IDENTIFICATION OF FREE HYDROXYL RADICALS IN RESPIRING RAT HEART MITOCHONDRIA BY SPIN TRAPPING WITH THE NITRONE DMPO

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1. Introduction

Rat heart mitochondria have been observed to generate steady state concentrations of hydrogen peroxide and superoxide radicals [1,2]. We have indirect evidence that these oxygen species could give rise to the formation of $OH^{\cdot}$ radicals [2,3]. Support for this hypothesis comes from the observation that mitochondria stimulated to activate molecular oxygen [2,4] accumulate products of a reaction between membrane lipids and highly reactive oxygen species. Furthermore the existence of an $OH^{\cdot}$ radical generating pathway might be envisaged from the disappearance of mitochondrial H_2O_2 in the presence of $O_2^{\cdot-}$ in RHM deficient in enzymes capable of destroying H_2O_2 [3].

Since difficulties are encountered in the direct detection of oxy-radicals by ESR the method of spin trapping would seem well suited for the detection of these short-lived free radical species. However, using this technique, we observed that the physical identification of ESR spectra as definite spin adducts does not necessarily reflect the identity or the existence of free oxygen radicals in the medium. Therefore, this study was undertaken to establish criteria to determine whether ESR signals detected with 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) may be attributed to the formation of free $OH^{\cdot}$ radicals in biological systems. Applying these criteria to rat heart mitochondria, it was found that these organelles were an active source of $OH^{\cdot}$ radicals, involving superoxide and hydrogen peroxide as precursors.

2. Materials and methods

Antimycin A, xanthine, xanthine oxidase, and catalase were purchased from Boehringer, Mannheim; 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) from Aldrich-Europe, Belgium. Rat heart mitochondria were prepared as in [5]. After the last centrifugation, the pellet was suspended in the buffer used for ESR measurements (KCl–phosphate (pH 7.4)). Purification of the DMPO spin trap was performed with ~10 g activated charcoal GR/10 ml 0.15 M KCl–0.5 mM KH_2PO_4 buffer (pH 7.4). Prior to the addition of 1 g DMPO-batch the medium was cooled to 0°C, and saturated with oxygen-free nitrogen. After 10 min continuous bubbling with nitrogen in the dark, the spin trap was recovered by filtration and stored at −20°C in a KCl–phosphate buffer equilibrated with nitrogen.

ESR measurements were performed using a quartz flat cell in a Brucker 418s-spectrophotometer.

3. Results

Preliminary experiments with the spin trap 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) resulted in ESR spectra which were indicative of the formation of DMPO–OH adducts (A_N = 1.495 mT; A_H^{β} = 1.495 mT) despite the absence of any known enzymatic or non-enzymatic source of reduced oxygen species.

The formation of DMPO–OH-related ESR spectra were observed under the following conditions (table 1):

(i) Heating an air-saturated DMPO–buffer solution for 15 min at 40°C in a protic solvent;
(ii) Decrease from pH 7.0 to an acid pH;
(iii) Incubation of the spin trap with an $O_2^{\cdot-}$ radical-generating system (xanthine/xanthine oxidase);
(iv) Incubation of the spin trap with a Fenton-type generating system (xanthine/xanthine oxidase + Fe^{3+}/EDTA);

Table 1
Investigation of conditions causing the formation of DMPO–OH adducts

Treatment of DMPO	Type of ESR-spectra observed	Inhibition by scavengers of OH-radicals (mannitol, ethanol)
None	None	–
Heating in protic solvents	OH-adduct	–
Heating in aprotic solvents	None	–
Acid pH-ranges	OH-adduct	–
Xanthine/xanthine oxidase	OOH- and OH-adduct	–
Xanthine/xanthine oxidase +Fe(III)–EDTA	OOH- and OH-adduct	+
UV-irradiation	OH-adduct	+

The spin trap concentration was 0.2–0.3 M. The involvement of free OH-radicals was examined by the addition of 0.2 M mannitol or 0.4 M ethanol

(v) Exposure of the spin trap to UV-irradiation (1 min; pH 7.4).

The generation of DMPO–OH-related ESR-spectra under conditions iv and v could only be prevented if scavengers of OH· radicals were added to the system. Purification of the material (see section 2) obtained under conditions 1–3 also resulted in a complete disappearance of DMPO–OH-related ESR spectra. Furthermore, when DMPO was heated in an aprotic solvent (acetonitrile) spectra of OH adducts could not be observed. The unexpected formation of DMPO–OH spin-adducts was accompanied by a change from a clear to a pale yellow one. From these observations the unexpected appearance of DMPO–OH adducts may be due to chemical reactions catalysed by contaminating compounds. Therefore, an attempt was made to prove the purity of the spin traps by means of thin-layer chromatography. The chromatograms in fig.1 show a separation of the DMPO trap into two distinct spots, when the solution was exposed either to heating or to acid pH. After purification the spin trap ran homogeneously. This also occurred when DMPO was heated in aprotic solvents. The identity of the separated material was determined by eluting the different chromatographic spots with KCl buffer and measuring ESR spectra after heating. Spectra related to the formation of DMPO–OH adducts could only

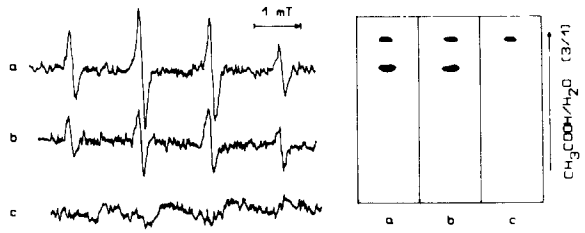

Fig.1. ESR spectra and corresponding thin-layer chromatograms (cellulose) of (a) DMPO following heating in protic and (c) aprotic solvents, and (b) of DMPO at acid pH.

be detected with the faster running compound. The same result was obtained when homogeneously running spots were investigated. Material from the slower running spots did not exhibit ESR signals attributable to any DMPO adduct formation. Fig.2 shows that the use of xanthine + xanthine oxidase in the absence of iron initially resulted in the formation of ESR species which can be attributed to DMPO–OOH adducts (A_N = 1.425 mT; A_H^β = 1.13 mT; A_H^γ = 0.14 mT). Within 13 min, this initial adduct appeared to decompose to DMPO–OH species. Superoxide dismutase (SOD) was found to interfere with this process by inhibiting the formation of the DMPO–OOH adduct. This observation agrees with [6], and indicates another source of generation of DMPO–OH adducts without the obligatory formation of free OH· radicals. Further support for this interpretation comes from the fact that OH· radical scavengers failed to prevent decomposition of DMPO–OOH to DMPO–OH adducts.

Purified DMPO solutions were used to test rat heart

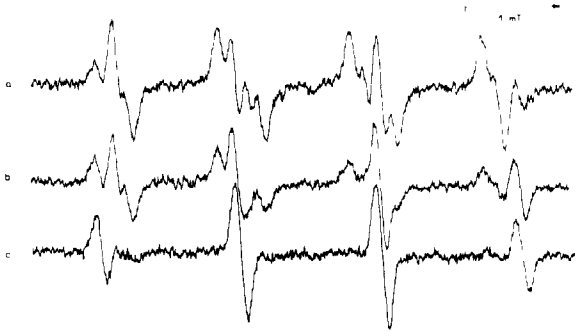

Fig.2. Transition of the ESR spectrum from the DMPO–OOH adduct to the DMPO–OH adduct as a function of time: (a) 5 min, (b) 9 min and (c) 13 min after mixing the $O_2^{\cdot-}$-generating system (5×10^{-5} M xanthine/50 μg xanthine oxidase/ml) with 0.2 M DMPO.

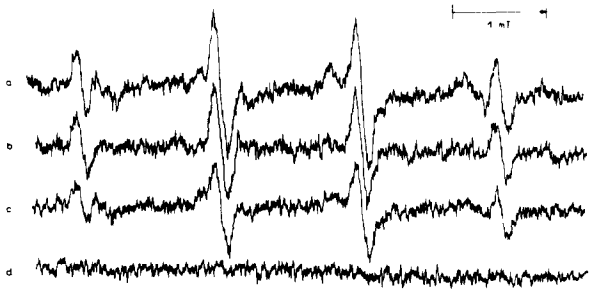

Fig.3. ESR spectra of the DMPO–OH adduct formed by the trapping of OH˙ radicals from respiring mitochondria; no addition (a), + SOD (b), + catalase (c), + SOD + catalase (d). Mitochondria were uncoupled by freezing and subsequent thawing. Mitochondrial protein (1.5 mg) was suspended in 300 μl reaction medium (0.15 M KCl–0.5 mM KH_2PO_4–buffer (pH 7.4)) in the presence of 6 mM succinate, 1 μg antimycin A and 0.3 M DMPO.

mitochondria for their capacity to generate OH˙ radicals. This biological system is able to reduce oxygen monovalently via minor side reactions of the respiratory chain [1] and is stimulated in uncoupled mitochondria in the presence of antimycin A or when RHM respire succinate under state 4 conditions [1]. Under both conditions, steady state concentrations of $O_2^{\cdot-}$ and H_2O_2 are established [2]. The use of spin trapping techniques showed the formation of ESR spectra which can be attributed essentially to the formation of DMPO–OH species, but characteristics of DMPO–OOH adducts were also detected (fig.3a). This became more evident when $HO_2^{\cdot}$ radicals were decomposed by the addition of exogenous SOD (fig.3b). The hyperfine splitting characteristic of the $HO_2^{\cdot}$ adduct disappeared, and the intensity of DMPO–OH adduct-related signals decreased by ~20%. The reduction was more pronounced in the presence of catalase (35%) while catalatic activity did not suppress the formation of DMPO–OOH-related signals (fig.3c). The combined addition of these enzymes completely inhibited both types of ESR spectra (fig.3d). This was also the case when mannitol or ethanol was added to the system. However, in the presence of these latter compounds, new types of ESR spectra appeared which may be attributed to DMPO adducts from the products of reactions of the hydroxyl radicals with the respective scavengers.

The involvement of mitochondrial electron flow as an essential stage in the formation of OH˙ spin adducts was assayed by competitive inhibition of succinate on addition of malonate. Since OH˙ radical formation was measured with uncoupled rat heart mitochondria in the presence of antimycin A redox changes of the respiratory component before the antimycin A stop were followed at different malonate/succinate ratios and compared with the intensities of DMPO–OH adduct formation. Fig.4 shows the inhibition of succinate-induced reduction of cytochrome b_{566} at increasing malonate concentrations. The non-linear curve of dependence of cytochrome *b* oxidation on malonate-related inhibition of electron flow via respiratory components inversely follows the curve for OH˙ radical formation. When the malonate/succinate ratio was 10 both the reduction state of cytochrome *b* and the intensity of DMPO–OH adduct formation were found to be inhibited by 80%.

4. Discussion

This paper provides evidence that the identification of short-lived radicals by characterizing DMPO spin adducts may lead to false results concerning the existence and the identity of free radical species. The determination of hyperfine splitting constants can be taken as an evidence for the formation of DMPO–OH adducts but does not necessarily reflect the involvement of free OH˙ radicals. For instance, neither scav-

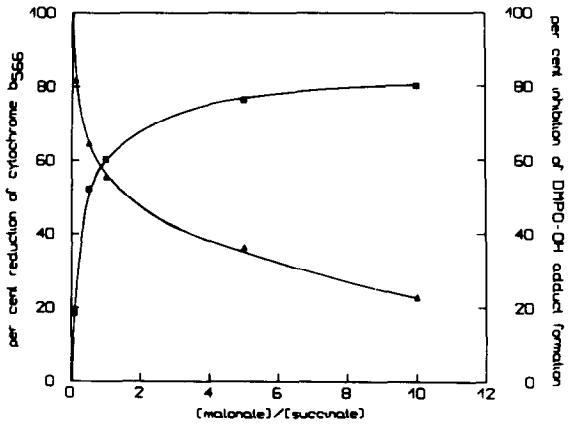

Fig.4. Involvement of the activity of electron transfer components in the formation of free OH˙ radicals. Quantitative changes in DMPO–OH adduct formation (■) was calculated from the intensities of the related ESR spectra. Values for redox changes of cytochrome b_{566} (▲) have been taken from double beam spectra (Aminco DW-2 spectrophotometer) at 575–566 nm.

engers of OH· radicals nor enzymatic decomposition of the essential precursors by SOD and/or catalase are effective in preventing DMPO–OH formation in heated DMPO preparations or in spin trap solutions at acid pH. Thus, it must be concluded that formation of DMPO–OH· is possible without the existence of free OH· radicals. In this case it is very likely that contaminating compounds serve as mediators for intermolecular radical reactions of the spin trap via decomposition to nitrone compounds. Support for this concept comes from the fact that thin-layer chromatography of the treated material revealed two separate spots, while the spin trap ran homogeneously after purification. Furthermore, as shown by ESR measurements, chromatographic separation of the treated spin trap indicates the existence of intact and decomposed DMPO molecules. The chemical properties of a nitrone spin trap (DMPO) may allow a multiplicity of reactions in water including the formation of nitroxide radicals as well as OH· radical functions by hydrolysis and subsequent disproportionation [7]. The existence of such sequences of intermolecular reactions with decomposed nitrone compounds in our system appears very likely while in water-free solutions (acetonitrile) DMPO seems to remain chemically stable. This may be concluded from the lack of chromatographic separation as well as from the absence of DMPO–OH-related ESR spectra. The rapid transition of DMPO spin adducts with $H_2^{\cdot}$ radicals to the more stable OH· spin compound may partly follow reaction sequences, similar to those discussed above, since formation of free OH· radicals could not be detected. Chemical reaction sequences involving decomposed nitrone compounds as possible sources for DMPO–OH-related ESR spectra can be excluded in mitochondrial suspensions for the following reasons:

(i) OH· spin adduct formation is sensitive to OH· radical scavengers, indicating the existence of free OH· radicals in the system;
(ii) Malonate-dependent modulation of electron flow via respiratory components was shown to regulate the intensity of OH· radical spin adduct formation; while
(iii) Catalase together with SOD was able to suppress production.

These observations indicate the role of the respiratory chain in the monovalent transfer of electrons to molecular oxygen and also that superoxide together with H_2O_2 may be required as precursors for OH· radicals in respiring mitochondria.

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