$^{19}F^-$ NUCLEAR MAGNETIC RELAXATION BY SUPEROXIDE DISMUTASE AS AN ENZYMIC METHOD FOR THE DETECTION OF SUPEROXIDE ION

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1. Introduction

Detection and measurement of O_2^- flows generated in biological systems is still a major problem due to the low values involved and the lack of specificity of the methods used. Generation of O_2^- in biological systems is usually demonstrated by the formation of chromophoric (e.g., Fe(II) cytochrome, the nitroformate anion, formazan, adrenochrome) [1] or ESR-probed (spin traps) [2] products from the reaction with suitable scavengers. These procedures are subjected to a number of interferences and, more generally, imply the use of rather high concentrations of reactive redox compounds as scavengers. In some instances, production of O_2^- may be induced by the presence of the detecting system itself. This work deals with a novel method for detection of O_2^- that uses the redox molecule with maximal specificity for O_2^-, i.e., the enzyme superoxide dismutase itself. In this way sensitivity is also greatly enhanced and the risk of perturbing biological systems with high concentration of reactive compounds is minimized.

2. Theory

The catalytic reaction of Cu–Zn superoxide dismutase involves alternate reduction (reaction (1)) and reoxidation (reaction (2)) of the Cu ion at the active site by O_2^- with $k_1 = k_2 = 2.3 \times 10^9\ M^{-1} . s^{-1}$ [3]. Because of this exceptionally high value, enzyme concentrations as low as 10^{-7} M are able to dismutate O_2^- very efficiently and almost independently of pH in the range pH 5–10 [4].

By measuring the paramagnetic contribution of the oxidized enzyme to the NMR relaxivity of $^{19}F^-$ – T_{1p}^{-1} –, it was shown [5] that in turnover conditions $[E_{Cu^{2+}}] \simeq [E_{Cu^+}]$, as expected from the identical values of $k_1 = k_2$. At $\geqslant 10^{-7}$ M superoxide dismutase, the steady-state approximation can be applied for the O_2^- concentration because the rates of reactions (1) and (2) are expectedly much higher than the rate of non-enzymic O_2^- dismutation and of O_2^- generation by biological systems. Therefore it results:

$$\frac{d\,[O_2^-]}{dt} = R_0 - k_1 \frac{[E_{Cu^{2+}}]}{2}\,[O_2^-] - k_2 \frac{[E_{Cu^+}]}{2}\,[O_2^-] \simeq 0 \qquad (1)$$

where R_0 is the rate of O_2^- generation and $([E_{Cu^{2+}}]/2) + ([E_{Cu^+}]/2) = [E_0]$ is the enzyme concentration.

Since $k_1 = k_2$ (1) the O_2^- concentration at steady-state is:

$$[O_2^-]_{ss} = R_0/k_1\,[E_0].$$

If at the start of O_2^- generation the enzyme is in the oxidized form the rate of approach to the enzyme steady-state condition $[E_{Cu^{2+}}] \simeq [E_{Cu^+}]$ is:

$$\frac{d\,[E_{Cu^{2+}}]}{dt} = k_2 \frac{[E_{Cu^+}]}{2}\,[O_2^-]_{ss} - k_1 \frac{[E_{Cu^{2+}}]}{2}\,[O_2^-]_{ss} = -\frac{R_0}{[E_0]}\left([E_{Cu^{2+}}] - [E_0]\right) \qquad (2)$$

which means that starting from the oxidized enzyme

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the steady-state condition $[E_{Cu^{2+}}] \simeq [E_{Cu^{+}}]$, is attained by a first-order process with:

$$\tau_{1/2} = 0.69\ [E_o]/R_o$$

Since $[E_{Cu^{2+}}] \propto {}^{19}F\ T_{1p}^{-1}$ [6] and ${}^{19}F\ (T_{1p}^{-1})_{ss} \simeq 1/2\ (T_{1p}^{-1})_{ox}$, from the integration of eq. (2) results:

$$\ln \frac{[E_o]}{[E_{Cu^{2+}}] - [E_o]} = \ln \frac{(T_{1p}^{-1})_o}{2T_{1p}^{-1} - (T_{1p}^{-1})_o} = (R_o/[E_o])\,t \qquad (3)$$

where $(T_{1p}^{-1})_o$ is the paramagnetic contribution at the start of O_2^- generation ($t = 0$). Since at the steady-state, because of the presence of 0.5 M F^-, $E_{Cu^{2+}}$ is slightly higher than $[E_o]/2$ [5], the following equation must be considered instead of eq. (3):

$$\ln \frac{(T_{1p}^{-1})_o - (T_{1p}^{-1})_{ss}}{T_{1p}^{-1} - (T_{1p}^{-1})_{ss}} = (R_o/[E_o])\,t \qquad (4)$$

The same equations hold when at the start of the O_2^- production the enzyme is present in the reduced form.

3. Results and discussion

The addition of an O_2^--producing system, such as xanthine oxidase oxidizing xanthine, to a solution containing F^- and the oxidized form of Cu–Zn superoxide dismutase brought $[E_{Cu^{2+}}]$ to the steady-state value $[E_{Cu^{2+}}] \simeq [E_{Cu^{+}}]$ and this process was followed through the decrease of $^{19}F^-$ relaxivity (fig.1A). The plot of the data, according to eq. (4) (fig.1, right ordinate) gives a straight line (fig.1A′) from the slope of which $(R_o/[E_o])$ a rate of O_2^- gener-

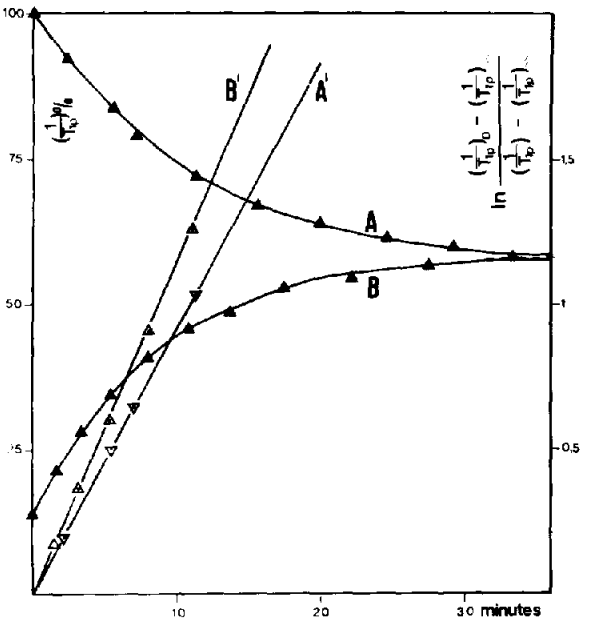

Fig.1. Time course of the approach to the steady-state $[Cu^{2+}]$ as measured by $^{19}F^-$ relaxivity by oxidized (A) or reduced (B) superoxide dismutase (4.7 and 4.0 μM in A and B, respectively). A′ and B′ are the respective first-order plots. ^{19}F relaxivity data and reduced enzyme were obtained as in [5]. The reaction medium contained 0.5 M KF and 1×10^{-4} M EDTA in 0.03 M potassium phosphate (pH 7.35). O_2^- was generated by addition of 5×10^{-4} M xanthine and 5×10^{-8} M xanthine oxidase at 25°C.

Table 1
Rate of O_2^- generation by the action of varied amounts of xanthine oxidase on 5×10^{-4} M xanthine, as measured by the $^{19}F^-$ NMR relaxivity–superoxide dismutase method as compared with the cytochrome *c* method

pH	Superoxide dismutase (μM)	$R_o/[E_o]$ ($min^{-1} \times 10^2$)	R_o ($^{19}F^-$ NMR) ($M^{-1} \cdot min^{-1} \times 10^8$)	R_o (cyt. *c*) ($M^{-1} \cdot min^{-1} \times 10^8$)
7.35	4.8	2.7	9.5	10.2
7.35	5.1	9.3	47.4	50
7.35	0.4	3.2	1.3	n.m.
7.35	10	19.8	190	201
5.60	1.6	16	25	25.3
8.60	1.4	5.3	72	75

n.m., not measurable

ation of 4.3×10^{-7} M . min^{-1} was calculated.

In a parallel experiment where Fe(II) cytochrome *c* was substituted for superoxide dismutase a constant and comparable O_2^- flow of 3.9×10^{-7} M . min^{-1} was measured from the increase of absorbance at 550 nm [7]. It was also verified starting from $[E_{Cu^+}] \gg [E_{Cu^{2+}}]$ that the same steady-state condition was reached, fig.1B, and that the rate of O_2^- generation (4.7×10^{-7} M . min^{-1}), calculated from the relative first-order plot (fig.1B'), was in good agreement with the value obtained using the oxidized enzyme.

In the experimental conditions used, the rate of reoxidation of E_{Cu^+} by molecular O_2 were negligible as compared with the rates of reactions (1) and (2) [5]. Table 1 reports some indicative results obtained in various conditions. In general O_2^- flows that may be detected by the proposed method range, for superoxide dismutase between 2×10^{-7}–10^{-4} M and for the time constants for the process of approach to steady-state measurable by $^{19}F^-$ relaxivity ($\tau_{1/2}$ = 10–2×10^3 s), between 5×10^{-9} and 5×10^{-4} M . min^{-1}. This means that O_2^- flows more than an order of magnitude lower than those safely detectable by the cytochrome *c* method were measurable. Furthermore, contrary to most of the current methods for O_2^- detection, the ^{19}F method is independent of pH. Finally, it should be recalled that a method, such as the above, that uses the specific enzyme in catalytic amounts for the detection of its substrate is clearly a much less perturbing and more reliable approach for the measurement of fluxes of O_2^- in biological systems as it involves very low concentrations of a protein with predictable and controllable reactivity. Other advantages of the method are its applicability to turbid or highly coloured samples and the redox stability of F^- as compared to nitroxide spin traps, nitroblue tetrazolium, tetranitromethane, cytochrome *c* or epinephrine.

Possible limitations of this method may in principle arise from the interference from relevant redox systems that are present in biological systems. In fact, because of the redox potential of the Cu–Zn superoxide dismutase, $E^\circ = 0.42$ V [8], a variety of compounds could in principle reduce the enzyme. Reducing agents characterized by $E^\circ < 0.42$ V such as cysteine, glutathione, cystamine, NADH, 1,4-dihydroxybenzene, were tested in strictly anaerobic conditions and were found to reduce the oxidized enzyme at much slower rates as compared to those of table 1, such as even low O_2^- fluxes could be detected in the presence of high concentrations of natural reducing agents.

To conclude, the validity of the $^{19}F^-$ NMR relaxation–superoxide dismutase method for the detection of O_2^- fluxes in biological materials, appears to be established as far as specificity, lack of interference and sensitivity are concerned.

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