First observation of very long-lasting emissive polarization in EPR spectra of Fremy's salt when reduced by ascorbic acid. An unusual aspect of the radical pair mechanism

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Emissive EPR spectral lines were observed in experiments dedicated to investigating the efficiency of ascorbic acid in reducing Fremy's salt. The emission was detected by CW spectroscopy and was exceptionally long lasting, being observed as long as 20 minutes. Fremy's salt emission lines were observed at the end of the redox reaction. The Fremy's salt absorption signal was totally quenched, and was replaced by an emission signal. This feature was observed under different experimental conditions. The inversion proceeded simultaneously for the three lines, which keep their relative intensities and hyperfine separation. The emission signal was also detected together with the normal absorption signal of ascorbyl free radical. No emission spectra were observed by using other nitroxides, or by using as reducing agent 1,4-dithio-DL-threitol instead of ascorbic acid. The observed spin polarization is accounted for on the basis of a Radical Pair Mechanism (RPM). The amount of polarization is related to the diffusion constant D, which decreases with increasing viscosity. By adding glycerol to the solution, enhanced polarisation was indeed observed, as expected, which confirms the proposed RPM polarisation mechanism. We report here for the first time, to our knowledge, the phenomenon of long-lasting emissive EPR lines.

Introduction

Potassium nitrosodisulfonate $K_2NO(SO_3)_2$ or Fremy's salt is a relatively stable water-soluble nitroxide. It was studied as a model of the peroxyl radicals because it reacts with ascorbic acid in a similar way.[1] Moreover nitroxides and peroxyl radicals are isoelectronic and they both have large dipole moments.[2] For these reasons, Fremy's salt can be a useful tool in understanding the reduction mechanism induced by ascorbic acid.

Fremy's salt is easily detected by EPR spectroscopy and in solution it shows the typical three line of the nitroxide spectrum. It has been used in many studies on biological systems such as in EPR imaging,[3] in evaluating the radical scavenging activity[4] and in EPR kinetic studies.[1,5,6]

In biological systems, nitroxides are reduced by various biological agents,[7] but the reaction is known to be relatively slow compared to that mediated by ascorbic acid.[8] Ascorbic acid seems to be the only significant biological reductant for nitroxides,[9] and it is often used in solution to test nitroxide stability.[10–13] Furthermore it has been used in many applications such as the study of the stability of liposome-encapsulated nitroxides[7] and the study of the kinetics of spin labelled human lipoprotein reduction.[14]

In many metabolic pathways, specific enzymes use ascorbate as a very effective reductant.[15–18] In these cases, although it is able to donate two electrons, it mainly functions as one-electron donor, the resulting species being the ascorbate radical anion, also called ascorbyl radical $A^{-\bullet}$. In Fig. 1 the possible transformations of ascorbate species are illustrated.

At physiological pH ascorbic acid (AH_2) is mainly in the mono anion form (AH^-), which is deprotonated, because the pK_1 of the ascorbic acid is 4.04. At higher pH values, AH^- can lose the second proton ($pK_2 = 11.34$), leading to the dianion form A^{2-}, which, although it represents an exceedingly small fraction at physiological pH, is a more efficient electron donor.[18,19] ESR studies on radicals derived from ascorbic acid have demonstrated that $A^{-\bullet}$ is the most stable species formed over the pH range 0–9. The neutral radical $AH^\bullet$ has been observed only in very acidic media. In non-acidic media rapid deprotonation to A^- take place and $AH^\bullet$ was called the "elusive" neutral ascorbate radical.[15,20] Moreover, the reduction potential to generate this species is prohibitively high ($E^{01} = +0.766$ V).[18]

Non-enzymatic oxidation of ascorbate is reported to proceed in two successive one-electron steps,[2,21] that is, the ascorbate radical anion $A^{-\bullet}$ further donates the second electron to the acceptor, leading to the neutral dehydroascorbate form A, in accordance with the overall equation:

$$AH^- + 2\,[(SO_3K)_2NO^\bullet] \longrightarrow A + 2\,[(SO_3K)_2NO^-] + H^+$$

A = twice oxidized, twice deprotonated ascorbic acid.

In a recent work, aimed at investigating the efficiency of ascorbic acid in reducing Fremy's salt,[6] emissive EPR spectral lines were detected by CW spectroscopy. The emission was exceptionally long lasting, being observed for several minutes.

As the reducing agent was added to a Fremy's salt solution, it almost instantaneously quenched a fraction of the EPR nitroxide signal. The further reduction of the EPR probe proceeds slowly. Towards the end of the reaction, the Fremy's salt absorption signal intensity approaches zero and and is replaced by an emission signal (Fig. 2). Where the amplitude is represented with a negative sign, it describes an oscillation below the zero line. This indicates a non-equilibrium electron spin distribution, that is a distribution between the $m_S = -1/2$

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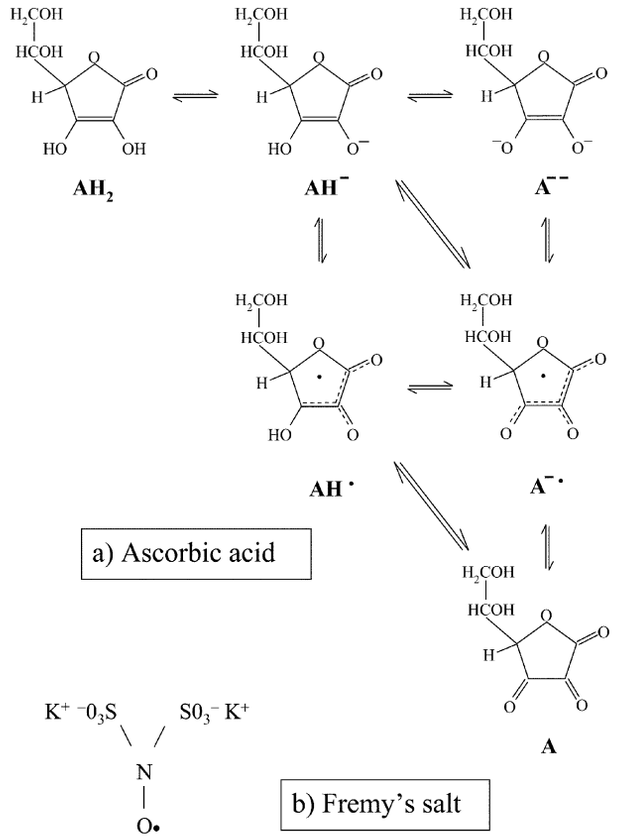

Fig. 1 a) Interconversion of ascorbate species. Protonation reactions are shown horizontally and electron transfer reactions are shown vertically. The two pK values for deprotonation of ascorbic acid leading to the semidehydroascorbate (AH^-) and dehydroascorbate (A^{2-}) are pK_1 = 4.04 and pK_2 = 11.34. Ascorbyl radical is $A^{-\bullet}$. b) Fremy's salt chemical structure.

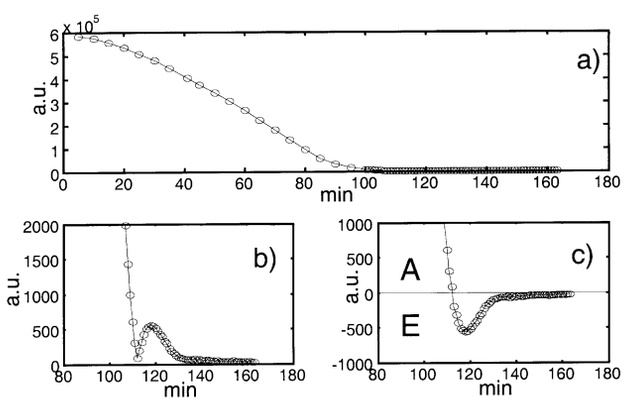

Fig. 2 a) Peak to peak amplitude of Fremy's salt central line as a function of time, during interaction with ascorbic acid with zero order kinetics. b) Final part of the reaction: after about 110 minutes from the start, the EPR absorption signal approaches zero and is replaced by an emission signal. The emission signal lasts about 20 minutes. The points after the first minimum represent the intensities of emission signals. c) The amplitudes of the emission signals are here plotted with a negative sign.

and m_S = 1/2 Zeeman sublevels that differs from the Boltzmann distribution. This phenomenon is referred to as spin polarisation. EPR lines of polarised species may occur in enhanced absorption (A*) or emission (E), depending on the sign of the population difference.[22]

Unlike in the present case, normally observed electron spin polarised signals are fast transient, with lifetimes of the order of microseconds or less and they are detected with TR-EPR (Time Resolved EPR) by pulsed photoexcitation.[22] The unusual long lasting polarisation is explained on the basis of CIDEP (Chemically Induced Dynamic Electron Polarisation) or ESP (Electron Spin Polarization) effects, due to free encounters of radical pairs.

The observation of long lasting spin polarisation during the reaction gives further insight into the intermediate steps of the ascorbic acid redox reaction.

Results

The reduction reaction of Fremy's salt by ascorbic acid has been previously studied at various relative concentrations of the reactants and at different pH values.[6] The reducing agent was found to immediately quench a fraction of the Fremy's salt by a fast reaction. The EPR signal drops to about 50% of that of a reference signal of a solution of Fremy's salt alone at the initial concentration. The kinetics of this reaction step could not be followed because a few minutes dead time where required for instrumental settings.

The data reported in Fig. 2a refer to the following slower decay, when the EPR signal decreases with zero order kinetics (Fig. 2a).

Emissive EPR lines were observed at the end of the kinetic decay, after the total quenching of the Fremy's salt absorption signal. An emission signal then appears at the same rate. It reaches a maximum and then it decreases and eventually disappears. The emission duration depends on the starting reactants concentration ratio and on the reaction rate. By changing the experimental conditions it was possible to clearly detect the emission for as long as 20 minutes (Fig. 2b).

Emissive Fremy's salt lines were detected under different instrumental conditions, *i.e.* different field modulation amplitude and microwave power. It was also verified that the buffer has no effect on the phenomenon, which is still observed even in a buffer-free reaction mixture.

The emission signal showed the same hyperfine structure of the Fremy's salt triplet and was found in all three hyperfine components of the spectrum. In Fig. 3, upper panel, a sequence of four spectra collected near the inversion time is shown. These spectra were detected with a 50 G (5 mT) scan range, in order to observe potential changes in the spectral parameters before and after the inversion phenomenon. The paramagnetic probe concentration decreases during the scan time (2 min), also within the same spectrum. For this reason in the first spectrum the EPR lines appear to diminish gradually. In the second spectrum the low field line is the last one visible in the absorption mode. The central line signal becomes indistinguishable from the noise and the high field line is the first which arises in the emission mode. The different line intensities in the same spectrum are due to the different times at which the lines are recorded, because of the scan rate. Repeated experiments have verified that the inversion proceeds simultaneously for the three lines, which retain their relative intensities and their hyperfine coupling constant values. Therefore, any multiplet effect on the spin polarisation is ruled out (see discussion).

Another attempt was made, using DTT as the reducing agent instead of ascorbic acid, but at the same concentration. The emission signal from Fremy's salt was not observed, while its reduction kinetics was slowed down.

Moreover, emission was not observed when two other nitroxides, PCA and TEMPO, were used and their EPR signals were followed during their reduction by ascorbic acid.

Since Fremy's salt in solution, when exposed to light at room temperature, undergoes a decrease in the EPR spectral intensity, a reference sample was tested for several days in order to exclude the inversion phenomenon being a consequence of the decay of the nitroxide alone. When the signal intensity was reduced to be comparable with that at which the spectral inversion occurs, no change of mode (absorption to emission) was observed in the Fremy's salt signal, which decayed without inversion.

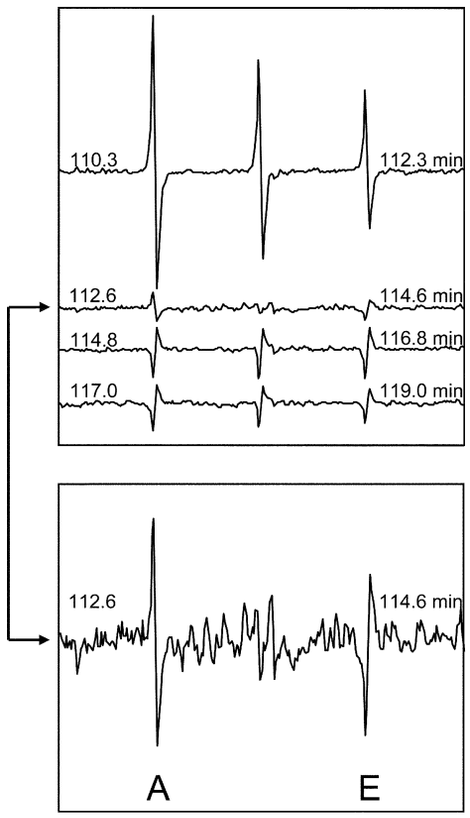

Fig. 3 Upper panel: sequence of four spectra showing the Fremy's salt triplet collected after 110 minutes of reaction, close to the inversion time. Each scan lasts 2 minutes. About 113 minutes after mixing with ascorbic acid, during the second scan, the EPR signal changes from an absorption to an emission signal. In the following two scans, the amplitude of the emission lines increases up to a maximum, then decreases. Lower panel: detailed view of the second spectrum. The lower field line is in absorption mode (A), the middle line obscured by the arising spin polarization, and the higher field line is completely emissive (E). The presence of A and E lines in the same spectrum is only due to the different times of recording, and not to an A/E multiplet effect.

During the ascorbic acid reduction, another EPR signal was observed consisting of a 1 : 1 doublet with 1.82 G hyperfine splitting, which is assigned to the ascorbyl free radical.[15,24] This signal was observed only at the end of the reduction process when it was no longer hidden by the stronger Fremy's salt signal. In Fig. 4, while the Fremy's salt signal changes from absorption to emission (spectra A and B respectively), only the high field line of the small doublet (1.82 G hyperfine constant) of ascorbyl free radical is evident. The low field line of the doublet near $g = 2$ is overlapped by the central Fremy's salt line. The simultaneous observation of EPR signals having opposite polarisations confirms beyond any doubt that the nitroxide signal corresponds to emission and it is not an instrumental artefact. The two lines of the ascorbyl free radical signal are visible alone (spectrum C) when the emission signal disappears.

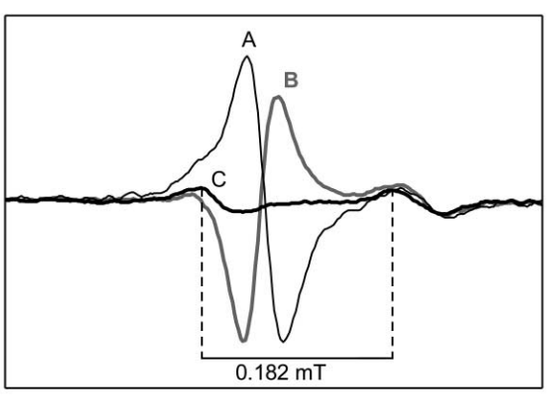

Fig. 4 EPR signals of the central line (g = 2) of Fremy's salt superimposed onto the doublet of ascorbyl free radical (0.182 mT). While the Fremy's salt signal changes from absorption (A) to emission (B), the ascorbyl signal is always present in absorption mode. After the total decay of the emission signal the ascorbyl signal is detected alone, as shown in bold (C), for reference.

The amount of polarization generated in paramagnetic species by chemical reactions in the presence of magnetic fields can be related to the diffusion constant D (see discussion), which decreases with increasing viscosity. For this reason, various amounts of glycerol (10, 20, and 30%) were added to the reaction mixture and a corresponding increase in the emission signal intensity was observed (Fig. 5).

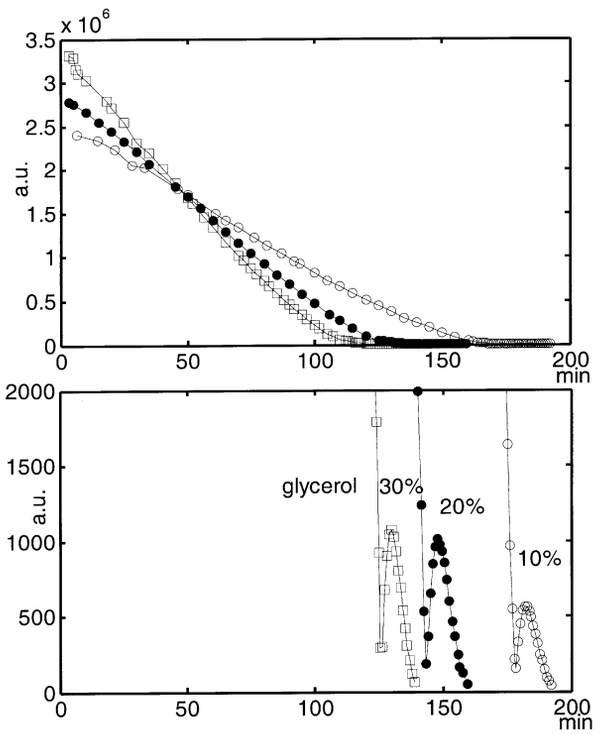

Fig. 5 Effect of glycerol in the emission phase. Upper panel: decay of Fremy's salt signal in the presence of 30% (□), 20% (●), 10% (○) glycerol. Increasing glycerol content seems to slightly hamper the initial fast quenching and to speed up the later reduction kinetic of Fremy's salt. Lower panel: signal amplitudes are shown on an expanded vertical axis scale. Emission lines appear at the end of the reaction, at different times. The different intensities account for different degrees of polarization, according to the theory for diffusion-controlled processes in viscous media.

The added glycerol has the effect of speeding up the decay, as can be seen in Fig. 5. In contrast, the quenching step is slightly hampered at higher viscosity. This step determines the initial intensity for each reaction, that is the amount of residual Fremy's salt, which then reacts in the slow phase. The first step is so fast that it is undetectable under the present experimental conditions, because the mixing occurs outside the cavity and a few minutes are needed to record the first signal.

The maximum amplitude reached by the emission line is larger for the most viscous solution and diminishes with decreasing viscosity. An approximately twofold enhancement factor was also observed for the most viscous solution with respect to the normal viscosity solution (without glycerol). This result confirms that the amount of emissive polarization depends on the lifetime of the solvent cage of the radical pair involved in the interaction.

Discussion

Fremy's salt reduction by ascorbic acid, as measured by the EPR line intensity of Fremy's salt signal, proceeds in two steps. Initially there is a sudden loss, then a "slower" step has been recorded. The data in Fig. 2 and Fig. 5 show the slower decay step, when the EPR signal decreases with a reaction rate independent of the Fremy's salt concentration.

It can be assumed that the first step, whose effect is revealed by the first EPR signal intensity of Fremy's salt, corresponds to a first electron/proton transfer from the species ascorbate AH^- which is oxidized to the radical anion $A^{-\bullet}$. This intermediate radical has been detected by EPR spectroscopy in the experiments, when the Fremy's salt probe was almost completely reduced (see Fig. 4).

In previous work,[6] the same reaction mixture was analysed by UV spectroscopy. It was found that the ascorbate absorbance has a maximum decrease in the first minute, indicating the loss of the first electron/proton. This confirms the above assumption.

Once the ascorbyl radical ($A^{-\bullet}$) has been formed, it reacts again with Fremy's salt in a second electron transfer process. This step appears to be the radical pair interaction, which accounts for the observed spin polarization.

By using the symbols in Fig. 1, the formation and decay of ascorbate radical anion intermediate can be outlined by the following equations:

FIRST STEP (formation of ascorbyl)

$$AH^- + (SO_3K)_2NO^\bullet \longrightarrow A^{-\bullet} + (SO_3K)_2NO^- + H^+$$

SECOND STEP (decay of ascorbyl)

$$A^{-\bullet} + (SO_3K)_2NO^\bullet \longrightarrow A + (SO_3K)_2NO^-$$
$$2A^{-\bullet} + H^+ \longrightarrow AH^- + A$$

The radical pair interaction will take place in the second step, first equation, involving the ascorbyl radical and the Fremy's salt radical. At the same time the decay of ascorbyl by reverse dismutation, second step, second equation, gives again the reagent species AH^-, thus altering the reagents/ products ratio.

The reducing agent was indeed found to be very effective in reducing Fremy's salt, although their stoichiometric reaction ratios are unfavourable: 2.5 mM ascorbic acid towards 17 mM Fremy's salt.

In general, ESP can be generated by a radical pair mechanism (RPM),[22,23,25,26] a triplet mechanism (TM)[27] and a radical triplet pair mechanism (RTPM).[28–30] In the present case, TM and RTPM are ruled out, since they require an excited triplet state molecule, which is not present in the ascorbate/Fremy's salt system.

A RPM operates when a radical pair (RP) is formed in a solvent cage, from a precursor molecule that can be either in a singlet or in a triplet state. A chemical reaction giving diamagnetic products can occurs in the cage only if the RP spin function is singlet (S), because spin should be conserved in the elementary reaction steps. If the RP is a triplet (T) the radicals escape into the solution bulk and eventually give rise to products (escape products).

In the present case RPs are formed by reactive free diffusing radicals in solution (free pairs) and their overall behaviour follows that of RPs formed by triplet precursor molecules, because the recombination reaction depletes the solution of singlet RPs.

When the radicals forming the pair have different Larmor frequencies ω_A and ω_B, transitions take place between the singlet state S and the $M_S = 0$ triplet component T_0 of the pair. These S/T_0 transitions give rise to spin polarized radicals if the S and T energies are separated by an exchange interaction $2J$ of the order of magnitude of $\hbar(\omega_A - \omega_B)$. This condition could be met during the diffusion of the radicals, since J depends on the radical separation r.[22]

The frequency difference may be due either to g factors or to hyperfine couplings. When the g difference is the cause of S/T_0 mixing, one radical becomes polarised in emission (E) and the other in absorption (A) . When hyperfine coupling is responsible for the mixing, the EPR spectrum of radicals escaping from the cage has the low field lines with A polarization and the high field lines with E or *vice versa* (multiplet effect), depending on a number of factors: triplet or singlet character of the precursor, sign of the hyperfine coupling constant and sign of J. Often a mixed polarisation pattern is observed, deriving from both g and hyperfine terms. This is indicated for example as E*/A, if emission prevails over absorption. S/T_0 transitions are responsible for most of the observed polarisation of transient radicals.

In our case, the nitrogen hyperfine coupling of Fremy's salt is sufficiently large to overcome any g factor difference at X-band, which corresponds to a field difference of the order of 0.1 mT, as can be seen in Fig. 4. Nevertheless, we did not observe any multiplet effect: all the nitroxide lines occur in emission with the same amount of polarisation. This rules out S/T_0 mixing as a source of spin polarisation.

For a triplet precursor, spectra completely in emission are obtained if effective mixing occurs between S and the triplet component T_{-1}. Crossing between S and the triplet component T_1 is also possible, but it requires a positive exchange interaction J and for this reason it is observed in only a very few cases. In order to observe spin polarisation, the RP partners should reside for a sufficiently long time at shorter distances, where $2J$ is close to the Zeeman interaction. ESP is generated because S/T_{-1} transitions transfer spin population from the non-reactive T_{-1} component of the RP to the chemically reactive state S. Since T_{-1} is formed by radical β spins, the solution becomes depleted by those spins and the EPR transitions of both radicals appear in emission, independently from the nuclear spin component. The S/T_{-1} mixing is promoted by the hyperfine coupling term, which should be large for effective S/T_{-1} transitions, as occurs in the case of the nitroxide triplet.

The experimental observations are explained through a chemical reaction between Fremy's salt and ascorbyl radical, to give a diamagnetic product. Nevertheless, when we observe the EPR lines due to the ascorbyl radical, its EPR signal is not polarized in emission as would be expected for S/T_{-1} mixing.

An explanation could be that, in contrast to the nitroxide radical, the ascorbyl radical is continuously renewed in solution with the same probability for α and β spins. This is in agreement with its dismutation reaction (second equation of second step), which describes the double decay of ascorbyl to the final product A, as well as its back-reaction to the first reagent species AH^-.

As expected by theory, the amount of polarisation should depend on the time of residence of the radical pair at the distance where S/T mixing is more effective. This is accounted for by the following equation,[31] relating polarisation $P = (n_\alpha - n_\beta)/(n_\alpha + n_\beta)$ and the diffusion constant D.

$$P = \pi A^2 r_c J/(4g\mu_B \lambda D)$$

where A is the hyperfine constant in Gauss and r_c and λ are parameters which define the dependence of the exchange interaction on the inter-radical separation r: $J = J_0 \exp(-\lambda(r - r_c))$.

Since D decreases with increasing viscosity, an increase of polarisation is expected upon adding glycerol to the solution, as was indeed observed and shown in Fig. 5.

Conclusions

The time evolution of the chemical reaction of Fremy's salt nitroxide with ascorbic acid has been investigated by recording the EPR signal. When the reaction is almost complete, the EPR signal changes from absorption to emission and its intensity finally decays to zero.

This behaviour is explained through the formation in solution of radical pairs consisting of free diffusing Fremy's salt radicals and ascorbyl radicals. A RPM with S/T_{-1} mixing accounts for the observed long lasting spin polarisation in full emission of the Fremy's salt EPR lines.

Mixing of T_{-1} and S is effective in underpopulating the T_{-1} state ($T_{-1} = \beta\beta$), because it makes this level more reactive. In solution, the total number of β radicals is decreased, and this process generates emission spectra. The amount of polarisation P in this case depends on the diffusion coefficient D of the solution. The increase of the emission signal intensity when glycerol is added to the solution confirms the proposed radical pair mechanism. Since only a fraction of the Fremy's salt radicals interact by the RP mechanism, their EPR lines in emission and absorption are added, because they are completely superimposable. The emissive polarisation can thus be observed only at the end of the reaction, when the fraction of nitroxide radicals giving the absorption signal is no longer able to mask the inverted (*i.e.* emission) lines.

This kind of long-lasting emissive phenomenon is here reported, to our knowledge, for the first time.

Experimental

Materials

Potassium nitrosodisulfonate, $K_2NO(SO_3)_2$, Fremy's salt and the other used nitroxides PCA (2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylic acid) and TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) were from Aldrich Chimica (Milano, Italy). Ascorbic acid, HEPES (*N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid]) sodium salt, and TRIS [tris(hydroxymethyl)aminomethane], were from Sigma Chemical Co. (St. Louis, MO). DTT (1,4-dithio-DL-threitol), glycerol and other chemical compounds, reagent-grade, were from Fluka AG (Buchs, Switzerland). All the solutions were made in bidistilled water.

The stock solution, 33 mM, of the inorganic nitroxide Fremy's salt was prepared according to Okazaki *et al.*[32] It was stored protected from air and light at 4 °C. The ascorbic acid stock solution, 50 mM, was prepared daily by dissolving the ascorbic acid in water. Before each measurement, Fremy's salt and ascorbic acid were diluted in a 0.1 M Na_1/Na_2-phosphate buffer (pH 7.5) as appropriate. As sample tubes, 50 µl quartz micropipettes were used.

Instruments

The EPR X-band spectrometer (9.5 GHz) was a conventional assembly of Bruker units (Bruker Spectrospin S.r.l., Milano, Italy): a magnet B-M8, a resonant cavity 4108 TMH/9101, and a microwave bridge ER040XR, equipped with the field controller BH15. Under typical conditions the spectrometer operated at a central magnetic field near 3480 G (348 mT), scan range 5 G (0.5 mT), sweep time 35 s, time constant 100 ms, modulation frequency 100 kHz, modulation amplitude 0.5 G (50 µT), microwave power 280 mW, attenuation 10 dB. When recording the whole Fremy's salt triplet 50 G (5 mT) scan range and sweep time 120 s were used.

In EPR experiments, Fremy's salt was mixed to a final concentration 17 mM with ascorbic acid 2.5 mM. This ascorbic acid concentration was chosen from a range of tested concentrations in order to optimize the rate of the studied reaction.[6] Repeated EPR spectra were recorded soon after mixing, at room temperature. To determine the level of Fremy's salt probe during the reaction with the scavenger, the peak-to-peak amplitude of each spectrum was calculated. The time delay for the first spectrum recording was about one minute and it was due to the mixing of the reactants outside the cavity, to the sample positioning as well as to the adjustment of EPR instrumentation.

A large number of reactions were recorded, in most of them only the central line of the Fremy's salt triplet was observed. In some cases the reaction was followed by recording the whole triplet spectrum, to verify the possible presence of emission or enhanced absorption effects on the low and high field lines and a possible change in hyperfine constant or in the relative intensities of lines.

Addition of glycerol to the solution was tested in seven experiments at three concentrations (10, 20, and 30%).

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