusively to the lipid phase, - a function of obvious importance in an aqueous environment of high relative volume (one version of the “bacterium in the Pacific ocean” problem).[19]

The reaction between $SQ^{\cdot -}$ and O_2 is generally considered to be second order. When the potential of the donor couple is suitably matched (as with the $SQ^{\cdot -}$ at the Q_o-site of the bc_1 complex), a direct electron transfer has been assumed. Since O_2 also partitions favorable into the lipid phase, co-partition would allow O_2 and $SQ^{\cdot -}$ to react effectively unless the latter were sequestered away from access to O_2.[13] Both local concentration and $SQ^{\cdot -}$ stability depend strongly on local environment, exploited in the evolution of catalytic sites, some aspects of which can be studied in model systems. The reaction of quinones with superoxide in dimethyl sulfoxide (DMSO) has often been used for generation of quinone anion-radicals.[20] In anhydrous DMSO, the superoxide radical created from KO_2 is quite stable, but it slowly disproportionates in the presence of water in the reaction: $2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$. In the presence of substituted 1,4-benzoquinones, the superoxide species disappears, and is replaced by semiquinone at lower concentration. In previous work, EPR spectra have shown only anionic $SQ^{\cdot -}$ radicals generated on reaction with KO_2 in anhydrous DMSO at room temperature. No evidence was found for formation of any paramagnetic oxygen species. In the present work we have studied the reaction of quinones having different lengths of isoprene chain with superoxide in a DMSO-water mixture. We report detection of a paramagnetic state that is not a simple $SQ^{\cdot -}$, and investigate its nature.

EXPERIMENTAL

Radicals were formed in frozen glassy mixtures of DMSO with water (80/20 volume %). In the absence of quinone, the reaction mixture after addition of KO_2 showed a pH of 13–14. For radical generation, the solution of the quinone in DMSO (~5×10^{-2} M final [Q]) was added to KO_2 (0.2 M final conc.) placed on the bottom of thin EPR tube. The sample was quickly cooled and frozen. Then the required amount of water was placed above the frozen quinone solution and also frozen. The tube was connected to vacuum line, and the dissolved oxygen gas was removed from the combined unmixed system. After pumping, the frozen sample was carefully melted so as to allow mixing to generate the superoxide substrate. The reaction of quinone with superoxide could be observed through the formation of O_2 bubbles. The sample was vigorously shaken and frozen again. The freeze and melt procedure was repeated several times until no appearance of gas bubbles was observed on melting. Then

the tube with the frozen reaction mixture was flame-sealed under vacuum and kept at 77 K. The instrumentation, pulse sequences and spectral processing for X-band continuous-wave (CW) EPR, 2-pulse Electron Spin Echo (ESE) (π/2-τ-π-τ-echo), 4-pulse 2D Electron Spin Echo Envelope Modulation (ESEEM) experiment (also called HYSCORE) (π/2-τ-π/2-t_1-π-t_2-π/2-echo), and Davies pulsed Electron Nuclear Double Resonance (ENDOR) were as previously described.[4–6]

RESULTS

Figure 1 shows field-sweep two-pulse Electron Spin Echo (ESE) spectra in the samples reacted under the same conditions with different quinones (see Experimental section). Field-sweep ESE generates a spectrum similar in shape to the absorption spectrum produced on integration of the normal continuous-wave EPR derivative. A narrow singlet line with width ~ 0.8–1 mT, typical of the anion-radicals of quinones, is observed in the samples with duroquinone or with ubiquinones having a short isoprene tail (geranyl, UQ_2) or without tail (UQ_0) at temperature T<90 K. In contrast the field-sweep ESE spectrum of the sample with UQ_{10} is a broad line with the width ~20 mT. The shape of this signal shows an axial g-tensor anisotropy with principal values $g_{\|}$ =2.108, $g_{\perp}$ =2.004 (species I). The ESE signal from species I is only observed at T<30 K. The spectrum did not change when isotopically labeled reagents such as d_6-DMSO, D_2O or $H_2{}^{17}O$ were used for the sample preparation, suggesting that the properties of the paramagnetic species were not significantly modified by interaction with solvent molecules.

Kinetic experiments show that in the absence of quinone, the superoxide, initially at ~0.2 M, decays slowly, presumably by disproportionation, with $t_{½}$ ~ 5 min, consistent with the low rate constant previously determined at the high pH measured.[21] However, when quinone was present, a more rapid decay that is complete by the time of the first measurement (~1 min) was observed. The residual radical species is then either the anionic $SQ^{\cdot -}$, or species I, depending on the nature of the quinone, at ~0.02 M. The disappearance of a large fraction of the radical species suggests that this more rapid reaction also involved a disproportionation, possibly of $SQ^{\cdot -}$.

In order to obtain additional structural information about the environment of species I formed in the sample with UQ_{10} we have applied high-resolution EPR techniques, ENDOR and 2D ESEEM (HYSCORE).

The ENDOR spectrum of species I in DMSO/H_2O shows one splitting ~7–8 MHz with broad lines, and multiple splittings of smaller value. In contrast to the field-sweep spectrum, deuteration of water or DMSO influences the shape of the corresponding ^1H ENDOR spectra, indicating that the interactions of the unpaired electron spin with the protons of water and DMSO molecules significantly contribute to the spectra. On the other hand, the proton splittings at ~1.5 MHz and 7 MHz were still observed in the spectrum of species I prepared in fully deuterated d_6-DMSO/D_2O solvent.

Two-dimensional HYSCORE spectra were obtained in the same solvents. In the absence of deuterium nucleus the ^1H HYSCORE spectrum shows two pairs of cross-peaks $\mathbf{1_A}$ and $\mathbf{2_A}$. Only one pair of cross-peaks $\mathbf{1_B}$ remains in the solution containing deuterated water. However, two pairs of cross-peaks $\mathbf{1_C}$ and $\mathbf{2_C}$ were present in the solution with deuterated DMSO, indicating their source as exchangeable water protons suitable for formation of hydrogen bonds with the oxygen atoms of species I. Comparison of the spectra A-C allows us to conclude that cross-peaks $\mathbf{1_B}$ and $\mathbf{2_C}$ overlap significantly and thus form cross-peaks $\mathbf{2_A}$ in the spectrum (A). In the fully deuterated solvent, observation of the proton peaks was largely suppressed via cross-suppression effect due to the presence of very intensive

deuterium signals.[22,23] However, we were able to detect the presence of two cross-peaks $\mathbf{1_D}$ corresponding to the proton hyperfine coupling ~1.5–2.0 MHz (also seen in ENDOR spectrum) by increasing time $\boldsymbol{\tau}$ between the first and second pulses up to 400 ns, when the intensity of deuterium peaks is significantly suppressed.

The linear fitting of the cross-peaks $\mathbf{1_C}$ and $\mathbf{2_C}$ in the coordinates $(\nu_1)^2$ *vs.* $(\nu_2)^2$ has shown that they are parts of extended cross-ridges produced by the proton(s) with the isotropic a = ±1.7 MHz and anisotropic T= −/+10.6 MHz components of the axial hyperfine tensor with principal components (a+2T, a-T, a-T).[24] The parts of these ridges around the spectral diagonal is suppressed because the intensity varies with τ as $\sin(\pi\nu_\alpha\tau)\ \sin(\pi\nu_\beta\tau)$.[25] Simulations of HYSCORE spectra from two protons, with equivalent but differently oriented hyperfine tensors, have shown that two protons with anisotropic components as large as this would produce intense combination peaks. These, however, were not observed in our spectra. Thus we can conclude that species I forms only one hydrogen bond under our conditions. The spectra show an additional interaction with the DMSO protons with estimated $|T|$ ~ 4.4 MHz indicating their presence in the solvation shell, but not in H-bonds.

DISCUSSION

The g-tensor of species I is similar to the tensor reported previously for the superoxide radical $O_2^{\cdot-}$ in frozen solutions of potassium superoxide in DMSO/water mixtures with the same relative content of mixed components.[26] EPR spectra for $O_2^{\cdot-}$ have been obtained under a wide variety of conditions and it is clear that the g-tensor components, and especially g_z (z is the molecular axis), are markedly dependent on the environment. It is concluded that strong hydrogen bonds lift the degeneracy of the x-orbitals, with solvation being effectively confined to one of these orbitals.

The HYSCORE spectra show the presence of water proton(s) with relatively high anisotropic coupling $|T|$=10.6 MHz. A value close to this, $|T|$= 9.8 MHz, was reported for $O_2^{\cdot-}$ interacting with a hydroxyl proton on MgO surface.[27] Anisotropic hyperfine couplings are typically $|T|$~3 MHz for protons hydrogen bonded with anion-radicals of quinones.[28–30] In anion-radicals, the π unpaired spin density ρ_O at the carbonyl oxygen is about 0.2.[31] In contrast the spin density on $2p_\pi^x$ of each oxygen is ~ 0.495 in $O_2^{\cdot-}$.[32] This difference between spin densities would explain the high value of $|T|$~10 MHz for the anisotropic coupling to the proton H-bonded with $O_2^{\cdot-}$ as resulting from dipole-dipole interaction with unpaired spin equally delocalized over two oxygens. The value of the g-tensor and value of the coupling with water protons presumably involved in H-bonding found for species I allowed us to conclude that it is basically the $O_2^{\cdot-}$ radical.

A surprising result of this study is the observation of proton couplings in the species I sample with fully deuterated solvents. There is only one possible source of non-exchangeable protons in this sample, i.e. UQ_{10} molecule. Therefore, the observation of proton couplings up to 7 MHz indicates either a close proximity of species I to the quinone molecule or its tail, or the specific stabilization of species I near the quinone molecule. For example, the formation of K^+(crown) $O_2^{\cdot-}$---HOH units with g_z~2.1 in the presence of crown ether in a solution of KO_2 in DMSO/H_2O has been studied.[26] The formation of a similar species could be suggested in the presence of the quinone in solution but it is stabilized only in the case of UQ_{10}, perhaps by an interface between hydrophobic and hydrophilic phases. Alternatively, the data might be interpreted as indicating a covalent connection between the superoxide species and the quinone, accompanied by the transfer of the unpaired spin density onto the quinone molecule, including its proton-containing substituents in a quinone-peroxyl adduct.

A reversible binding of semiquinone with molecular oxygen was recently reported and studied in detail.[33] The reaction goes through the formation of the peroxyl paramagnetic adduct (Scheme I).[33] The effectiveness of the reaction depends on the provision of hydrogen bond donors to the semiquinone radical in the reaction phase. Clearly, such a reaction provides an alternative pathway for formation of superoxide.

One could suggest stabilization of this intermediate in our case. However, it is well known that the g values for ROO· radicals are largely independent of the nature of the attached R group, and commonly observed values g_1=2.035, g_2=2.008 and g_3=2.003 are inconsistent with the g-tensor observed in this work for species I.[34,35]

The species I observed above has not been reported in the EPR spectra from biochemical studies (superoxides are rapidly removed by superoxide dismutase or by reaction with cytochrome *c*), and no $ROO^{\cdot}$ species that might correspond to the intermediate above has been observed, so it is unlikely to be a major product. However, the $SQ^{\cdot -}$ generated at the Q_o-site of the bc_1 complex, for example, is seen only in the absence of O_2,[36] so we cannot exclude the possibility that when oxygen is present the reaction for formation of superoxide might proceed through an oxygen adduct as an intermediate that rapidly decays to products. Since the biological interest is in avoiding superoxide production, a more practical consideration is how flux through this pathway could be minimized. From this perspective, a mechanism that limits collision of $SQ^{\cdot -}$ with O_2, and which does not provide a favorable H-bonding partner to catalyze the reaction would be anticipated. In the normal forward chemistry at the Q_o-site, the $SQ^{\cdot -}$ is generated from an enzyme-substrate complex in which QH_2 is likely stabilized by H-bonds from the protein to both carbonyls.[16,37] However, the $SQ^{\cdot -}$ product of the first electron transfer likely dissociates from these partners as the reaction proceeds.[36,38] and is then rapidly removed, and the protein configuration likely changes so as to insulate any accumulating $SQ^{\cdot -}$ from the aqueous exterior.[39] The formation of $SQ^{\cdot -}$ is also strongly endergonic, and these features all likely contribute to limiting any collisional complex either for direct electron transfer or formation of an intermediate adduct.

Further work using both UQ_{10}, ^{13}C-labeled in either head group or tail, and ^{17}O labeled KO_2 and UQ_{10}, is needed to provide data allowing firm conclusions about the interaction of superoxide with UQ_{10} in this system.

Acknowledgments

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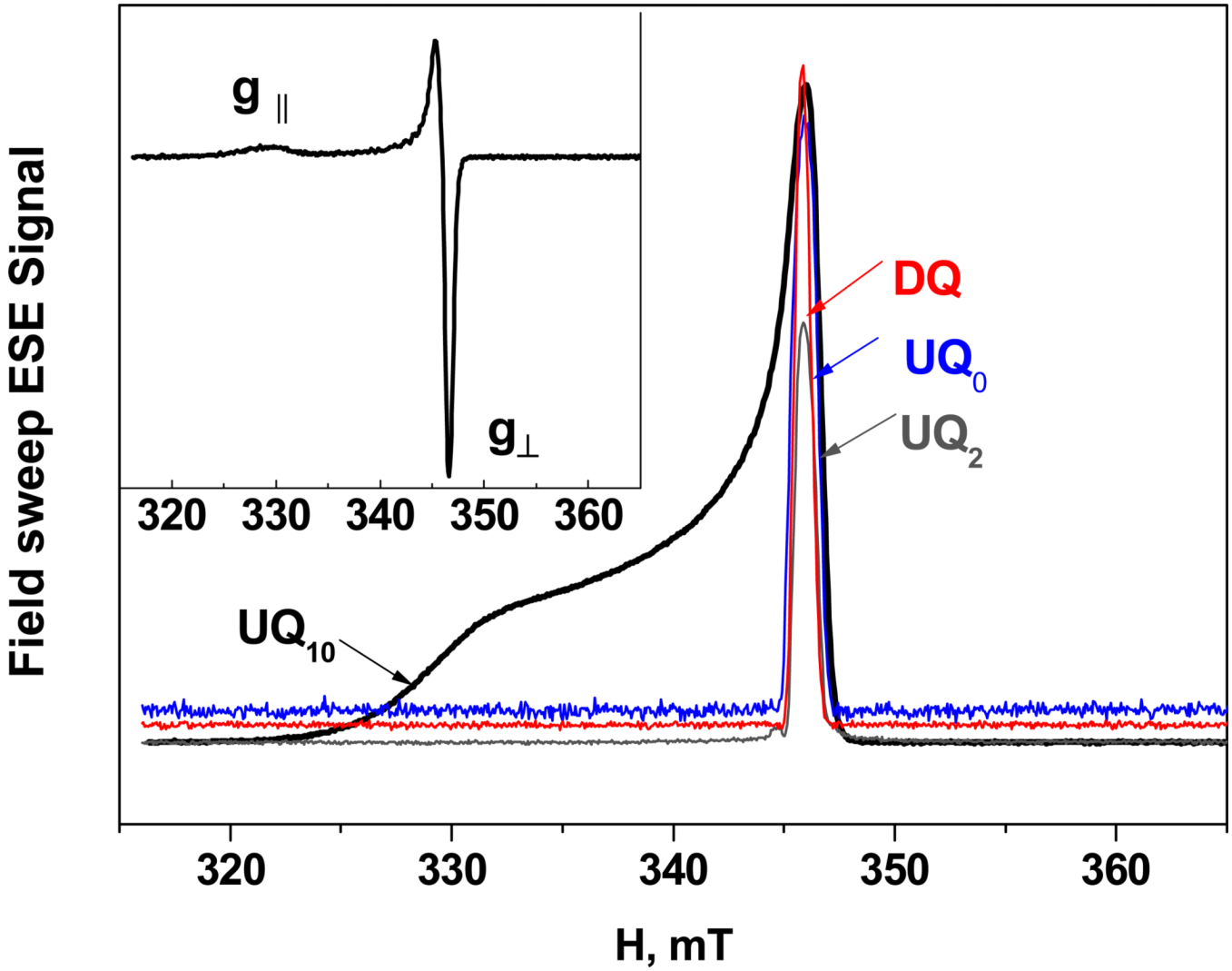


Figure 1.

Field-sweep X-band two-pulse ESE spectra of the radicals formed in the reaction of substituted benzoquinones with KO_2 in DMSO/H_2O mixture. Time between first and second microwave pulses is 400 ns, microwave frequency is 9.707 GHz, and temperature is 20 K. The insert shows the CW EPR spectrum (corresponding to the first-derivative of the field-sweep ESE spectrum) of the radical (species I) formed in the presence of UQ_{10} in DMSO/H_2O mixture. Abbreviations are: DQ, duroquinone; UQ_N, ubiquinone, with the subscript *N* indicating the number of isoprene units in the tail.

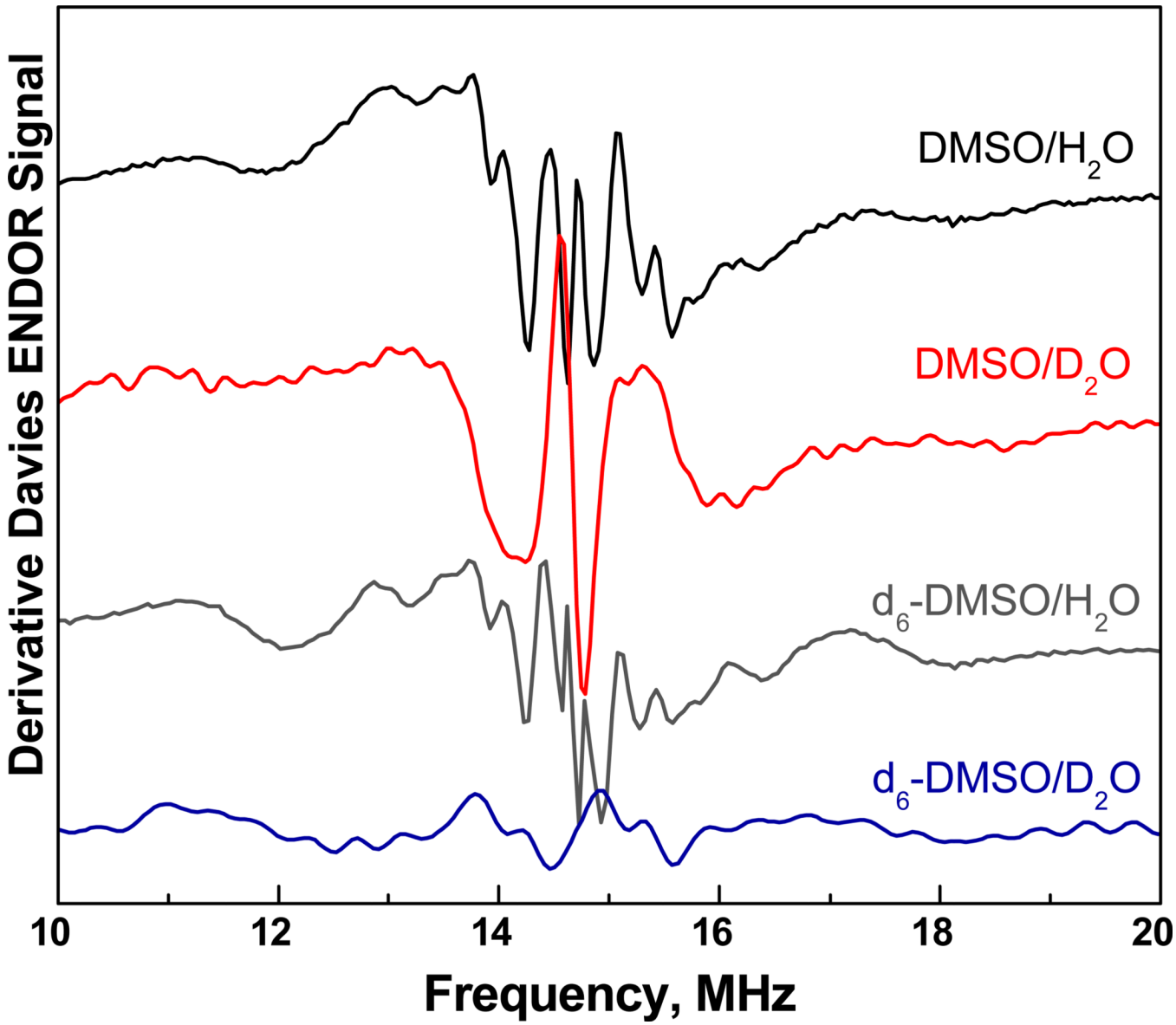


Figure 2.

^{1}H pulsed ENDOR spectra of the species I in DMSO/H_2O mixtures with different content of deuterated compounds. The spectra were measured using the Davies pulse sequence (π-t-π/2-τ-π-τ-echo), with a radiofrequency pulse applied during the time interval t, the length of microwave pulses t_{mw}=80, 40, and 80 ns, respectively, τ=400 ns, length of a radiofrequency pulse - 8 μs, microwave frequency is 9.6992 GHz, magnetic field is 344.5 mT (near $g_\perp$), temperature is 20 K.

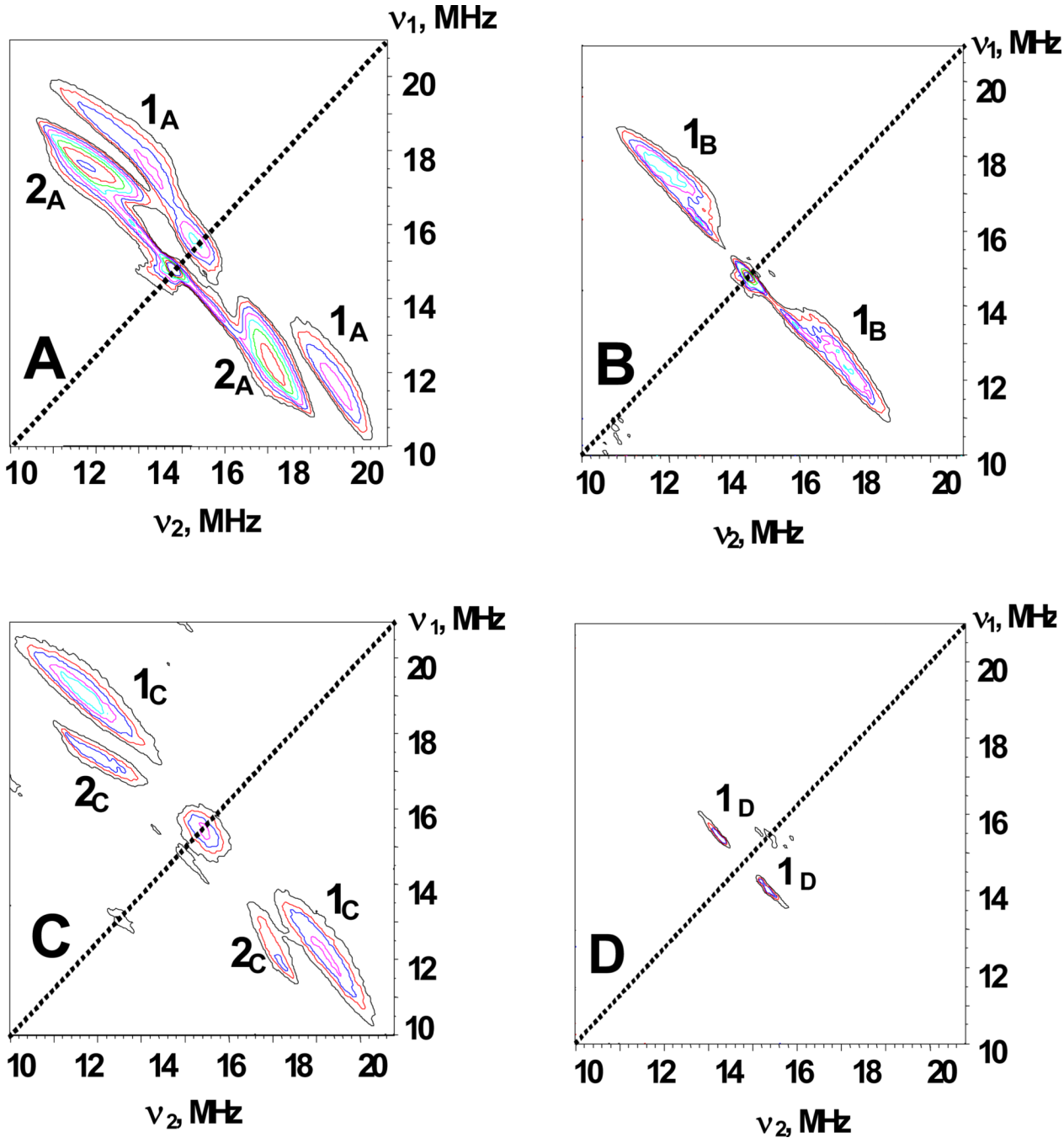
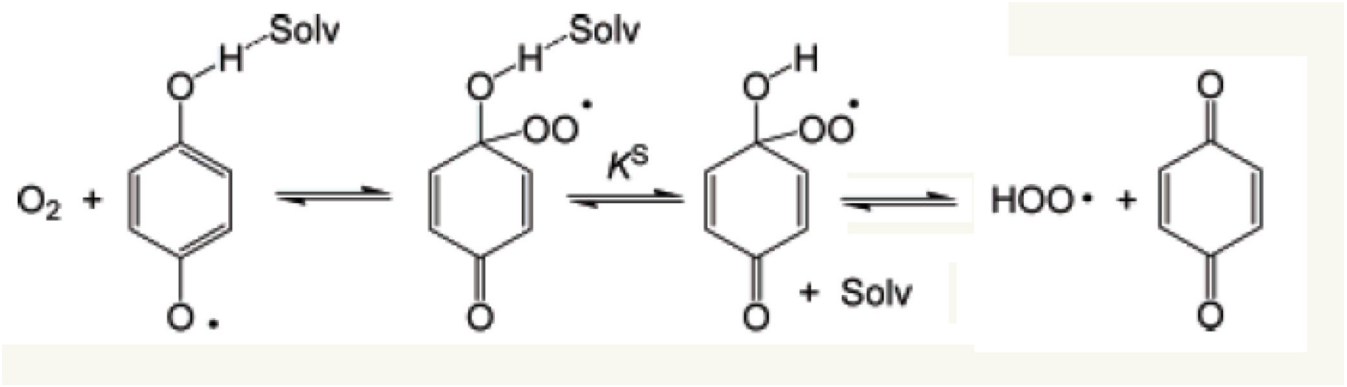


Figure 3.

^{1}H HYSCORE spectra of the species I in the following mixtures: (A), DMSO/H_2O; (B), DMSO/D_2O; (C), d_6-DMSO/H_2O; (D) d_6-DMSO/D_2O. In (A-C), τ=136 ns; in (D), τ=400 ns, magnetic field is 346 mT (A-C) (near $g_\perp$), microwave frequency is 9.670 GHz, temperature is 20 K.



Scheme I.