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Reactions of Superoxide Radicals with Curcumin: Probable Mechanisms by Optical Spectroscopy and EPR

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Reactions of superoxide-crown ether complex with curcumin have been studied in acetonitrile. Optical absorption spectra showed that curcumin on reaction with superoxide forms a blue color intermediate absorbing at 560 nm, which subsequently decayed in a few hours with the development of the absorption band corresponding to the parent curcumin. The regeneration was 100% at low superoxide concentrations (1:1, or 1:2 or 1:3 of curcumin:superoxide) but reduced to 60% at high superoxide concentration (>1:5). The regeneration of curcumin is confirmed by HPLC analysis. Stopped-flow studies in acetonitrile following either the decay of parent curcumin at 420 nm or formation of 560 nm absorption have been used to determine the rate constant for the reaction of superoxide with curcumin. EPR studies confirmed the disappearance of characteristic superoxide signal in presence of curcumin with the formation of new featureless signal with $g = 2.0067$. Based on these studies it is concluded that at low superoxide concentrations curcumin effectively causes superoxide dismutation without itself undergoing any chemical change. At higher concentrations of superoxide, curcumin inhibits superoxide activity by reacting with it.

Keywords: Curcumin; Superoxide; Absorption spectra; Free radical; EPR spectroscopy

INTRODUCTION

Curcumin (1,7-bis(4-hydroxy 3-methoxy phenyl)-1,6-heptadiene-3,5-dione) is a natural phenol found as a major pigment in the Indian spice turmeric. It shows remarkable pharmacological activity including anti-inflammatory, anti-carcinogenic, and antiproliferative activity.[1–9] Curcumin acts as a lipoxygenase substrate and also an inhibitor of CoX-1 and CoX-2 enzymes.[4,5] It is also an inducer of hemeoxygenase-1 in vascular endothelial cells.[6] It is considered as a potential chemo preventive agent and the clinical trials in this direction are in different stages.[1,2,7–9] In addition to all this it exhibits remarkable antioxidant activity.[9–11] One of the important factors responsible for all the activity of curcumin is its ability to scavenge reactive oxygen and nitrogen free radicals.[10–16] The active sites available for the free radical attack are the two phenolic OH groups (4-hydroxy 3-methoxy phenyl) and the methylenic group of the β-diketone (heptadiene-dione) moiety.[15] From biochemical experiments, it has been shown that curcumin scavenges hydroxyl radicals, superoxide radicals and peroxyl radicals. Using pulse radiolysis and flash photolysis techniques, the rate constants for the reactions of curcumin with perxoyl, alkoxyl, superoxide and other radicals have been determined.[13–15] Superoxide radical is one of the reactive oxygen free radicals generated biochemically under physiological and pathophysiological conditions. It is produced by the enzymes xanthine oxidase, NADPH oxidase etc.[17] In the human body, out of the total amount of oxygen available <1% is converted into superoxide radicals by the mitochondrial respiratory chain.[18,19] Under normal conditions its effect is reduced by the presence of the enzyme superoxide dismutase (SOD).[17] Superoxide radicals are not very reactive towards biomolecules but they are converted into

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powerful oxidizing radicals in presence of trace metals.[17] Thus, one of the most important characteristics of an antioxidant is its ability to neutralize the effect of superoxide radicals. There are also some reports in the literature on the reactions of curcumin with superoxide.[14,16,20] Using spin-trapping techniques Das and Das showed that curcumin quenches singlet oxygen but from the same studies, the authors reported that curcumin is not a very effective quencher of superoxide anion.[16] Chignell *et al.* have reported that excited curcumin molecules can act as a source of oxygen radicals.[21] Recently, Toniolo *et al.* have showed that curcumin reacts effectively with electrochemically-generated superoxide and the reaction is mainly by the proton loss from curcumin to superoxide.[20] In this paper, we present the reactions of superoxide ion with curcumin in acetonitrile. The superoxide ion is solublized by complexing with crown ether. Acetonitrile being an aprotic solvent is best suited for such superoxide reactions. The dynamics of the reaction was followed using absorption spectroscopy, stopped-flow spectrometer and EPR spectroscopy.

EXPERIMENTAL PROCEDURES

Curcumin, potassium superoxide (KO_2), Dicyclohexano-18-crown-6-ether, were purchased from Sigma/Aldrich, USA. Spectrograde acetonitrile and dimethyl sulfoxide from Spectrochem India, Mumbai were used as received. Superoxide-crown ether complex was prepared according to the procedure given in references [22,23]. Potassium superoxide was weighed quickly in a previously weighed sealed tube and was quickly added to equivalent moles of 18-crown-6-ether in acetonitrile solvent. It was stirred in sealed condition for 30 min. In order to minimize the effects of light on these solutions, all the experiments were carried out in dark. The concentration of superoxide was determined by using the extinction coefficient of $1460\,M^{-1}\,cm^{-1}$ at 255 nm.[24] Absorption spectrophotometric studies were carried out using JASCO V-530 spectrophotometer. Stopped-flow studies were carried out using SX-18 MV stopped-flow spectrometer from Applied Photophysics, UK in the single mixing mode. Here the two mixing syringes contain acetonitrile solutions of superoxide-crown ether complex and curcumin separately and the two solutions were mixed in the stopped-flow cell. The absorption changes at any suitable wavelength after the mixing were monitored as a function of time. The dead time of the instrument is 1.3 ms. EPR spectra were recorded on Bruker ESP 300 spectrometer operated at X-band frequency (9–10 GHz) using 100 KHz field modulation. DPPH sample was used as a field marker. The spectra were recorded at 77 K using a liquid nitrogen dewar insert.

RESULTS

The absorption spectrum of 20 μM curcumin in acetonitrile shows broad absorption in the wavelength region from 300 to 500 nm with maximum absorption at 420 nm (Fig. 1a). It was found to be

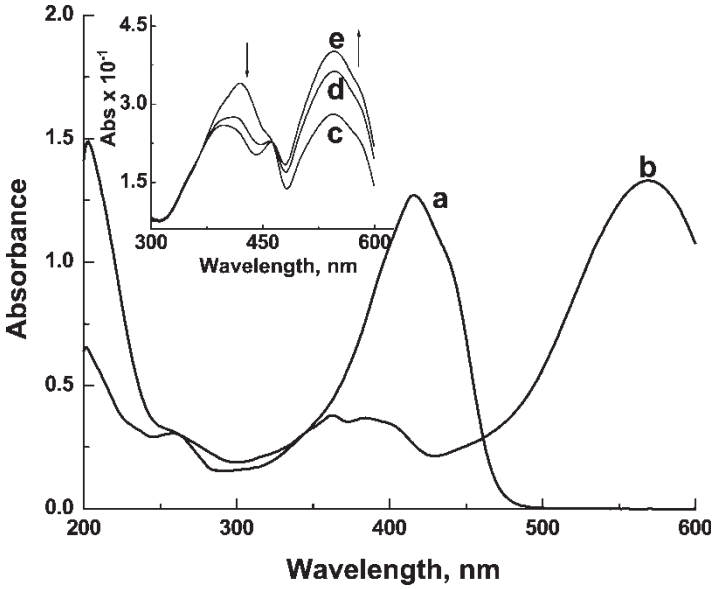


FIGURE 1 (a) Absorption spectrum of 20 μM curcumin in acetonitrile and (b) Absorption spectrum of 20 μM curcumin on mixing with 190 μM KO_2-crown ether complex in acetonitrile. Inset shows time resolved spectra at time points $c = 1.250$ s, $d = 12.5$ s, $e = 50$ s after mixing 20 μM curcumin with 160 μM KO_2-crown ether complex in acetonitrile in the stopped-flow cell.

unaffected by the addition of crown ether. When superoxide solution (30–190 μM) was mixed with curcumin, the solution turned intense blue instantly and the absorption at 420 nm decreased with the formation of a new species having absorption maximum at 560 nm (Fig. 1b). The intensity of the 560 nm absorption increased with increasing concentration of superoxide. When the molar ratio of curcumin and superoxide was more than 1:8 (Curcumin:KO_2) the absorption at 420 nm disappeared completely and no further increase in the 560 nm absorption was seen. However, on purging the solutions with dry argon the absorbance at 560 nm was found to increase by 20%. The 560 nm band is attributed to the formation of deprotonated curcumin, by independently following the absorption spectral changes in crown ether complexed curcumin in acetonitrile as a function of pH. The increase in OD in absence of oxygen indicates that oxygen reacts with the ionized curcumin.

Stopped-flow studies between superoxide and curcumin were carried out in acetonitrile to determine the kinetics of the reaction. Inset of Fig. 2 shows absorption-time plots indicating the decay of parent curcumin absorption at 420 nm after mixing with superoxide. Followed by the decay of 420 nm absorption is the build up of 560 nm absorption. By fitting the growth and decay traces to single exponential function, the rate constants were determined, which were found to increase with increasing concentration of superoxide and curcumin. From the linear plot for increase in the rate constant with superoxide concentration (Fig. 2), the bimolecular rate constant for the reaction of superoxide as crown ether complex with curcumin in acetonitrile was determined to be 4.7 $(\pm 1.1) \times 10^2\,M^{-1}\,s^{-1}$. Earlier using pulse radiolysis technique, the rate constant in aqueous solutions was determined to be $4.6 \times 10^4\,M^{-1}\,s^{-1}$.[14] The crown ether complexed superoxide shows much less reactivity as it is encapsulated inside the crown ether and therefore participates in the reactions much slowly. Time resolved spectra (inset of Fig. 1) showed the spectral changes corresponding to the simultaneous decrease in the 420 nm absorption and increase in the 560 nm absorption with time, along with an isosbestic point at 460 nm. The 560 nm absorption showed only a marginal decay in the maximum detectable time scale of 1000 s. Therefore, no kinetic studies could be performed for the decay of 560 nm absorption using stopped-flow spectrometer. However, these kinetic studies were performed on a spectrophotometer by following absorption changes over period of few hours.

The blue colored solution formed after the reaction of superoxide with curcumin, absorbing at 560 nm, slowly turned into red and finally became yellow over a period of 3 h. Figure 3 gives the time dependent changes in the absorption spectrum of the reaction product formed by the reaction of 20 μM curcumin and 70 μM superoxide-crown ether complex in acetonitrile. It can be seen that the 560 nm absorption decreased gradually with simultaneous build up of the 420 nm absorption reaching maximum after about 3 h. This indicates

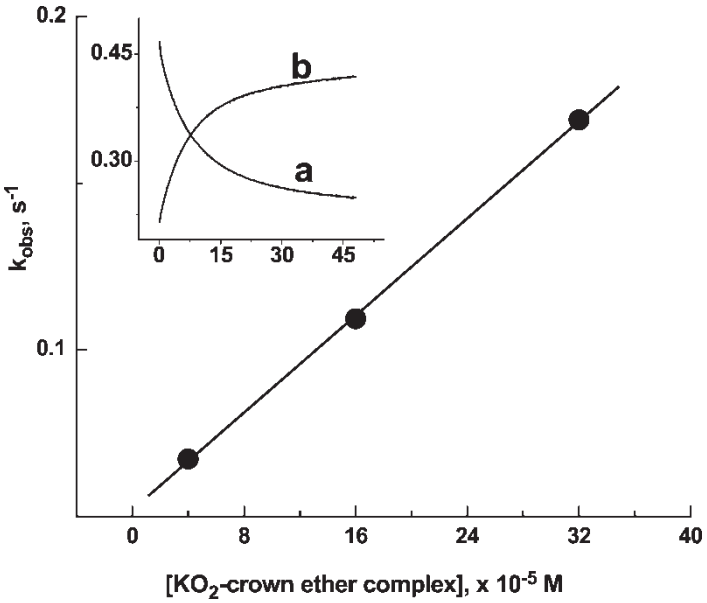


FIGURE 2 Variation of observed rate constant obtained either by the decay of 420 nm absorption or by the formation of 560 nm absorption on reaction of 20 μM curcumin with superoxide as a function of superoxide concentration (40–320 μM). Inset: absorption-time plots showing (a) decay of parent curcumin absorption at 420 nm (b) formation of the product absorption at 560 nm after mixing 20 μM curcumin with 160 μM superoxide.

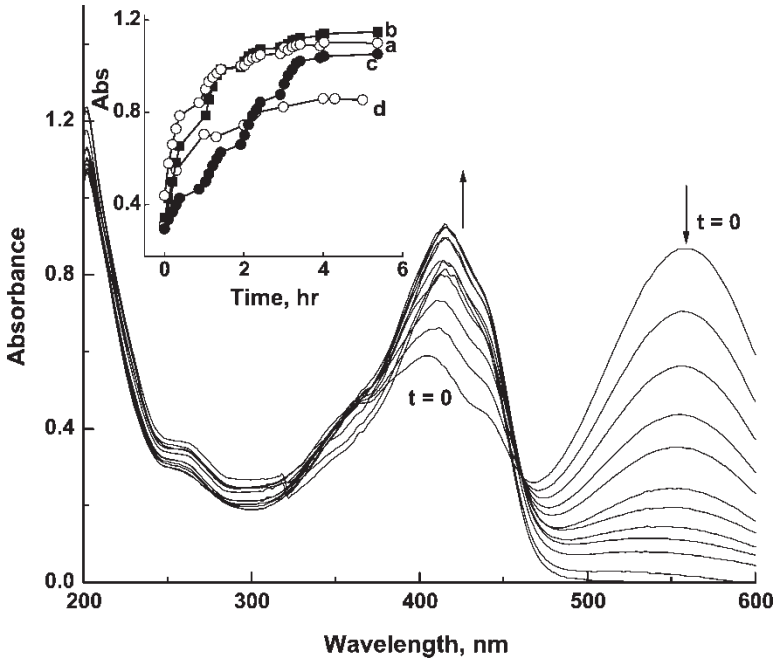


FIGURE 3 Time resolved spectra showing the decay of the blue colored species (560 nm) and simultaneous regeneration of parent curcumin (420 nm). The solution is prepared by mixing 20 μM curcumin and 70 μM superoxide solution in acetonitrile. Inset shows percentage of curcumin regenerated at different concentration of superoxide, (a) 30 μM, (b) 50 μM (c) 70 μM and (d) 114 μM.

that the new intermediate formed on reaction of superoxide with curcumin, is subsequently converted back to curcumin. From the increase in absorbance at 420 nm, it is found that the amount of curcumin regenerated depends on the concentration of superoxide also. Inset of Fig. 3 shows the amount of curcumin regenerated at different concentrations of superoxide. At low concentration of superoxide (30–70 μM), when the ratio of curcumin to superoxide was 1:1 or 1:2 or 1:3, the regeneration of curcumin is almost 100%. The results indicate that at this stoichiometry curcumin reacts by proton loss as observed in case of other antioxidants like α-tocopherol and the 560 nm band corresponds to the deprotonated curcumin, which slowly picks up the dissolved protons from acetonitrile to regenerate the parent curcumin. However, the regenerated curcumin decreased with increasing superoxide concentration and became nearly 60% at higher concentration of superoxide, i.e. 114 μM (ratio of curcumin to superoxide is >1:5). The decrease in the percentage regeneration of the parent at higher concentration of superoxide suggests that the reaction with superoxide causes chemical changes in ionized curcumin producing other radical species, which may undergo radical–radical reactions to produce different reaction products. The regeneration of curcumin was also confirmed by HPLC analysis using PCX column and acetonitrile as mobile phase (flow rate = 0.6 ml min^{-1}), where the regenerated solution showed curcumin peak at the characteristic retention time of the standard (6.55 min).

When small amount of water was added to the reaction product, the blue color disappeared instantly and the parent curcumin was regenerated completely. This may be due to the fact that water being a protic solvent, the protonation reaction is accelerated and the regeneration of curcumin is instantaneous. Similar experiments were carried out in DMSO. Here also a blue colored solution was formed initially but the regeneration to curcumin was observed only after a very long time (>10 h). DMSO is aprotic like acetonitrile but is more polar than acetonitrile. It is also an excellent acceptor of protons due to the presence of lone pair of electrons on DMSO oxygen and which may have delayed the reprotonation process significantly.[25]

We have also tested the reaction of superoxide with dimethoxy curcumin. In this compound the phenolic OH group is blocked by methylation. When acetonitrile solution of dimethoxy curcumin was mixed with superoxide solution no blue coloration or change in the absorption spectra was noticed. Even after increasing the superoxide concentration to 1 mM, only a decrease of 7% in the absorbance at 420 nm (parent absorption) was observed. These studies gave a direct indication that the phenolic OH group participates in the formation of blue color solution and also in reaction with superoxide.

EPR SPECTRAL STUDIES

EPR spectral studies on the reaction product of superoxide ion with curcumin were carried out to know the probable mechanism of the reaction. Inset of Fig. 4 (spectrum *g*) shows the EPR spectrum of 3.4 mM superoxide solution in acetonitrile at 77 K. It showed axially symmetric spectrum (with $g_{\|} = 2.0867$ and $g_{\perp} = 2.0053$) characteristic of superoxide ion and the *g* values of superoxide ion agreed well with that in acetonitrile solution.[24] The features of superoxide signal in the ESR spectrum disappeared completely after the addition of 0.8 mM curcumin in acetonitrile, suggesting that curcumin reacted completely with superoxide ion. This blue colored solution was immediately frozen and the EPR spectrum was recorded at 77 K, which showed a new signal without any hyperfine structure corresponding to a *g* value of 2.0067 (spectrum a of Fig. 4). This solution was brought to room temperature and the EPR spectra recorded at 77 K after time intervals of 20, 30, 45, 60 and 80 min (Spectra *b*, *c*, *d*, *e* and *f*, respectively, of Fig. 4). During the course of reaction, the color of the solution changed from intense blue to dark red and finally yellow. The intensity of the new EPR signal decreased exponentially as the regeneration of curcumin progressed. No HO_2 radical spectrum could be detected probably due to its short lifetime. Lack of any hyperfine structure in the EPR signal made it difficult to predict the exact nature of the curcumin radical. Bors *et al.* have reported EPR spectrum of the phenoxyl radical of curcumin where only a weak, broad, featureless spectrum is reported.[26] Comparing the nature of the spectra (Fig. 4a) with that by Bors *et al.* it is assumed that the reaction of superoxide with curcumin may have produced similar phenoxyl radicals. Although, it is not possible to find exact nature of the product, the EPR studies gave direct evidence that curcumin acts as a scavenger of superoxide ion and neutralizes the radical by converting to non-radical species.

DISCUSSION

Curcumin is a well-known lipid soluble antioxidant with potential of scavenging several reactive oxygen free radicals. Its reactions with many oxidizing free radicals have been studied in detail using steady state, biochemical and time resolved methods. The reactions of superoxide anion

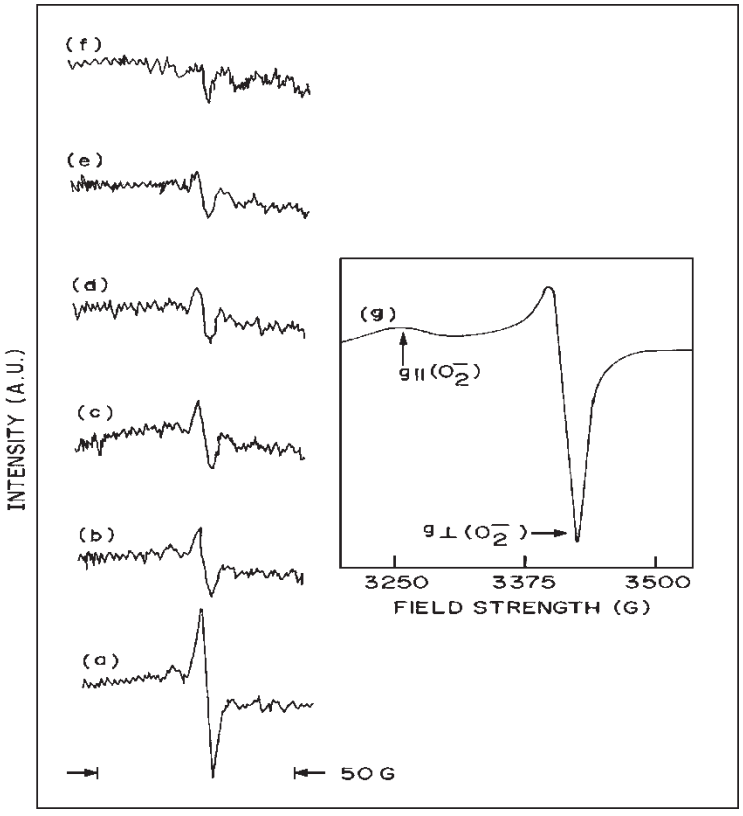
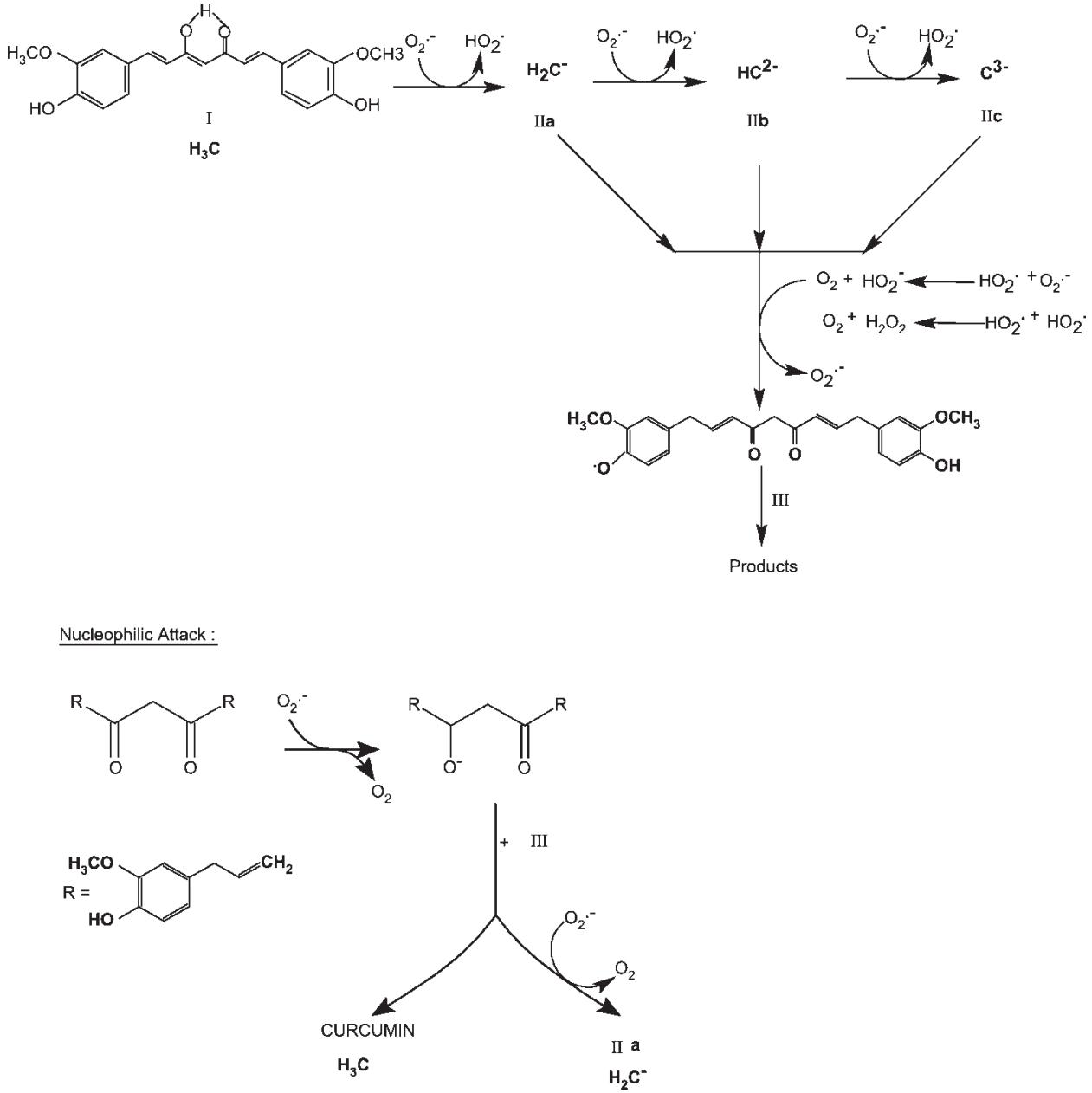


FIGURE 4 EPR spectra of the radical generated from the reaction of 0.8 mM curcumin and 3.4 mM KO_2-crown ether complex in acetonirile at 77 K. (a) Immediately (b) 20 min, (c) 30 min, (d) 45 min, (e) 60 min and (f) 80 min after mixing. Inset: (g) shows the characteristic spectra of 3.4 mM superoxide in acetonitrile at 77 K.

with curcumin have been the subject of debate, and so far many of the results were based on indirect studies. Methods based on enzymatic production of superoxide may also be due to the inhibition of superoxide production. Hence it is necessary to follow the studies employing methods based on direct reaction of superoxide with curcumin. In this paper, superoxide anion as potassium salt is stabilized (with the help of crown ether and an aprotic solvent like acetonitrile) for sufficiently long time. Such reactions can be followed over a period of a few hundreds of seconds. Superoxide reacts with substrates either by oxidation or reduction. During oxidation it is converted to hydrogen peroxide and the potential for such process is 1.05–1.06 V vs NHE.[27–29] During the reduction it is converted back to oxygen and the reduction potential for such process is 0.33 V vs NHE.[27] Some reports in the literature indicate that curcumin is not reactive towards superoxide,[16] while some other reports suggest that curcumin can generate superoxide under photo excitation conditions.[21] Using pulse radiolysis, it has been shown that in aqueous solutions curcumin can scavenge superoxide with bimolecular rate constants of 4.7 $(\pm 1.1) \times 10^2\,M^{-1}\,s^{-1}$ in acetonitrile and $4.6 \times 10^4\,M^{-1}\,s^{-1}$ in aqueous solutions, which is comparable to that of well-known antioxidants like α-tocopherol.[16,27,28] Recently, Toniolo *et al.* reported that curcumin exhibits electro catalytic activity on reacting with superoxide and the reaction proceeds by proton loss only.[20] In the present paper, we studied the reaction of superoxide solublized as crown ether complex with curcumin using optical spectroscopy and EPR spectroscopy. The results show confirmatively that curcumin reacts with superoxide radicals. It has been reported in the literature that in solvents like acetonitrile superoxide behaves like a strong base and reacts with substrates by abstracting protons. Curcumin has three ionizable protons (three pK_a) corresponding to the two phenolic OH groups and methylenic CH_2 group.[15] Thus one molecule of curcumin can donate protons to three superoxide molecules converting superoxide into hydroperoxide radicals. The hydroperoxide radicals undergo disproportionation producing less reactive hydrogen peroxide and oxygen. The blue colored intermediate produced during this reaction is attributed to the formation of deprotonated curcumin. This ionized curcumin can slowly pick up protons dissolved in acetonitrile converting the deprotonated curcumin back to parent curcumin in 3 h time. Accordingly, the regeneration of curcumin was 100% when the stoichiometry of superoxide is up to three times that of curcumin. The complete regeneration of curcumin at low superoxide concentrations prompted us to suggest that at low superoxide concentration curcumin is very effective as a catalytic agent like SOD, which enhances the dismutation of superoxide to convert into less reactive species such as hydrogen peroxide. However, the regenerated curcumin decreased with increasing superoxide concentrations and it decreased to almost 60% when the stoichiometry was 1:5 indicating some radical–radical reactions leading to the loss of parent curcumin. The complete loss of superoxide by reaction with curcumin was confirmed by EPR studies, where the characteristic EPR spectrum disappeared by the addition of curcumin. The new signal formed in such reaction is featureless and matches qualitatively with that of curcumin phenoxyl radical. Based on these observations, we propose that the reaction between curcumin and superoxide ion proceeds not only by proton loss but other free radical processes such as hydrogen abstraction and nucleophilic addition also take place and are responsible for the overall reactivity. Accordingly, the possible mechanism for the neutralization of superoxide radicals by curcumin and its regeneration is given in Scheme 1 and explained below.

Superoxide radicals abstract three protons successively from curcumin (I) to give its phenoxide ion (IIa, IIb, IIc) and HO_2 radicals. The HO_2 radicals undergo disproportionation and also can react with excessive superoxide ions producing oxygen. The phenoxide ions (IIa, IIb, IIc) can undergo easy oxidation compared to the protonated form by transferring an electron to the oxygen molecule to produce phenoxyl radical (III). The formation of phenoxyl radicals by the direct oxidation of the phenoxide ion by superoxide is although thermodynamically favorable may not take place due to repulsion between two negatively charged species. The other possible reaction pathway is that the diketo group in curcumin undergoes nucleophilic addition and accepts an electron from superoxide ion to give a radical anion (IV), which can react with the phenoxyl radicals (III) to finally regenerate curcumin (I) with the conversion of superoxide into oxygen molecule. Some phenoxyl radicals (III) may undergo radical–radical reactions to give new products. All these reactions contribute to the complete neutralization of superoxide radicals. The regeneration of curcumin after such neutralization process can be of significant importance as it gives evidence for catalytic activity and may work as a mimic for SOD enzyme. Considering the fact that acetonitrile acts as an excellent mimic for the lipid membrane and curcumin being a lipid soluble compound these studies are exceptionally important in explaining the unusual and remarkable antioxidant activity of curcumin.



SCHEME 1

Acknowledgements

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