REACTIVE OXYGEN SPECIES: ELECTRON DONOR–HYDROGEN PEROXIDE COMPLEX INSTEAD OF FREE OH RADICALS?

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1. Introduction

Since the discovery of superoxide dismutase (EC 1.15.1.1) by McCord and Fridovich in 1969 [1], more and more researchers have focussed on 'oxygen activation and oxygen toxicity' and an increasing number of reports on this topic have appeared [2–6]. The oxygen species described as responsible for more or less deleterious effects on biological processes or tissues comprise the superoxide anion ($O_2^{\cdot -}$) or its corresponding acid ($HO_2^{\cdot}$, $pK = 4.8$), hydrogen peroxide (H_2O_2), the free OH-radical ($OH^{\cdot}$), organic alkoxy- or peroxy-radicals ($RO^{\cdot}$, $ROO^{\cdot}$), certain metallo-oxygen complexes (perferryl, percupryl) and singlet oxygen ($O_2^1\Delta g$). The free OH-radical, besides singlet oxygen, probably represents the most powerful oxidant ($E'_0 = +2.33$ V) among the above-mentioned species, exhibiting a lifetime of probably <1 μs and diffusion-limited rates of reaction with most organic molecules [7]. These properties render its reactions close to the site of its generation.

Reactions inhibited, or biological damage ameliorated, by both catalase and superoxide dismutase as well as by polyalcohols, benzoate, α-tocopherol (and many other compounds) have been interpreted as being driven by free OH-radicals due to its synthesis from H_2O_2 and $O_2^{\cdot -}$ according to the (iron-catalyzed) Haber-Weiss cycle (i.e., Fenton-type reactions):

$$O_2^{\cdot -} + \text{Fe (chelate)}^{3+} \rightleftharpoons O_2 + \text{Fe (chelate)}^{2+}$$

$$H_2O_2 + \text{Fe (chelate)}^{2+} \longrightarrow OH^{\cdot} + OH^- + \text{Fe (chelate)}^{3+}$$

$$\text{Sum: } O_2^{\cdot -} + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + O_2$$

(Haber–Weiss reaction)

The relevance of the above reactions for biological systems has frequently been questioned [4,7], since:

(i) The uncatalyzed Haber-Weiss reaction as well as the Fenton reaction do not seem to proceed with rates fast enough to yield appreciable concentrations of $OH^{\cdot}$ necessary to account for observed 'in vivo' activities;

(ii) The electrophilicity together with the short lifetime of the OH-radical render oxidations by this species extremely unspecific thus randomizing its effects in biological systems yielding general inactivations of enzymes and membraneous systems.

Here we report on an enzymic system which might reflect both the unspecific and specific reactivities of an oxygen species generally refered to as the free OH-radical. This system consists of NADPH + H^+ (reduced by glucose 6-phosphate and glucose 6-phosphate dehydrogenase) and ferredoxin–$NADP^+$-reductase (EC 1.18.1.2), a flavoprotein specific at the $NADP^+$/NADPH + H^+ site but unspecific at the second cofactor site thus accepting several redox compounds with appropriate redox potentials besides its 'in vivo' substrate, ferredoxin.

2. Materials and methods

Ethylene formation from methionine in the presence of pyridoxalphosphate was followed as in [8–10]. Ferredoxin [11], ferredoxin–$NADP^+$-reductase [12] and superoxide dismutase [13] were prepared from spinach, autotrophically cultured [14] *Euglena gracilis* cells and from dried green peas, respectively. Catalase, glucose 6-phosphate dehydrogenase and glucose 6-phosphate were obtained from Boehringer, Mannheim. Reaction conditions and concentrations were as outlined in tables 1 and 2.

3. Results and discussion

During monovalent oxygen reduction catalyzed by either ferredoxin (fd) or anthraquinone-2-sulfonate (AQ), driven by NADPH + H^+ and ferredoxin–$NADP^+$-reductase, both $O_2^{\cdot-}$ and H_2O_2 are formed. The rates of oxygen uptake and H_2O_2 and $O_2^{\cdot-}$-formation with this system are faster in the presence of AQ (E_0' = −0.38 V) as compared to fd (E_0' = −0.43 V) due to thermodynamic reasons (E_0' for the $O_2/O_2^{\cdot-}$ couple has been determined as −0.33 V; that of the $NADP^+$/NADPH + H^+ couple as −0.32 V). The detector system for the $OH^{\cdot}$-like oxidant, methionine–pyridoxalphosphate (most probably as the Schiff-base), is not oxidized by H_2O_2 or $O_2^{\cdot-}$ but seems to react readily with a Fenton-type oxidant or pulse-radiolytically generated free OH-radical forming ethylene which can easily be determined gas-chromatographically [8–10].

In the above enzymic system, both 20 μM fd and AQ stimulate ethylene formation in 0.2 M phosphate buffer (pH 7.6) by ~300–500-fold.

While the fd-catalyzed system is fully active in both 0.2 M phosphate buffer and 0.2 M Tris–HCl buffer around neutral pH-values, the AQ-catalyzed system is only active in phosphate buffer and by >90% inhibited in Tris–HCl buffer. The operating mechanisms, however, seem to be similar in both systems as shown in table 1.

As shown in table 1, both systems are strongly inhibited by catalase and propylgallate, but stimulated by scavengers of the free OH-radical, as mannitol, α-tocopherol and formiate. Superoxide dismutase inhibits the AQ system but stimulates the fd system.

Assuming that the first reaction of the reduced cofactors in the presence of air consists in the monovalent reduction of oxygen yielding $O_2^{\cdot-}$, fd_{ox} and anthrasemiquinone ($AQH^{\cdot}$), the following reaction would logically be the formation of H_2O_2 after the spontaneous dismutation of $O_2^{\cdot-}$. If we assume that either fd_{red} or $AQH^{\cdot}$ can donate one electron to H_2O_2 forming free OH-radicals, we also have to assume that the electron donor forms a complex with H_2O_2 ($AQH^{\cdot}$–H_2O_2 or fd_{red}–H_2O_2). This complex might be the methionine oxidizing species. After electron abstraction from the substrate methionine–pyridoxalphosphate the complex may decay yielding AQ or fd_{ox}, 2 OH^- and CH_3–$\dot{S}^+$–CH_2–CH_2–R.

The differential effects of superoxide dismutase can be explained in terms of oxidation of fd_{red} by $O_2^{\cdot-}$ and thus stimulation by superoxide dismutase or reduction of AQ by $O_2^{\cdot-}$ yielding $AQH^{\cdot}$ and thus inhibition by superoxide dismutase. The sole requirement of reducing equivalents and H_2O_2 for ethylene formation from methionine–pyridoxalphosphate can be demonstrated by running the fd-catalyzed reaction under oxygen limiting (unpurified nitrogen) conditions (table 2).

The results presented in table 2 demonstrate that H_2O_2 in the presence of an appropriate one-electron donor seems to function as an 'OH-like' oxidant. Since this reaction is neither inhibited by high concentrations of superoxide dismutase nor by 'classical' OH-

Table 1
Ethylene formation from methionine: Effects of radical scavengers, superoxide dismutase and catalase

Cofactor and buffer	No. additions	+Catalase (100 U)	Superoxide dismutase (80 U)	Mannitol (0.1 mM)	α-Tocopherol (0.1 mM)	Formiate (0.1 mM)	Propylgallate (0.1 mM)
AQ in phosphate buffer	330[a]	2	30	600	800	740	9
fd in Tris buffer	500	8	750	680	980	740	90

[a] Rates represent pmol/h ethylene produced

Reaction conditions: 2 ml contained: 10 μmol glucose 6-phosphate; 50 μg glucose 6-phosphate dehydrogenase; 1 μmol $NADP^+$; ferredoxin–$NADP^+$-reductase with 0.2 mg protein isolated from *Euglena gracilis*; 20 nmol indicated cofactor; 20 μmol methionine; 0.2 μmol pyridoxalphosphate; 100 μmol indicated buffer (pH 7.6); the reaction was conducted for 20 min at 22°C in Fernbach flasks with ~14 ml, sealed with serum rubber stoppers; ethylene was withdrawn from the headspace of the reaction vessel and determined gas-chromatographically

Table 2
Ethylene formation from methionine: Effects of oxygen limitation and hydrogen peroxide

Conditions (see table 1) cofactor, 20 nmol fd	Ethylene formed (pmol/h)
Air	90
Air + 5 mM H_2O_2	500
Nitrogen	190
Nitrogen + 5 mM H_2O_2	2200

scavengers we have to assume that neither $O_2^{\cdot-}$, H_2O_2 nor the free OH-radical but rather 'reduced' hydrogen peroxide seems to function as a specific (and not randomly inactivating or oxidizing) oxidant for methionine-pyridoxyalphosphate $O_2^{\cdot-}$ seems to act as a reductant for AQ thus providing the 'active' semiquinone; the inhibition by superoxide dismutase of certain oxidations and destructions thus need not necessarily indicate the aggressiveness of $O_2^{\cdot-}$ but merely the inhibition of the formation of these 'active semiquinones' as already indicated [15]. The reported model of methionine oxidation in a fd_{red}–H_2O_2–methionine complex bears some analogies to the free radical–methionyl cluster of the hydrogen peroxide compound of cytochrome *c* peroxidase, as described in [16].

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